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Research Article

Improved Visualization of *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae)—Part II: Alimentary Canal Components and Measurements

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Alphitobius diaperinus (Panzer) (Coleoptera: Tenebrionidae) is a pest of stored food products and problematic to every type of poultry production facility. Larvae and adults can ingest and harbor foodborne and poultry pathogens. Determining the efficiency of this insect's capacity to transmit disease is critical to improving management of *A. diaperinus* on poultry facilities and providing a safe food supply for human consumption. However, a deficiency exists in the literature reporting measurements of the gut and its defined segments. Previous reports include line drawing depictions, which aid little in the determination of the pathogen reservoir potential of these insects. Advances in technology allowed more accurate visualization and precise measurement of gross anatomical features of the alimentary canal. A photographic depiction to aid the researcher in the visualization of anatomical features and accurate measurements of the alimentary canal for these insects is presented here.

1. Introduction

The of high-density poultry feeding operations to increase production output in order to meet market demand has changed the environment in which poultry is raised. The close quarters and high bird density favors the survival of arthropod pests. *Alphitobius diaperinus* (Panzer) (1797) (Coleoptera: Tenebrionidae), a pest of stored food products, is a common and persistent pest in every type of poultry production facility: breeders, grow-out, caged-layers and pullets. These insects have adapted well to the artificially controlled environment within poultry houses and what were once only minor pests in low density flocks have become large infestations in high-density rearing facilities. Their presence generates economic and management concerns. For example, *A. diaperinus* survive on the floor of a broiler production house in the accumulated mix of bedding material, excreta, feathers, spilt feed, carcasses, and other debris, referred to as litter. The high density of birds in production results in increased litter moisture, both from the excreta and automatic drinkers. Combined with the controlled temperature in the houses these conditions are highly conducive to beetle survival and population expansion.

Chickens and turkeys readily feed on *A. diaperinus*, and young birds preferentially ingest larvae, even in the presence of starter feed [1, 2]. Chicks fed solely *A. diaperinus* larvae for 9 days gained 37% less body weight than chicks on starter feed and, in addition, showed signs of stress [2]. This weight was not recovered when returned to starter feed through 14 days of age [2]. The omnivorous diet of *A. diaperinus* also means that they can compete with the birds for their feed. Furthermore, *A. diaperinus* can ingest and harbor foodborne and poultry pathogens (reviewed in: [3]). Consequently, *A. diaperinus* represents a health issue to the birds and to the humans which consume the birds [4].

During their life cycle *A. diaperinus* larvae migrate into the insulation of the building walls and the soil beneath the litter for pupation and eventual eclosion. Their tunneling

behavior disrupts the compacted earth floors and dense wall insulation [5]. Their activity in the walls reduces the building insulating capacity, causing enough damage to raise energy costs 67% and require replacing of insulation every two to three years [6–9]. Their activity in the compacted earthen floors, on which bedding is spread, results in an irregular and hollowed floor surface [6]. This hollowing can retain bedding and reduce the effectiveness of litter clean outs by tractor loaders. Therefore, *A. diaperinus* also represent a structural pest for the producer. Economic effects of *A. diaperinus* infestations on poultry production are difficult to quantify. Of financial concern for the producer is the issues that these insects cause facilities structural damage, affect bird growth and health, and vector poultry diseases.

A primary concern for the consumer is that these insects vector foodborne pathogens. Determination of the efficiency of this insect's capacity to harbor pathogens is critical to improving management of *A. diaperinus* in poultry facilities and providing a safe food supply for human consumption. Understanding the anatomy and physiology of the insect to model pathogen movement and transport within the insect is vital [10–12]. Line drawings of the alimentary canal for larvae and adult *A. diaperinus* have been provided in previous studies, but these aid little in determining reservoir potential of the alimentary canal [13–15]. No single reference exists with a photographic depiction to aid the researcher in the visualization of anatomical features and measurements of the alimentary canal for these insects. Improved reference images are needed to more accurately describe the larval and adult *A. diaperinus* alimentary canal, and these are presented here.

2. Experimental

2.1. Beetles. The Southern Plains Agricultural Research Center (SPARC) starter colony of *A. diaperinus* was from a colony originally isolated from a poultry farm located in Wake County, NC. The SPARC colony has remained in production since 2004. The adult colony was reared in 1000 mL wheat bran (Morrison Milling Co., Denton, TX) in plastic containers (15 × 15 × 30 cm) with screened bottoms. Insects were provided a 6 cm^2 sponge, placed atop a piece of aluminum foil, and moistened with deionized water as needed, and a 0.5 cm thick slice of a medium-sized apple was replenished twice per week. Fishmeal (30 mL; Omega Protein, Inc., Hammond, LA) was added to the wheat bran once per week, and new wheat bran was added as it was depleted by dropping through the screened bottom of the cage. Eggs were collected as needed on layered black construction paper (6 × 6 cm) and transferred to a separate container; emergent larvae were maintained as described above in a solid bottom container, until pupation. Pupae were transferred to a screened bottom container and emerging adults reared as described above. The entire colony was maintained at 30°C in an 8 : 16 hr (light : dark) photoperiod.

2.2. Morphometrics of Insects and Alimentary Canal

2.2.1. Insect Measurements. Immediately before dissection for removal of alimentary canals, as described below, male and female adults and late instars were measured using imagery software described by Esquivel [16]. Head capsule widths were also recorded for larvae to determine stadia, and, based on previous head capsule width measurements [17], the larvae used in this study were 7th instar or older and the adults were more than 4 weeks after eclosion.

2.2.2. Alimentary Canal Measurements. To determine size and capacity of the sections comprising the alimentary canals, intact alimentary canals were removed from male (n = 5) and female (n = 5) adults and late instars (n = 10). Equipment and dissection methodologies described by Esquivel [16] were slightly modified for excision of alimentary canals. Briefly, individual specimens were examined under an Olympus SZ60 dissecting stereomicroscope (Olympus, Kalamazoo, MI, USA). Lumenera INFINITY software and INFINITY 1–3 C camera (Lumenera, Ottawa, ON, Canada) were interfaced with a computer to record images and measurements of each specimen. Because the adults and larvae *A. diaperinus* were smaller than those insects examined previously, dissection technique, pins, and forceps varied, as described below.

2.2.3. Adults. Beetles were taken from rearing cages and placed in a vial at −20°C for ca. 15 min. Individual adults were removed from the vial and pinned (no. 00, BioQuip, Rancho Dominguez, CA, USA) dorsolaterally through the right elytron and through the body. Positioning of the pinning site was closer to the right margin of the abdomen to prevent piercing of the alimentary canal. Pinning at this location also provided an "anchor" during the dissection process. The beetle was then pinned into one of the "dissection wells" [16]. Distilled (RO) water was added to the well to facilitate dissection and excision of the alimentary canal.

The technique to remove the wings and abdominal dorsal cuticle was similar to Esquivel [16], with the exception that the right pair of wings was not removed and the cuticle was cut only at the left lateral margin of the abdomen. Two pair of forceps (no. 55 Rubis, BioQuip, Rancho Dominguez, CA, USA) were used to grasp the thorax dorsally at the midline and break each half open, allowing access to the ventral connective tissue between the head and the thorax. This connective tissue was severed to allow removal of the head intact and the alimentary canal was excised by teasing away the tracheae and connective tissue along the length of the body. The tissue between the ultimate and penultimate ventral abdominal plates was severed, allowing removal of the intact alimentary canal. The intact canal was then placed into a separate well and the head was grasped dorsally at midline and gently pried open. Pieces of the exoskeleton and tissue were teased away leaving only the mandibles and alimentary canal. Similarly, abdominal plates still attached around the rectum were teased away. The mandibles were pinned using minuten pins (BioQuip, Rancho Dominguez, CA, USA) and the rectum was grasped and pulled taut to lay the alimentary canal in a straight line, exercising care to not distend the canal past its normal length. A minuten pin held the rectum in place.

Following distension of the alimentary canal, measurements were recorded for the foregut (from the mouth—including buccal cavity, pharynx, and esophagus—to distal end of proventricular valve), the midgut (distal end of proventricular valve to distal end of pyloric valve), the small intestine (distal end of pyloric valve to enlargement of the intestine), the large intestine (enlarged intestine), and the rectum. Section assignments closely follow designations of McAllister et al. [13] and Snodgrass [18] except the rectum, which was not delineated or measured separately in those studies.

Total exterior body lengths ($n = 10$ per group) were measured along the anteroposterior axis for comparison to total alimentary canal lengths. For the adult, the head was measured from the anterior end of head to the first anterior thoracic segment. The thorax was measured from the first anterior thoracic segment to the anterior elytra attachment. The abdomen was measured from the anterior elytra attachment to the distal end of abdomen. For the late instar a measurement from the anterior end of head to the distal end of abdomen was performed.

2.2.4. Larvae. Late instars were taken from rearing cages and placed in a modified plastic centrifuge vial (JFE, unpublished data) and killed by exposure to ethyl acetate for 10–15 min. Dead larvae were placed in a dissection well and held down using a modified no. 00 pin (JFE, unpubl. data) allowing anchoring of the larva so that the alimentary canal was not pierced by the conventional pinning technique. Distilled water was added to the well to facilitate dissection and excision of the alimentary canal.

The dorsal cuticle of the larvae was cut along the left margin from the penultimate abdominal segment to the first thoracic segment. The cuticle was pulled to the right while removing tracheae and other tissue. Similar to the adults, the head was removed intact from the larva and the last abdominal segment (i.e., pygidium) was also removed intact. Removal of the alimentary canal from the body, subsequent clearing of the attached material (head and abdominal segments), distension of the alimentary canal, and measurement of alimentary canal sections were as described for adults.

Total exterior body lengths ($n = 10$) were measured along the anteroposterior axis for comparison to total alimentary canal lengths. For the late instar, a measurement from the anterior end of head to the distal end of abdomen was performed.

2.3. Data Analysis. Data were analyzed using commercially available statistical software (Prism ver. 5.01, GraphPad Software Inc., La Jolla, CA). Descriptive statistics were generated and are presented in table formats. Within each anatomical segment, life stage, and sex of insect, a means comparison of length was performed using a two-way ANOVA followed by Bonferroni posttests ($P < 0.05$).

3. Results and Discussion

The digestive tract is arranged into fore-, mid-, hindgut and rectum sections, which can be easily demarcated by visual examination. Figures 1(a) and 1(b) of *in situ* and extracted digestive tracts reveal the simplistic gut structure with a sigmoid bend distinctive to larval *A. diaperinus*. Conversely, adult *A. diaperinus* possess a more convoluted alimentary canal containing a complete loop before reaching the pyloric valve (Figures 1(c) and 1(d)). These images correspond with existing line drawn schematics presented by McAllister et al. [13] and Rahman et al. [14, 15] but provide more details regarding morphology and reservoir potential of the alimentary canal. Figure 2 displays the distended adult alimentary canal used during measurement of gut segments and insets of the proventricular valve and expanded views of the female and male rectum. Figure 3 displays the extracted larval alimentary canal demonstrating features used to delineating gut segments, with insets of the demarcating proventricular and pyloric valves, and the distended position of hindgut showing features used during measurement of the large and small intestinal segments. These delineating features are similar on both the larva and adult.

The alimentary canal of *A. diaperinus* is a tubular structure with similarities to beetles consuming stored grain products, as well as characteristics distinctive to its more omnivorous feeding habits when in a poultry production environment, as previously described by McAllister et al. [13]. Larvae in particular are known to be cannibalistic and have chitinase activity in their digestive secretions [19]. However, a study comparing the alimentary canal of *A. diaperinus* of individuals fed herbivorous or carnivorous diets determined no anatomical differences between the larvae on the two disparate diets [15]. Therefore our laboratory-raised insects were used as representative specimens for this study.

Adults of 2 to 4 months of age and late instar larvae were measured for this study. The mean head capsule width of the late instars ($n = 10$) used in this study was 1.084 ($\pm$ 0.013) mm and ranged from 0.960 to 1.320 mm; representing 7th to 9th instar. Barké and Davis [17] reported head capsule widths of 0.95, 1.08, and 1.28 mm, and Francisco and Prado [20] reported widths of 1.061, 1.208, and 1.339 mm for 7th, 8th, and 9th instars, respectively. Overall measurements of the alimentary canal and its sections resemble those reported by McAllister et al. [13] for fully tanned adults and 8th- to 11th-instar larvae. However, some variation was expected due to the likely differences in the range of ages of the insects used in the two studies.

The ten adult foreguts in this study, measured from the epipharynx to the posterior end of the proventricular valve, averaged 1.73 mm in length and the ten late instars foreguts averaged 2.36 mm (Table 1). No significant differences in the length of the foregut between male and female, nor between adult and late instar, were found. In comparison, foregut measurements from the mouth to the proventricular valve previously yielded mean lengths of 1.25 mm in adults and 1.5 mm in larvae (McAllister et al. [13]). However, measurements by McAllister et al. [13] were made using a dissecting scope and a calibrated ocular micrometer which may have affected precision.

The midgut was demarcated from the distal end of proventricular valve to the distal end of pyloric valve and measured 9.98 mm in larvae and 9.00 mm adults after distension.

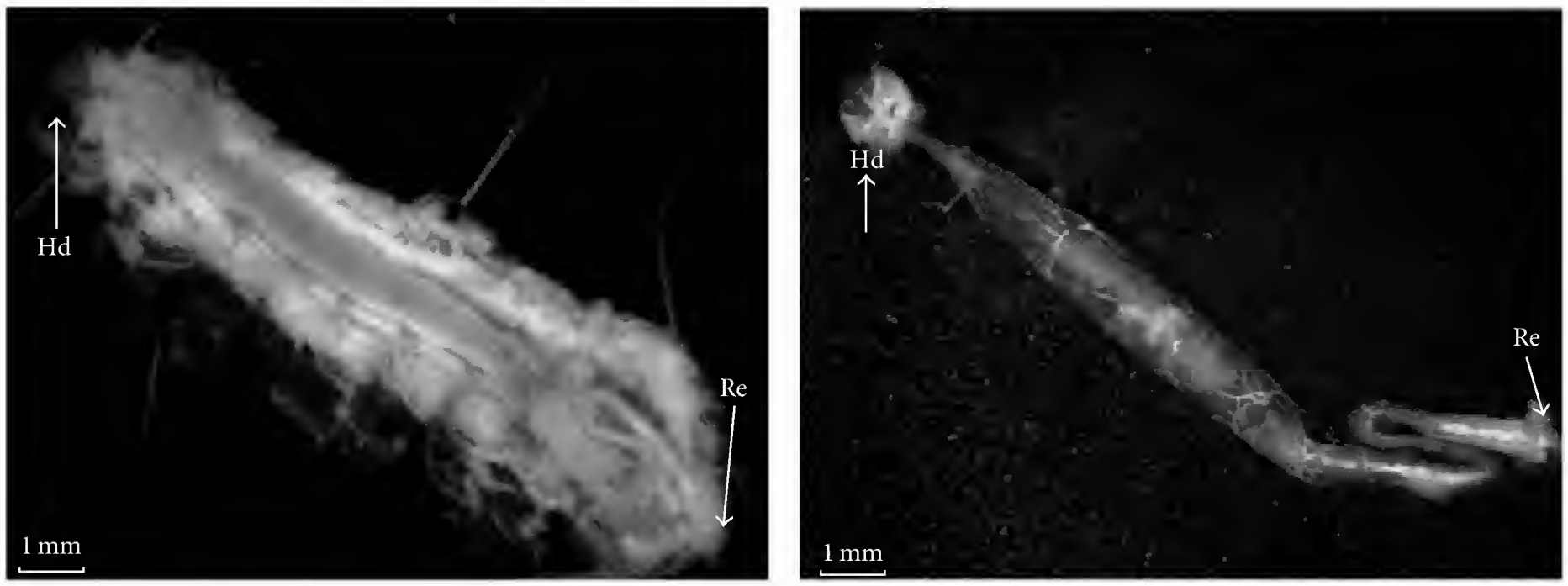

FIGURE 1: Alimentary canals for larval and adult *Alphitobius diaperinus*: ((a) and (b)), larval canal *in situ* and extracted, respectively; ((c) and (d)) adult canal *in situ* and extracted, respectively. Hd: head; Re: rectum.

TABLE 1: Mean length (mm) and standard deviation (SD) of alimentary canal of the *Alphitobius diaperinus* female and male beetles (>4 weeks post eclosion) and the late instars (7–9th). Measured by microscopy.

	Foregut		Midgut		Small intestine		Large intestine		Rectum		Total alimentary canal	
	Length	SD	Length	SD	Length	SD	Length	SD	Length	SD	Length	SD
Adult female	1.70	±0.30[a]	9.22	±0.71[a,b]	3.24	±0.35[a]	2.30	±0.18[a]	2.29	±0.32[a]	18.74	±1.11[a]
Adult male	1.75	±0.12[a]	8.77	±1.89[a]	3.14	±0.40[a]	2.43	±0.32[a]	1.26	±0.19[a,b]	17.35	±1.83[a]
Late instar	2.36	±0.38[a]	9.98	±1.94[b]	3.50	±0.46[a]	2.14	±0.50[a]	0.64	±0.10[b]	18.61	±2.30[a]
Adult*	1.73	±0.22	9.00	±1.37	3.19	±0.36	2.36	±0.25	1.78	±597.5	18.04	±1.61

* Mean compilation of adult male and adult female measurements.
[a-b]Sample groups (late instar, female, and male) with the same letter are not significantly different ($P < 0.05$) as compared within the anatomical segment of the gut (nonparametric 2-way ANOVA with Bonferroni posttests).

A significant difference was found in the length of the midgut between the male, which was shorter than that of the late instar. In comparison, McAllister et al. [13] defined the midgut as extending from the proventricular valve and terminating at the pyloric valve, measuring 7.5 mm in larvae and 4.1 mm adults.

The hindgut is divided into a small and large intestine. The small intestine was demarcated from the distal end of pyloric valve extending to the enlargement of the canal, signifying the start of large intestine, and measured 3.50 mm in larvae and 3.19 mm adults. The large intestine was demarcated from the enlargement of intestine to the origination of

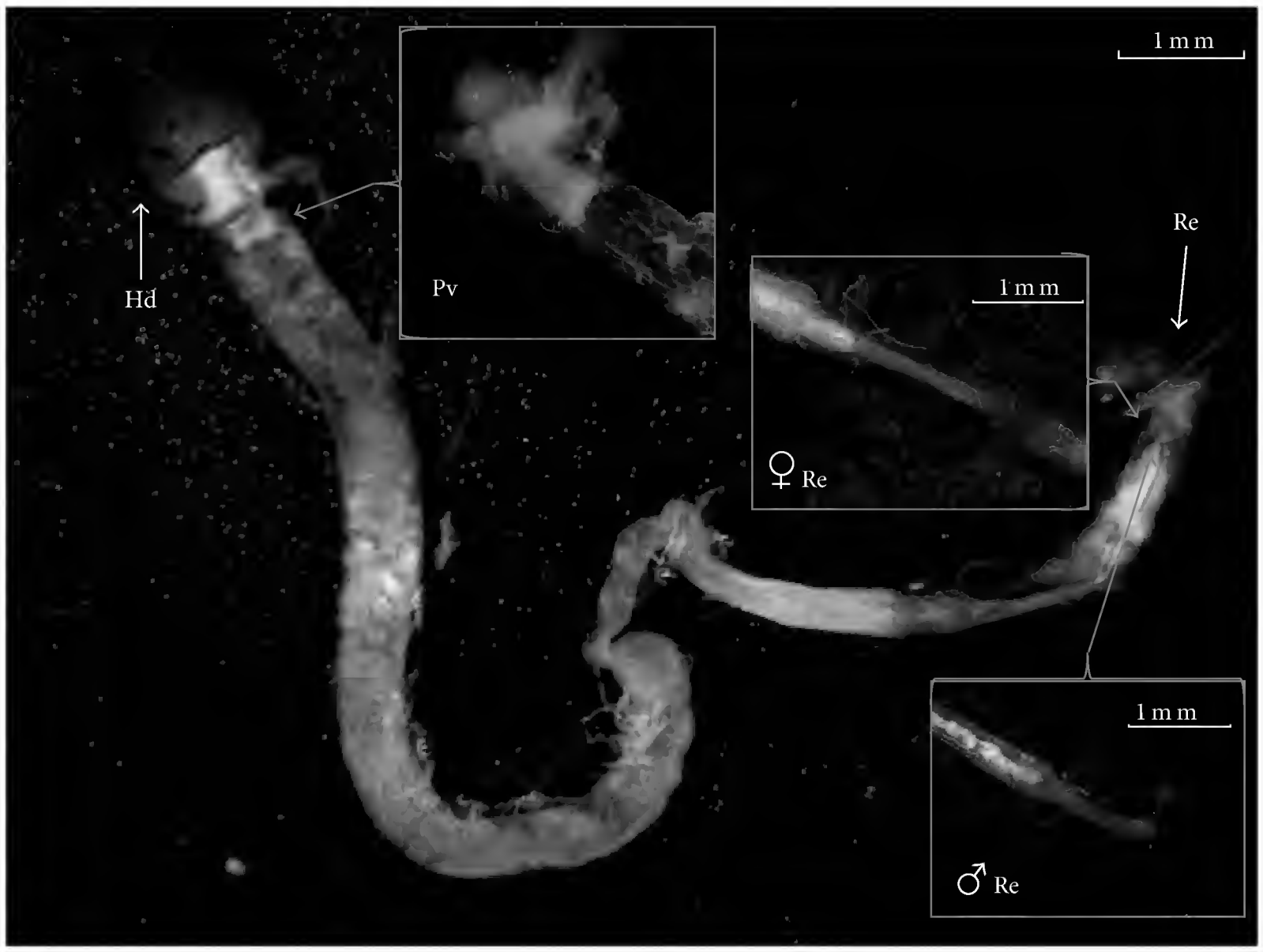

FIGURE 2: Distended alimentary canal of adult *Alphitobius diaperinus* canal; yellow lines indicate area of expanded detail for Pv: proventricular valve, male and female rectal areas. Hd: head; Re: rectum.

the rectum and measured 2.14 mm in larvae and 2.36 mm adults. No significant differences were found in the length of the hindgut between male and female, nor between the adult and late instar. In comparison, McAllister et al. [13] defined the anatomy in the larvae of the small intestine as a straight tube which begins near the pyloric valve and extends anteriorly to the posterior margin of the fifth abdominal segment for a total length of 2.0 mm. At that point, it reverses direction and extends posteriorly as the large intestine for a length of 2.9 mm. In the adult, the small intestine was described as a single loop beginning from the pyloric valve and extending to the posterior margin of the third abdominal segment for a length of 3.5 mm. It then extends posteriorly as the large intestine for a length of 2.4 mm.

The rectum was also measured as demarcated from the posterior end of large intestine to the anus and measured 0.64 mm in larvae and 1.78 mm adults. The female rectum was significantly longer than that of the late instar. Neither the ovipositor of the female nor the aedeagus of the male was included in these measurements; their anatomy is discussed in a counterpart paper in this journal issue [21].

The largest observed discrepancy was in the length of the midgut. McAllister et al. [13] reported lengths of 7.5 and 4.1 mm for larvae and adults, respectively, while current results indicated lengths of 9.98 and 9.00 mm for larvae and adults, respectively. Methodology differences between studies may account for these differences. In the current study, the alimentary canal was distended to normal length, to ensure a straight line measurement. However, methodologies in McAllister et al. [13] did not clearly indicate measurement technique and suggests measurements of the alimentary canal as it lay *in situ*. Discrepancies in measurements may also be attributed to definitions of sections comprising the alimentary canal. In the current study, measurements involving the proventricular and pyloric valves reached to the distal side of the valve. In contrast, language in McAllister et al. [13] suggests that measurements were taken from the proximal side of the respective valves. Inclusion of the valves in the measurements would likely bring their estimates closer to those reported in the current study.

According to Dunford and Kaufman [22] the average length of an adult *A. diaperinus* is approximately 5.8 to 6.3 mm, therefore the fore-, mid-, and hindguts are more than 2.5 times the length of the insect. Barké and Davis [17] noted that average adult female ranged from 6.75 to 8.00 mm and male from 5.50 to 7.00 mm; however, the method of measurement collection was not presented. Rahman et al. [14] used a micrometer to measure characteristics of the adult and determined the foregut was "about" 2 mm in length and the hindgut (including the rectum) was 0.9 cm. The entire canal was reported to be 3 times the body length, 21 mm in the female (body length of 7 mm) and 19 mm in the male (body length of 5 mm). The average length of an adult beetle, in this study, was 7.01 mm, and the average

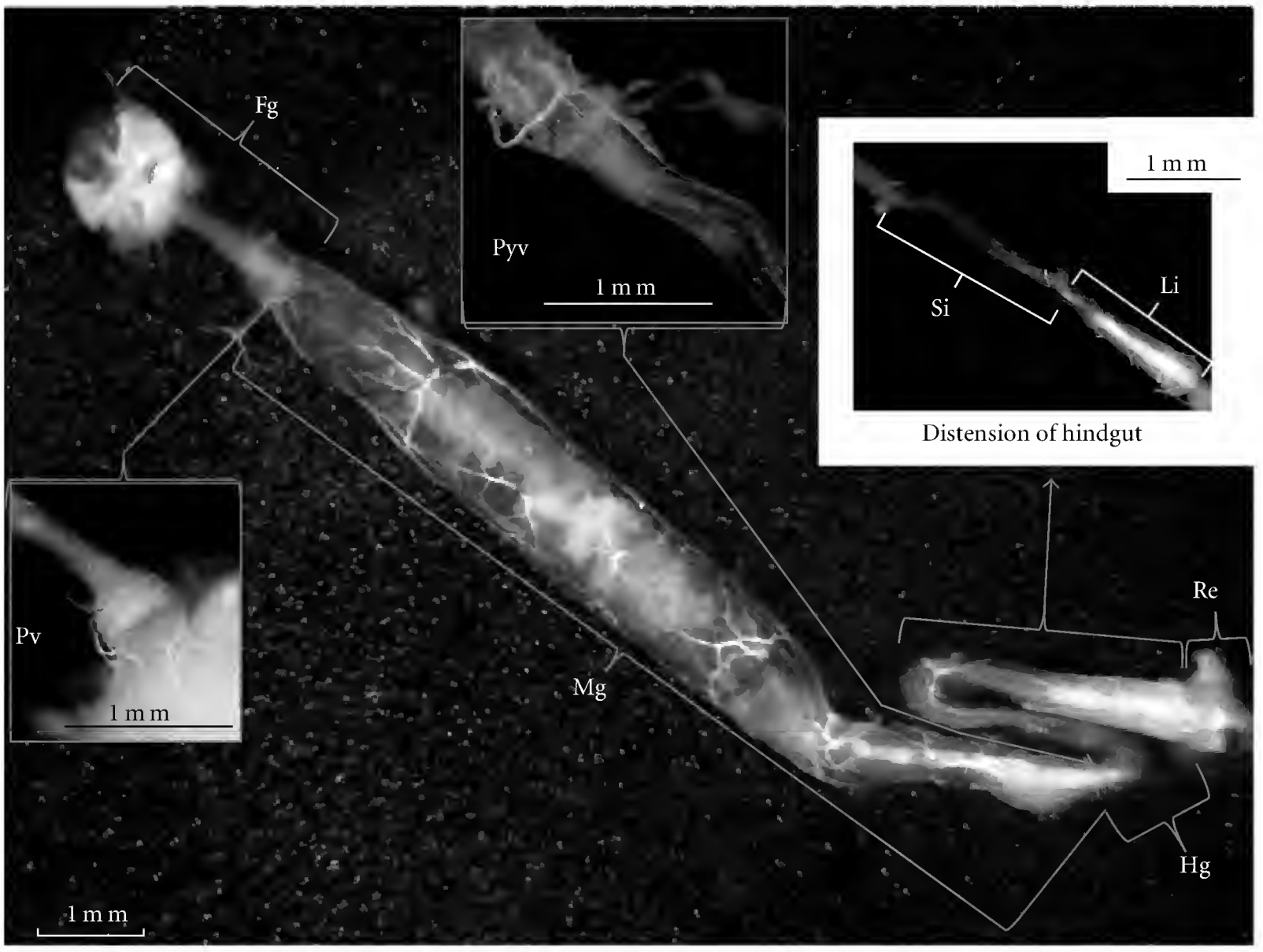

Figure 3: Components of larval *Alphitobius diaperinus* alimentary canal; yellow lines indicate area of expanded detail. Fg: foregut; Hg: hindgut; Li: large intestine; Mg: midgut; Pv: proventricular valve; Pyv: pyloric valve; Re: rectum; Si: small intestine.

Table 2: Mean length (mm) and standard deviation (SD) of segment and total body length of *Alphitobius diaperinus* beetles and total body length of late instars (7–9th). Measured by microscopy on the anteroposterior axis.

Segment	Female ($n = 10$)		Male ($n = 10$)		Late instars ($n = 10$)	
	Length	±SD	Length	±SD	Length	±SD
Head	1.094	±0.008[a]	1.165	±0.013[a]	—	
Thorax	1.355	±0.010[a]	1.382	±0.008[a]	—	
Abdomen	4.682	±0.030[b]	4.333	±0.020[b]	—	
Total	7.131	±0.041[c]	6.883	±0.031[c]	12.80	±1.169[d]

[a–d]Sample groups (late instar, female, and male) with the same letter are not significantly different ($P < 0.05$) (nonparametric 2-way ANOVA with Bonferroni posttests).

adult female ranged from 6.49 to 7.77 mm and male from 6.50 to 7.42 mm (Table 2). The mean alimentary canal length (foregut through rectum) was 2.6 times the length of the adult insect (Table 3).

The mean length of the late instars was 12.80 mm, ranging from 9.81 to 14.78 mm (Table 2), and the total alimentary length was 1.5 times the body length of the insect (Table 3). According to Dunford and Kaufman [22], the average length of a late instar was approximately 7 to 11 mm in length and the fore-, mid-, and hindguts were 1.6 to 2.5 times the length of the insect. Rahman et al. [15] determined larval alimentary canal length 1.5 times (21 mm) that of an 8th-instar body length (14 mm). They also stated that fore-, mid-, and hindgut measurements were 2, 12, and 7 mm, respectively; however the method of measurement collection was not presented. In addition, rectal lengths were included in the hindgut measurement.

4. Conclusions

A handful of studies have reported measurements of various parts of the alimentary canal of *A. diaperinus*. However, the exact method used for measurement and the anatomical structures used to define segment features were not always provided. No single study encompassed the scope of measurements on the same group of insects presented in this study. Advances in current technology also allowed more accurate visualization and precise measurement of gross anatomical features of the alimentary canal. These images and measurements provide additional perspective on the

Table 3: The length of the segments of the alimentary canal as percent of total alimentary canal length (ACL) and body length (BL) of the *Alphitobius diaperinus* female and male beetles (>4 weeks after eclosion) and the late instars (7–9th). Measured by microscopy.

	Foregut		Midgut		Small intestine		Large intestine		Rectum		Total alimentary canal
	ACL	BL	ACL	BL	ACL	BL	ACL	BL	ACL	BL	BL
Adult female	9.0	23.8	49.2	129.1	17.3	17.3	12.2	32.1	12.2	32.1	262.5
Adult male	10.1	25.5	50.6	127.4	18.1	18.1	14.0	35.2	7.3	18.3	252.0
Late instar	12.7	18.4	53.6	77.9	18.8	27.4	11.5	16.7	3.4	5.0	145.4
Adult*	9.6	24.6	49.9	128.3	17.7	45.5	13.1	33.6	9.9	25.4	257.1

* Compilation of adult male and adult female measurements.

pathogen reservoir potential of *A. diaperinus* and the magnitude of potential disease agents which could be harbored.

Acknowledgments

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Review Article

Sex Pheromones of *Stenotus rubrovittatus* and *Trigonotylus caelestialium*, Two Mirid Bugs Causing Pecky Rice, and Their Application to Insect Monitoring in Japan

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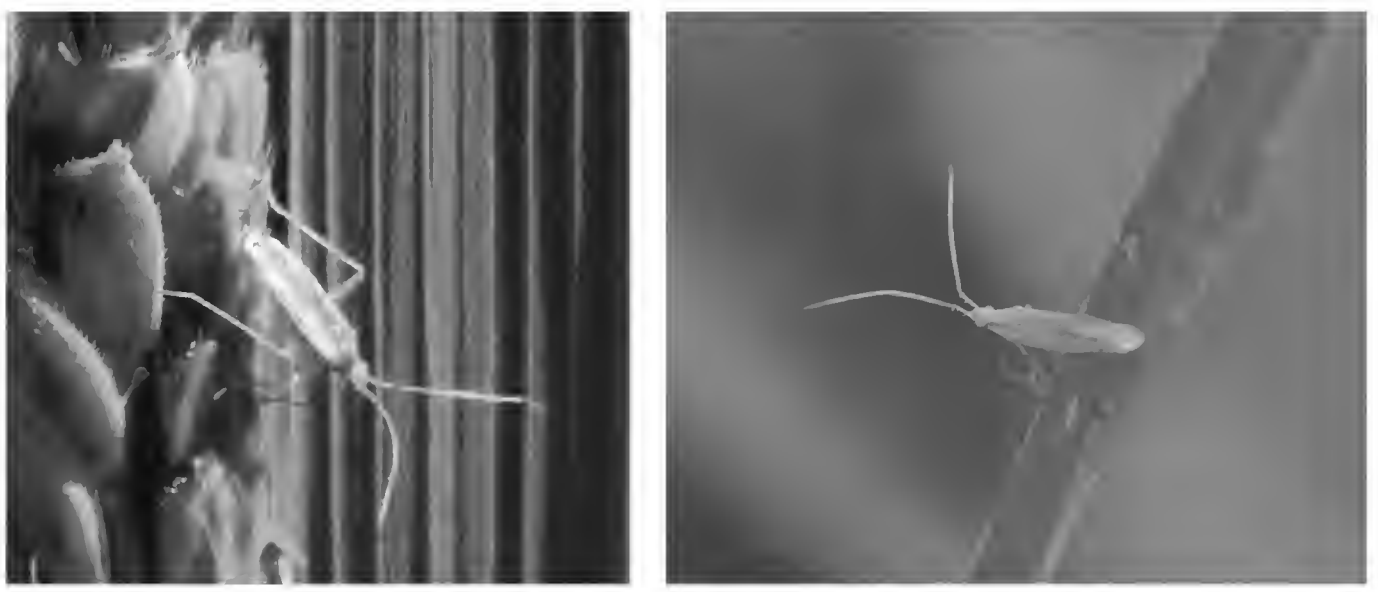
Two mirid bugs, *Stenotus rubrovittatus* and *Trigonotylus caelestialium* (Heteroptera: Miridae), are important pests that infest rice crops in many regions of Japan. Males of each species were attracted to traps baited with conspecific, unmated females. Hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal were identified as possible female-produced sex pheromone components for *S. rubrovittatus*, whereas hexyl hexanoate, (*E*)-2-hexenyl hexanoate, and octyl butyrate were found to be sex pheromone components for *T. caelestialium*. Pheromone doses and ratios were optimized for attraction of males of each species. Sticky traps set up close to or below the top of the plant canopy were optimal for monitoring these species, and trap catches were almost constant when traps were placed 7 or more meters in from the edge of a paddy field. Mixed lures, in which the six compounds from both species were loaded onto a single septum, or separate lures for each species, deployed in a single trap, were equally effective for monitoring both species simultaneously.

1. Introduction

The sorghum plant bug *Stenotus rubrovittatus* (Matsumura) (Figure 1(a)) and the rice leaf bug *Trigonotylus caelestialium* (Kirkaldy) (Heteroptera: Miridae) (Figure 1(b)) are major pests of rice, *Oryza sativa* L., in Japan [1]. They reproduce on graminaceous plants and invade rice fields after rice plant heading. Damage from bug feeding causes stained grains or kernel spotting, known as pecky rice (Figure 2) [2, 3]. Pecky rice contamination in brown rice, even in very small amounts (more than one stained grain per 1,000 brown rice grains), reduces rice quality under the Japanese rice quality regulations. This reduction in rice quality resulted in a price reduction to farmers of 8–16% in 2010. Damage due to heteropteran bugs has occurred in 30% of rice cultivation areas in Japan since 1999, and the total area of rice fields requiring pecky rice control currently amounts to 1,700,000–1,900,000 ha [1]. The range of *S. rubrovittatus* in Japan has been spreading since the 1990s [4], and this bug is now distributed from the southern part of Hokkaido to Kyushu [1]. *Trigonotylus caelestialium* is found in most parts of eastern Japan, but its damage to rice occurs mainly in the northern part of Japan.

Sweeping of vegetation with an insect net is one of the conventional methods of surveying for insect pests in rice. However, this is a time- and labor-intensive method and requires some knowledge and experience to determine the types of insects captured. In contrast, pheromone-baited traps are easy to use and can provide similar data on seasonal population dynamics and densities of specific species. Species-specific pheromone traps also eliminate the need for specialized training to detect and identify the target insects.

Attractant pheromones have now been identified for three major true bug species that cause pecky rice in Japan: *Leptocorisa chinensis* Dallas (Heteroptera: Alydidae) [5] and the two mirid bugs which are the subject of this paper. Here, we summarize the identification of the sex pheromones for these two mirid species, and the testing of their pheromones for insect monitoring.



(a) Sorghum plant bug *Stenotus rubrovittatus* (b) Rice leaf bug *Trigonotylus caelestialium*

FIGURE 1: Sorghum plant bug *Stenotus rubrovittatus* (Matsumura) (a) and rice leaf bug *Trigonotylus caelestialium* (Kirkaldy) (b) (Heteroptera: Miridae).

2. Sex Pheromones of the Mirid Bugs, *Stenotus rubrovittatus*, and *Trigonotylus caelestialium*

2.1. Mate Attraction and Mating Behavior. In the true bug family Miridae, orientation of males to conspecific females has been observed in several species [6–12], including *T. caelestialium* [13] and *S. rubrovittatus* [14]. In *T. caelestialium* and *S. rubrovittatus*, males were attracted only to conspecific females, and females were not attracted by either sex [13, 14]. These results indicated that females of these species probably produced female-specific sex attractant pheromones.

In *S. rubrovittatus*, courtship behavior by males consists of four steps: approaching a female, antennation (touching with the antennae), grasping (holding with the antennae), and mounting [15]. Male *T. caelestialium* exhibits similar behavioral steps, except for antennation [13]. In both species, calling behavior in females, as observed in another mirid bug, *Campylomma verbasci* [8], was not observed [13, 15].

2.2. Identification of Sex Pheromone Components

2.2.1. Stenotus rubrovittatus. Whole-body extracts of *S. rubrovittatus* females were attractive to conspecific males [16], and 16 peaks were detected from hexane extracts of whole female bodies by coupled gas chromatography-mass spectrometry (GC-MS) analysis (Table 1) [16]. The three most abundant components elicited responses from antennae of male bugs in gas chromatography-electroantennographic detection (GC-EAD) analyses (Figure 3) [17]. These three compounds were identified as hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal (Figure 4(a)). When the attractiveness of a 100 : 40 : 200 (μg) combination of hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal and subsets thereof were examined, a few males were attracted to the binary blend of hexyl butyrate and (*E*)-2-hexenyl butyrate, and no males were attracted to lures lacking either hexyl butyrate or (*E*)-2-hexenlyl butyrate [18]. Significantly more

FIGURE 2: Damaged (pecky rice; top row) and nondamaged (bottom row) brown rice grains.

S. rubrovittatus males were caught in traps baited with the three-component blend ($P < 0.05$) than in unbaited controls [18]. Extracts of female *S. rubrovittatus* contained at least 13 minor components, but lures impregnated with female extracts were no more attractive to males than the three-component blend [16], and none of the minor components enhanced the attractiveness of the lure when added individually to the three-component blend. These results suggest that the minor components, in the amounts found in the extracts of females, are not part of the sex attractant pheromone [16].

2.2.2. Trigonotylus caelestialium. Whole-body hexane extracts of *T. caelestialium* females were not attractive to conspecific males [19], even though live females had been shown to attract males. This suggested that attraction of males to extracts was being masked by other components of the extracts. Thus, extracts were fractionated by liquid chromatography on Florisil, successively eluting with hexane and 5%, 15%, 25%, and 50% ether in hexane. Ten components in the 5% ether in hexane fraction elicited responses from antennae of conspecific males in GC-EAD analyses [20].

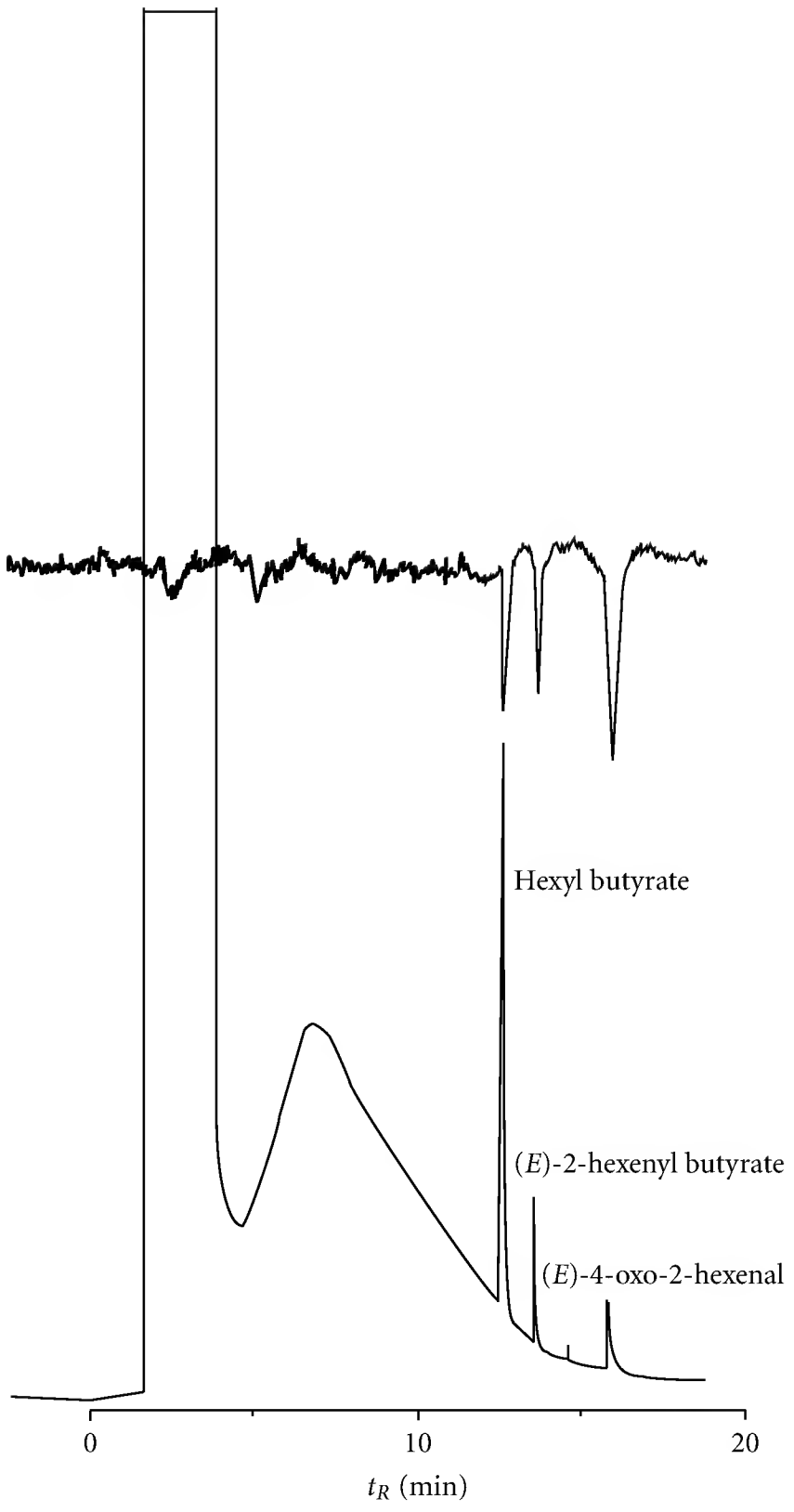


Figure 3: Coupled gas chromatography-electroantennographic detection (GC-EAD) chromatograms showing the responses from an antenna of a male *Stenotus rubrovittatus* (top trace) to a crude extract of unmated females.

Partial reconstruction of the mixture of EAD-active compounds determined that a six-component mixture of 5 μg hexyl hexanoate, 2.5 μg (*E*)-2-hexenyl hexanoate, 5 ng hexyl (*E*)-2-hexenoate, 150 ng octyl butyrate, 275 ng octyl hexanoate, and 275 ng (*E*)-2-octenyl hexanoate attracted males, whereas lures lacking either hexyl hexanoate or (*E*)-2-hexenyl hexanoate were not attractive [20]. Furthermore, a two-component blend of hexyl hexanoate and (*E*)-2-hexenyl hexanoate (Figure 4(b)) was attractive to males, and adding octyl butyrate (Figure 4(b)) enhanced the attraction [20].

2.3. Chemicals. The components identified as possible sex attractant pheromones of *S. rubrovittatus* [18] and *T. caelestialium* [20] are all commercially available in high purity, with the exception of (*E*)-4-oxo-2-hexenal. (*E*)-4-Oxo-2-hexenal was obtained readily in one step from commercially

Table 1: Compounds identified in extracts of female *Stenotus rubrovittatus* [16].

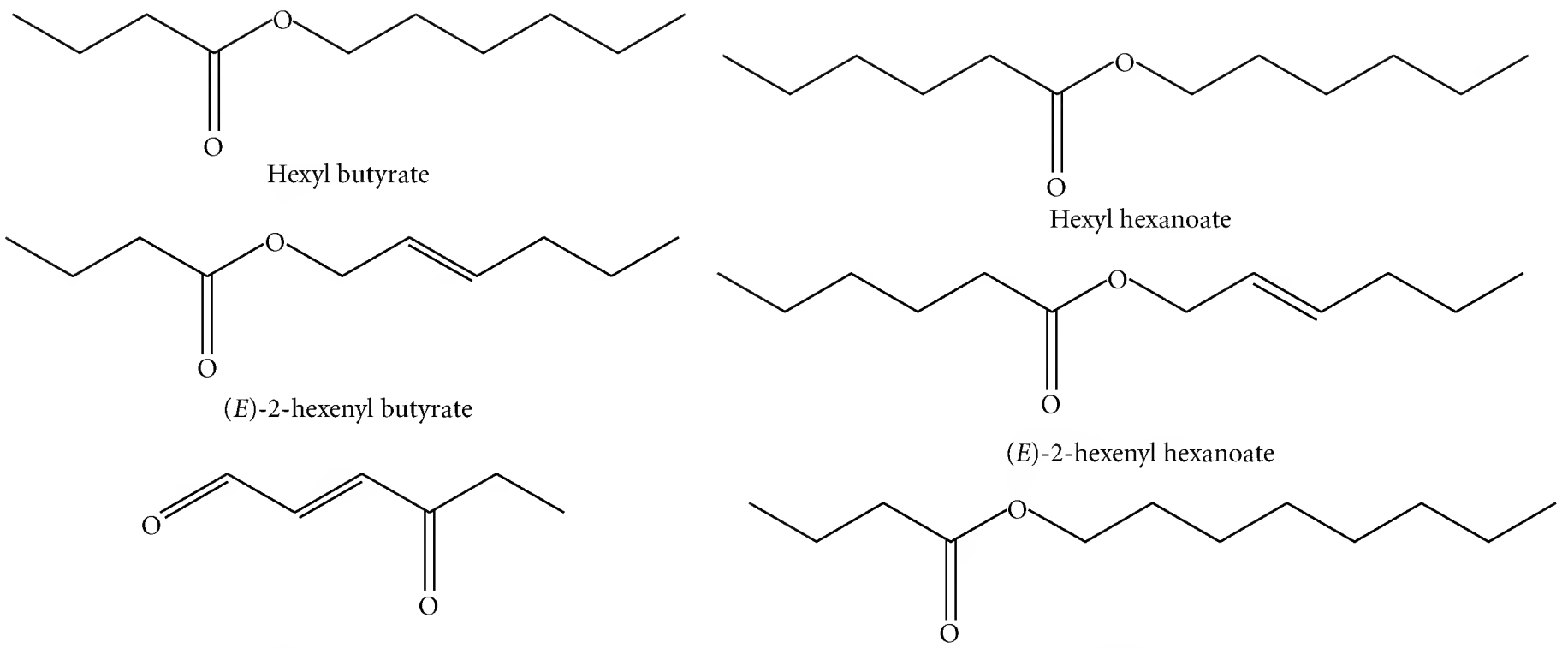
Compounds	$KI^{a}_{HPINNOWax}$	KI^{a}_{HP1}	Relative amount (%)[b]
Hexyl acetate	1,276	996	0.8
Pentyl butyrate	1,320	1,076	0.5
(*E*)-2-Hexenyl acetate	1,337	995	0.2
Hexyl propionate	1,342	1,091	0.2
4-Methylpentyl butyrate	1,374	1,142	0.8
Hexyl butyrate	1,417	1,192	100
Hexyl isopentanoate	1,447	1,228	0.2
(*E*)-2-Hexenyl butyrate	1,475	1,195	46.2
(*Z*)-3-Hexenyl butyrate	1,466	1,146	0.5
Hexyl pentanoate	1,516	1,274	0.2
Heptyl butyrate	1,520	1,276	0.1
Hexyl (*E*)-2-butenoate	1,562	1,224	0.1
(*E*)-4-Oxo-2-hexenal	1,599	958	5.4
Hexyl hexanoate	1,613	1,370	0.1
Octyl butyrate	1,620	1,374	0.1
Methyl tetradecanoate	2,014	1,684	2.0

[a] Kováts retention index [22] using HP-INNOWax ($KI_{HPINNOWax}$) and HP-1 (KI_{HP1}) columns.
[b] Values are percentages relative to the amount of hexyl butyrate.

available 2-ethylfuran [21], in high chemical purity (96.9% pure, as custom synthesized by Shin-Etsu Chemical Co., Ltd.). Although this compound is unstable in impure form, in our hands, pure (*E*)-4-oxo-2-hexenal was relatively stable in a freezer and could be used in a mixture of synthetic pheromones without further purification.

2.4. Lures. The rubber septa which are often used as pheromone dispensers for lepidopteran insects are not the most suitable pheromone dispensers for pheromones of some mirid bugs because most of the volatile pheromone components for mirid bugs are of relatively low molecular weight, and the components evaporate from septa too quickly. For example, for *Phytocoris relativus* and *Phytocoris californicus*, rubber septum lures that had been exposed in the field for 2 weeks were significantly less attractive than fresh lures [23, 24]. For *Lygus rugulipennis*, the effective lifetime of a rubber septum lure loaded with the same pheromone compounds as those of *S. rubrovittatus* was only a few hours, whereas polyethylene vials or sachets were found to give sustained release for at least 2 weeks [25]. Nevertheless, because rubber septa are easy to work with, they can be used as dispensers for short-term experiments testing variables such as pheromone blend ratio.

Experiments were carried out with both *S. rubrovittatus* [16, 18] and *T. caelestialium* [20, 26] to determine the optimal doses and ratios of pheromone compounds required for male attraction. For *S. rubrovittatus*, using a rubber septum pheromone dispenser, the release rate of (*E*)-4-oxo-2-hexenal from the lure was much faster than that of the butyrates [27]. Owing to the loss of significant quantities of



(a) Sorghum plant bug *Stenotus rubrovittatus* (b) Rice leaf bug *Trigonotylus caelestialium*

FIGURE 4: Structures of the pheromone components of *Stenotus rubrovittatus* (a) and *Trigonotylus caelestialium* (b).

(*E*)-4-oxo-2-hexenal during impregnation into the septum, the amount of (*E*)-4-oxo-2-hexenal released from the septum appeared to be substantially less than the amount that was loaded onto the septum. Thus, the ternary mixture formulated with the ratio found in the female extract (a 5 : 2 : 0.5 blend of hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal) was not attractive in preliminary bioassays, but ratios containing higher proportions of (*E*)-4-oxo-2-hexenal than found in the female extracts were found to attract males in subsequent bioassays [16]. As a result, a 5 : 1 : 10 blend of hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal at a total dose of 64 μg per septum was found to be most effective for attraction of males [16]. Analyses of the volatiles released from septa loaded with this blend showed that the ratio of compounds released from the lure was approximately 5 : 1 : 0.3, substantially different than the 5 : 1 : 10 loading rate.

Innocenzi et al. [27] observed that the release rate of (*E*)-4-oxo-2-hexenal drastically decreased when this compound was mixed with hexyl butyrate. They suggested that this phenomenon might be caused by chemical interaction between hexyl butyrate and (*E*)-4-oxo-2-hexenal, so they suggested that the butyrates and (*E*)-4-oxo-2-hexenal should be applied separately [25]. However, an experiment with *S. rubrovittatus* comparing catches in traps baited with lures containing the three-component blend versus catches in traps baited with a lure loaded with the two butyrates and a separate lure loaded with (*E*)-4-oxo-2-hexenal revealed that mixing the butyrates and (*E*)-4-oxo-2-hexenal made no difference [18]. Although the reason for this discrepancy between experiments is not clear, it may have been influenced by the purity of the (*E*)-4-oxo-2-hexenal used in the two experiments.

Extracts from female *T. caelestialium* were found to contain hexyl hexanoate, (*E*)-2-hexenyl hexanoate, and octyl butyrate in a ratio of 1000 : 414–491 : 5–11 [20]. Lures loaded with a 100 : 40 : 3 ratio of hexyl hexanoate, (*E*)-2-hexenyl hexanoate, and octyl butyrate at 4.29–14.3 μg per glass capillary tube (5 μL, 0.021 mm ID, 125 mm long) [20] or 10 μg per rubber septum (gray sleeve stopper, 8 mm outside diameter) [26] were most effective for attraction of male *T. caelestialium*.

The effective lifetime of rubber septum lures for these two species (*S. rubrovittatus*, 14 d [16]; *T. caelestialium* 30 d [26]) was generally shorter than those of the rubber septum lures used for many Lepidoptera. Experiments with alternate dispensers are in progress, with the aim of developing lures with longer effective field lifetimes.

3. Application of Synthetic Pheromone Lures for Monitoring Mirid Bug Populations

As a possible alternative to sweep-net sampling of vegetation in and around paddy fields, we have been investigating the potential for using pheromone-baited traps for monitoring *S. rubrovittatus* and *T. caelestialium*. As expected, we found that the pheromone traps attracted only conspecific males, and not females or nymphs, nor did they attract significant numbers of nontarget insects.

3.1. Trap Design. Two types of pheromone traps, a water-pan trap and a double-sided sticky trap (Figure 5), were tested for capturing both mirid species. Possible effects of trap color have not yet been examined. The water pan trap consisted of a plastic pan (~40 cm diam × 12 cm deep) filled with water, with a small amount of surfactant added to prevent trapped bugs from escaping. The lure was hung above the water on a wire frame. Double-sided sticky traps made up of two sticky boards (24 cm × 30 cm) were hung vertically, with the lure placed at the top [28].

(a) Water pan trap (b) Double-sided sticky trap

FIGURE 5: Typical water pan trap (a) and double-sided sticky trap (b) tested for catching mirid bugs.

For *S. rubrovittatus*, double-sided sticky traps were more effective in capturing males than water pan traps. Sticky traps caught an average of 2.1 ± 0.4 (±SE; $n = 12$) males per trap over 4 days, whereas significantly fewer males (0.8 ± 0.3, $n = 12$; t-test, $P < 0.01$) were captured in water-pan traps during the same time period.

For *T. caelestialium*, there was no significant difference in the effectiveness of the water pan or sticky traps [29, 30]. Water pan traps are cheaper than sticky board traps, but this cost saving is negated by the need to replenish the water frequently, especially in hot and/or dry areas. Thus, for practical use, sticky traps may be more suitable for monitoring both species than water pan traps. However, to be most effective, sticky board traps need to be replaced weekly. In field experiments with *S. rubrovittatus*, significantly more (2.4 ± 0.9; $n = 12$) males per trap were caught on new sticky traps than on sticky traps kept outdoors for 1 week before the experiments (0.3 ± 0.2 males; t-test, $P = 0.036$).

3.2. Optimizing Trap Location. Sticky traps placed below or near the top of the plant canopy were more effective than traps placed 30 cm above the canopy, for both *T. caelestialium* [28] and *S. rubrovittatus*. However, traps set below the canopy picked up a large amount of leaf litter and other detritus, rendering them less effective and more difficult to count. The effects of trap height have also been evaluated with the mullein bug *C. verbasci* in apple orchards [31], where it was found that more males were captured with traps higher (at 2.5-m) than lower (1.5-m) in the canopy.

The effect of trap position within a paddy field was tested by placing traps 0, 3, 7, 15, or 25 m in from the edge of the field (85 m long × 55 m wide). More males were captured in traps placed 3 m in from the edge of the field than in traps placed at the edge of the field. When the traps were set at or over 7 m in from the edge, the numbers of captured males were almost constant regardless of the distance from the edge [32]. Therefore, we recommend that monitoring traps be placed 7 or more meters in from the edge of a field.

3.3. Application for Monitoring the Seasonal Numbers of Bugs. We directly compared the effectiveness of pheromone trapping versus sweep-net sampling for monitoring mirid bug populations. Thus, insects were sampled with a 36-cm diameter sweep net, using 40 sweeps around a trap. For *T. caelestialium*, the number of adults captured by sweep-net sampling at 5-day intervals throughout the season increased from the middle of June to early or mid-July, then decreased, and increased again at the heading time of each rice variety. The seasonal trend of males caught in a trap set at the center of a paddy field (27 m long × 13 m wide) and the trend in the numbers of males captured by sweep-net sampling were similar (Figure 6) [28].

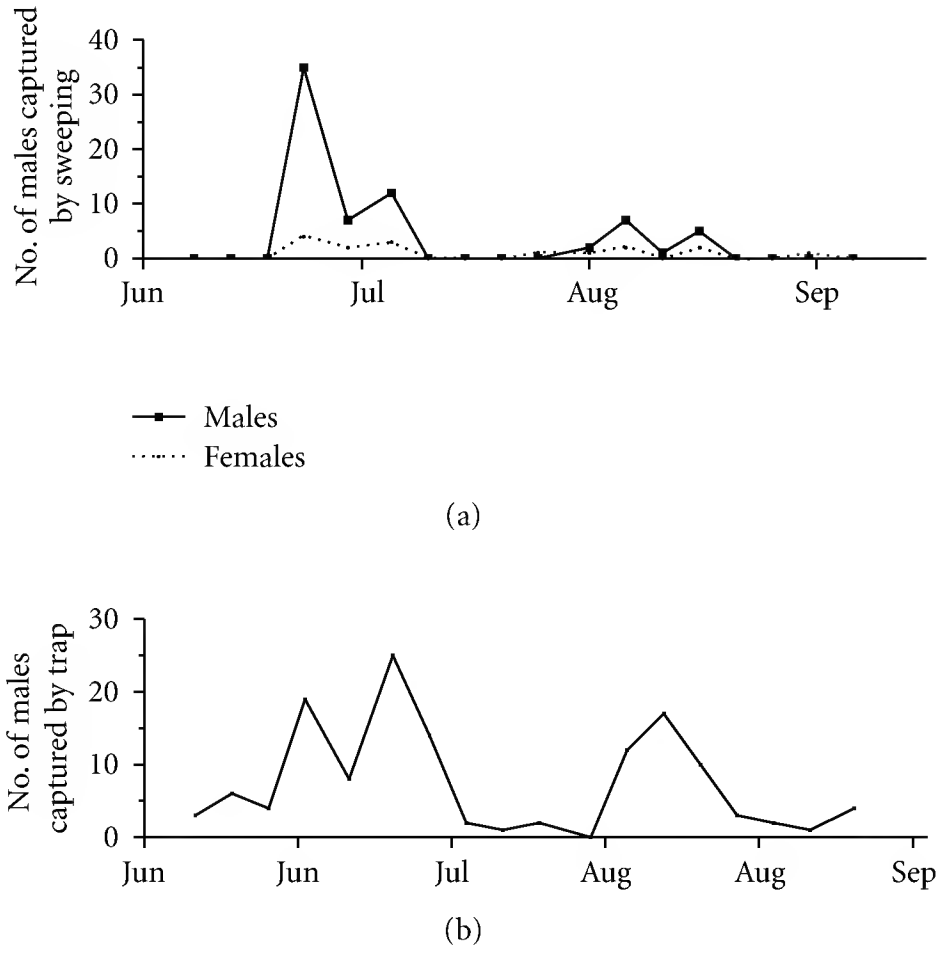


FIGURE 6: Comparison of the numbers of *Trigonotylus caelestialium* males caught by sweep-net sampling (a) or by a pheromone-baited sticky trap placed in the center of the paddy field (b). Sweep-net samples were taken 18 times at 5 d intervals. Data were modified from [28].

For *S. rubrovittatus*, catches in a trap set 10 m in from the edge of a paddy field (110 m long × 70 m wide) and

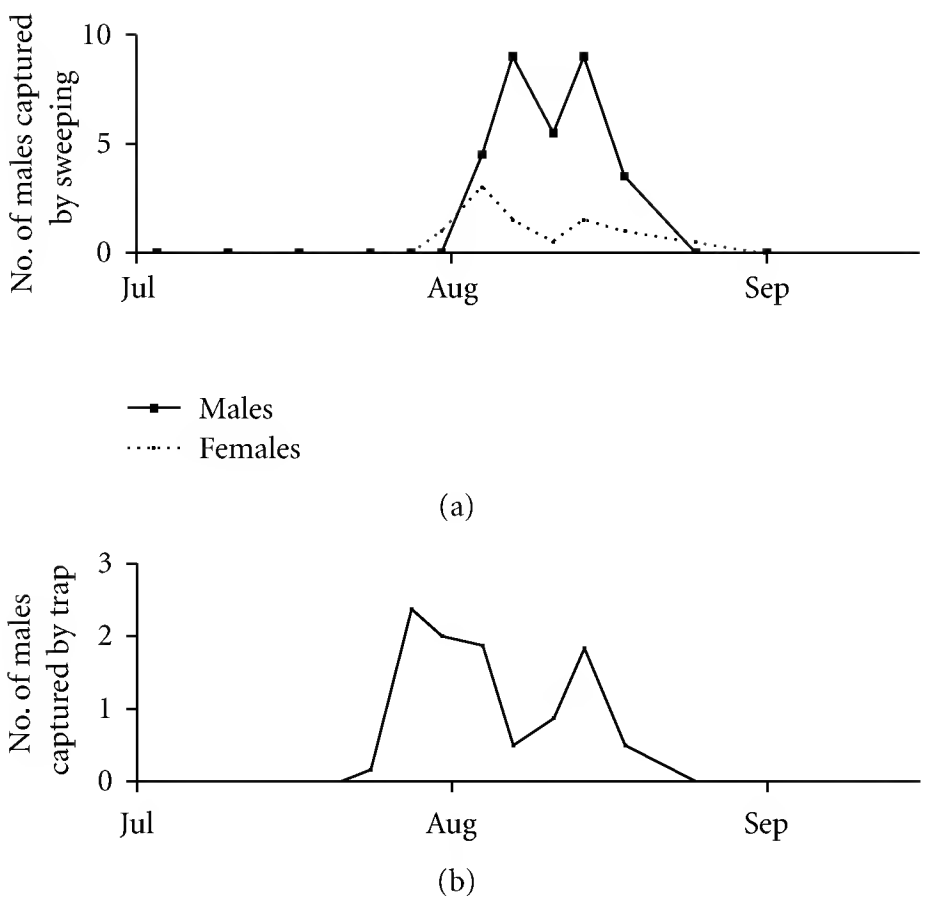


FIGURE 7: Comparison of the numbers of *Stenotus rubrovittatus* males caught by sweep-net sampling (a) or by a pheromone-baited sticky trap placed in the center of the paddy field (b). Sweep-net samples were taken 13 times at 7 d intervals. Data were modified from [32].

weekly sweep-net samples also showed similar seasonal patterns, with bugs being caught by both methods from about mid-July through late August (Figure 7) [32]. These results suggest that pheromone traps can be used as a tool for monitoring the seasonal population trends of these two mirid bugs in paddy fields.

Stenotus rubrovittatus and *T. caelestialium* are sympatric pests that infest rice crops in many regions of Japan. Synthetic pheromone lures for *S. rubrovittatus* do not attract *T. caelestialium* and vice versa. However, baiting traps with two separate lures (one for each species; combination lures) proved to be as effective as deploying separate traps for each species [33]. Even better, a mixed lure in which all six synthetic pheromone components (three from each species, see above) were loaded onto a single septum was as attractive to males of both species as the separate lures for each species, indicating no inhibition of attraction by any of the mixed pheromone components. These results suggest that mixed lures or combination lures can be used to monitor both species simultaneously, with a single trap. Furthermore, if necessary, *S. rubrovittatus* and *T. caelestialium* can be easily distinguished from each other by the color of the body and hind legs (Figure 1), even when stuck on sticky traps.

4. Chemical Ecology of Mirid Bugs

4.1. Acquisition of Pheromone Components. Crude pheromone extracts of mirid bugs can be prepared easily by brief soaking of individuals in a solvent such as hexane. However, the amounts recovered may be quite small, and, of course, the bugs are killed by the extraction process. Thus, as an alternative, a method for sampling pheromone components from living organisms may be more useful for qualitative and/or quantitative analyses of insect-produced semiochemicals. Adsorbents such as Porapak Q, Tenax TA, or activated charcoal have been widely used for collection of volatiles from living organisms. In a recent innovation to this general method, magnetic stir bars coated with polydimethylsiloxane (Twister, Gerstel, Mülheim an der Ruhr, Germany; 1 mm film thickness × 10 mm length) have been used to adsorb headspace odors released by a variety of organisms [34]. The Twister was originally designed for solventless sample collection followed by thermal desorption and online analysis by GC or GC-MS. However, analytes also can be recovered from the Twister by extraction with small volumes of organic solvents. For *S. rubrovittatus*, pheromone components were collected on the Twister for 1 d, followed by extraction in hexane (1 mL) [35]. Any desired number of replicate samples can be collected simultaneously by simply setting up the appropriate number of sampling chambers, each with its own Twister. Patterns of pheromone release from live individuals can be determined simply by changing the Twister at any desired time interval.

4.2. Effect of Age and Mating Status of Females on Their Attractiveness. Males of *S. rubrovittatus* were more frequently attracted to young virgin females than to old virgin females and were rarely attracted to mated females [14], probably due to differences in the release rates of pheromone between the different classes of females. That is, mated females released less pheromone (~0.67 μg in total) than unmated females (~1.54 μg), and young unmated females released more pheromone (~1.48 μg) than older unmated females (~0.79 μg) [35].

Interestingly, the amounts of volatile compounds released by females and the levels of compounds extracted from whole bodies with solvents did not appear to be correlated. The amounts of pheromone extracted from unmated females (3-d old) totaled about 5 μg and decreased with age to about 0.2 μg extracted from 18-d old females [35]. In contrast, the amount of pheromone extracted from mated females remained constant after mating until 18 d (total about 6–8 μg) [35].

For *T. caelestialium*, there was no evidence for daily periodicity in male attraction to females or mating [36], whereas male *S. rubrovittatus* were most attracted to females at night and in the morning [37]. Mating behavior of *S. rubrovittatus* was observed at any time of day, and males courted females regardless of the time of day [37]. However, female mating receptivity was higher in the morning than in the afternoon [38]. For *S. rubrovittatus*, mating behavior was sometimes initiated even when attraction of males to females was not observed, suggesting that, over shorter ranges, other signals may mediate the initiation of copulation.

4.3. Pheromone-Based Control. The efficacy of pheromone-based mating disruption has been investigated with *C. verbasci* in Canada [31] and *T. caelestialium* in Japan [39]. Captures of male *C. verbasci* in pheromone-baited traps were

greatly reduced when fields were treated with the complete, two-component sex pheromone blend, but reductions in trap captures were not observed when fields were treated with only one component of the pheromone [31]. In this experiment, decrease in trap captures was correlated with increased densities of pheromone dispensers.

In pheromone-based mating disruption experiments with the rice leaf bug *T. caelestialium*, treatment of grassy fields with pheromone-reduced captures of male *T. caelestialium* in traps baited with pheromone lures or with virgin females, and lowered population levels of *T. caelestialium* [39]. The total numbers of adults captured in the treated fields were 0–45% of those in the untreated fields, and the total numbers of nymphs sampled in the treated fields were 0–2.2% of those in the untreated fields [39].

However, *T. caelestialium* and *S. rubrovittatus* are polyphagous, and their host plant range includes a variety of graminaceous plants. Therefore, mated females of these species can invade treated areas from outside, even if mating behavior in treated areas such as paddy fields has been suppressed. Pecky rice damage results from mirid bugs invading paddy fields during the period when the rice ears are sprouting. The nymphs and adults present at the middle to end of the grain-filling period are the offspring of the adults that invaded early in the grain-filling period [39]. Decreasing the number of nymphs and adults during the grain-filling period should minimize pecky rice damage, and so pheromone-based control measures must remain effective for the duration of this period (~2 months). Furthermore, pheromone treatments are expensive (~9,300 yen/ha for the compounds alone, at a rate of 60 g/ha) and must be reapplied approximately monthly [39]. Thus, under the conditions used in the present study, mating disruption of *T. caelestialium* is not economically feasible for preventing pecky rice damage. It remains to be determined whether larger-scale application of mating disruption, and the resulting economies of scale, might make it possible to develop mating disruption of *T. caelestialium* and *S. rubrovittatus* as cost-effective management tools.

5. Conclusion

Our results suggest that pheromone-baited traps for two mirid bugs, *S. rubrovittatus* and *T. caelestialium*, may be able to replace sweep-net sampling with monitoring seasonal population dynamics of these two important pests of rice, particularly as traps baited with mixed or combination lures can be used to sample both species simultaneously. Sticky traps set up near the top of the plant canopy, and 7 or more meters in from the edge of the paddy field were optimal for monitoring. For *T. caelestialium*, mating disruption experiments showed that although the pheromones interfered with male attraction to lures and to females and appeared to suppress populations, the costs of treatment and the relatively small decrease in damage to the rice crop suggest that mating disruption of this bug may not be a practical technique for preventing pecky rice damage.

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Research Article

Taxonomic Position of the Oriental Species of *Mesosa* (*Mesosa*) (Coleoptera, Cerambycidae, Lamiinae, Mesosini)

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Twelve oriental mesosine species which had been belonged to the nominotypical subgenus of the genus *Mesosa* Latreille, 1829, are transferred to the subgenus *Dissosira* Pascoe, 1865 of the genus *Agelasta* Newman, 1842, as follows: *A. (D.) perplexa* (Pascoe, 1858); *A. (D.) columba* (Pascoe, 1859); *A. (D.) rufa* (Breuning, 1935); *A. (D.) catenatoides* Yamasako and N. Ohbayashi, nom. nov. [replacement name for *A. (D.) laosensis* (Breuning, 1935) already occupied by Pic (1925)]; *A. (D.) gardneri* (Breuning, 1938); *A. (D.) nigropunctata* (Breuning, 1938); *A. (D.) konoi* (Hayashi, 1956); *A. (D.) yonaguni* (Hayashi, 1962); *A. (D.) nigrostictica* (Breuning, 1967); *A. (D.) siamana* (Breuning, 1974); *A. (D.) praelongipes* (Kusama and Irie, 1976); *A. (D.) kumei* (Takakuwa, 1991).

1. Introduction

The genus *Mesosa* was erected by Latreille [1] on the basis of two Palearctic species, *Cerambyx curculionoides* Linnaeus, 1761 and *Lamia nebulosa* Fabricius, 1781, of which the former was designated as the type species of the genus. Later, Breuning [2] divided the genus *Mesosa* into six subgenera as follows: *Mesosa* (*Mesosa*) Latreille 1829; *M.* (*Aphelocnemia* [sic]) Stephens, 1831; *M.* (*Saimia*) Pascoe, 1866; *M.* (*Anthlyboscila*) Thomson, 1868; *M.* (*Perimesosa*) Breuning, 1939; *M.* (*Metamesosa*) Breuning, 1939. Recently, Yamasako and Ohbayashi [3] erected a new subgenus, *M.* (*Lissomesosa*), and they also [4] synonymized *M.* (*Anthlyboscila*) with *Agelasta* (*Dissosira*) Pascoe, 1865. As a result, the genus *Mesosa* now consists of six subgenera and includes more than 80 species.

Among the subgenera, *Mesosa* (*Mesosa*) is the nominotypical subgenus and comprised of 19 species until now. However, as a result of our close examination of the external and male genital features, they are considered to be polyphyletic and could be separated into some species groups. Of those, one group, including the name-bearing type *M. curculionoides* (Linnaeus, 1761), is the true nominotypical species group and is distributed throughout the Palearctic region. On the other hand, the 12 oriental species are a different phyletic line from the Palearctic species group and are closely related to the subgenus *Dissosira* Pascoe, 1865, of the genus *Agelasta* Newman, 1842.

As we have already pointed out in a previous paper (Yamasako and Ohbayashi [4]), the hitherto distinguishable feature between *Mesosa* and *Agelasta*, the rounded or truncated prosternal process in lateral view, is not a suitable characteristic, and many species in the genus *Mesosa* have been confused with the genus *Agelasta* because of this problem. According to other external features and the endophallic structures, the 12 species distributed in the Oriental region should be transferred to *Agelasta* (*Dissosira*) in spite of their rounded prosternal process. Therefore, we herein propose them to be transferred from *Mesosa* (*Mesosa*) to *Agelasta* (*Dissosira*). This is the ninth part of our studies on the Asian Mesosini.

2. Materials and Methods

This study was conducted based on the dried specimens preserved in the following public collections, our private collections, and also the collections of friends.

BMNH: The Natural History Museum, London, UK.

EUMJ: Ehime University Museum, Matsuyama, Japan.

MNHN: Muséum National d'Histoire Naturelle, Paris, France.

ZSM: Zoologische Staatssammlung München, Munich, Germany.

The verbatim label data indicated by double quotation marks ("") are given for the type materials, and the line breaks of the label are indicated by a slash (/).

The observational method and the corresponding terms of endophallus should be referred to Yamasako and Ohbayashi [5].

3. Systematics

Agelasta (Dissosira) Pascoe, 1865

Type species. *Agelasta catenata* Pascoe, 1862 (Figures 1(d)–1(f), 2(c)-2(d), 3(a)-3(b), 6(a), 7(a)–7(d), and 9(a)).

Dissosira Pascoe, 1865. 124, note [6].

Chaeromorpha (*Dissosira*): Aurivillius, 1922: 145 [7].

Agelasta (*Dissosira*): Breuning, 1939: 482 [2].

Anthriboscyla Thomson, 1868. 165 [8]; type species: *Anthriboscyla mima* Thomson, 1868.

Mesosa (*Anthriboscyla*): Breuning, 1939: 411 [2].

Pseudaemocia Breuning, 1935. 269 [9]; type species: *Pseudaemocia rufa* Breuning, 1935 syn. nov.

Mutatocoptops (*Pseudaemocia*): Breuning, 1939: 506 [2].

Redescription (Modified the Diagnosis of Yamasako and Ohbayashi [4]). Body ovoid in shape; eyes subdivided; lower lobes relatively large, slightly wider than long. Antennal tubercles hardly elevated. Antennae well long and thick; each segment without apical spine, fringed beneath by suberect short setae; scape well long, slightly thickened apically, with a well-developed cicatrix on the apex; third segment distinctly longer than scape and fourth, respectively. Pronotum wider than long, with some indistinct discal tubercles; each side near apical margin usually with a small dull projection. Prosternal process with extremity usually well swollen posteroventrally, almost truncate in lateral view, but sometimes hardly swollen and more or less roundly sloped in lateral view. Mesosternal process with a well-developed tubercle on the center near the apex and roundly projected anteroventrally, almost truncate in lateral view.

Elytra without basal high bosses and lacking long suberect hairs. Legs with mesotibiae without distal notch on anterior side.

Endophallus. Endophallus well long and slender, approximately three times as long as median lobe, divided into BPH, MPH, and APH, with three kind spicule like sclerites as MSp, LSp, and SSp. BPH nearly 0.2 times as long as endophallus. MPH nearly 0.7 times as long as endophallus, subdivided into two membrane subdivisions as almost fused MT+CT and PB by a distinct constriction. APH well swollen and oval bursiform, nearly 0.2 times as long as endophallus, with ED on dorsal side, usually with AA which is lingulate shape and laid near ED, without AS.

MSp usually distributed in nearly apical half of MT+CT. LSp usually distributed in nearly basal half of MT+CT; dorsal side ones thick and short, arranged into two irregular longitudinal lines, unidentate in apical area, thence becoming thicker, denser, and multidentate toward basal area; ventral side ones rudimentary unidentate, and disappeared in some cases. SSp unidentate, short, and small, covered almost entire area of PB. MSp area and LSp area adjacent. LSp area and SSp area are separated but close to each other.

Remarks. Up to the present time, the genus *Agelasta* includes over 70 species which are separated into nine subgenera. *Agelasta* (*Dissosira*) has mainly been distinguished from the other subgenera by the following features (Breuning [2]): (1) pronotum without five distinct tubercules on disk, (2) elytra with basal margin not forming transversal edge, without high bosses near base and long suberect setae throughout, (3) humeri weakly projected laterad, (4) prosternal process well truncated in lateral view.

As already indicated by Yamasako and Ohbayashi [4], 24 known species belonging to the subgenus *Dissosira* could be separated into some species groups based on the external and male genital features. The redescription above is based on the type species group of the subgenus (Yamasako and Ohbayashi [4]).

The Species Transferred from Mesosa (Mesosa) to Agelasta (Dissosira). The following 12 species which are classified into *Mesosa* (*Mesosa*) are distinctly different from the type species of the subgenus, *M.* (*M.*) *curculionoides* (Figures 1(a)–1(c), 2(a)-2(b)) in the external features and the endophallic structures. These characteristics well coincide with *Agelasta* (*Dissosira*) except for the rounded prosternal process.

3.1. *Agelasta (Dissosira) perplexa* (Pascoe, 1858), Comb. Nov. (Figures 3(c)-3(d), 6(b), 7(e)–7(h), and 9(b))

Mesosa perplexa Pascoe, 1858: 243 [6].

Pachyosa perplexa: Matsushita, 1933: 344 [10].

Mesosa (*Mesosa*) *perplexa*: Breuning, 1939: 401 [2].

Saimia alternans Schwarzer, 1925: 60 [11].

Mimocoptops? *formosana* Pic, 1925: 30 [12].

Diagnosis. Body black, covered with ocher pubescence. Occiput with four narrow longitudinal black bands. Antennae with each basal part of third to the last segments with white pubescent annulations though it is very narrow on fifth, seventh, and ninth segments. Pronotum with three longitudinal narrow black bands on disk. Elytra with two light yellowish

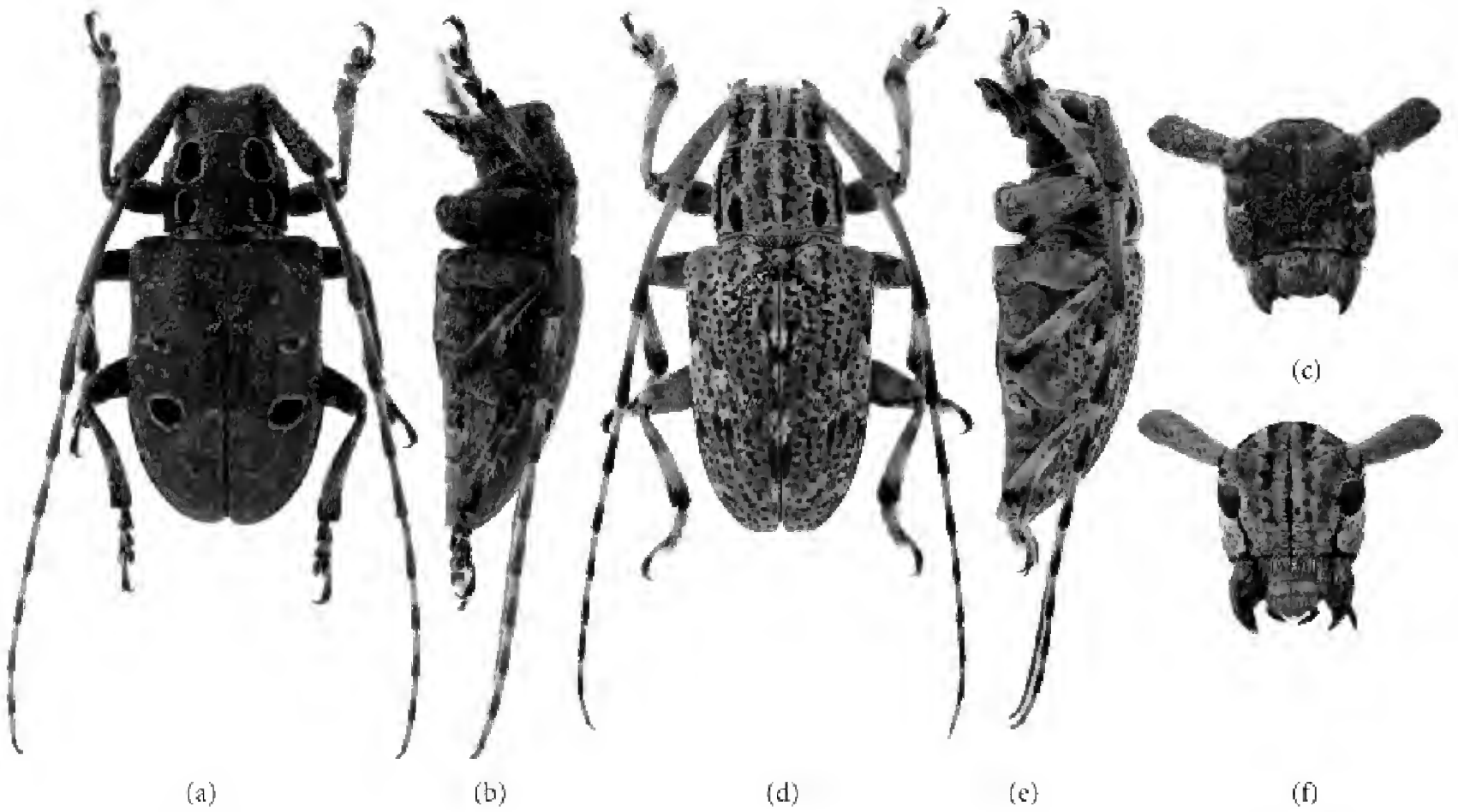

FIGURE 1: Comparison of the type species of *Mesosa* (*Mesosa*) and *Agelasta* (*Dissosira*). (a–c) *M.* (*M.*) *curculionoides*; (d–f) *A.* (*D.*) *catenata*; (a, d) male habitus in dorsal view; (b, e) ditto in lateral view; (c, f) ditto in frontal view.

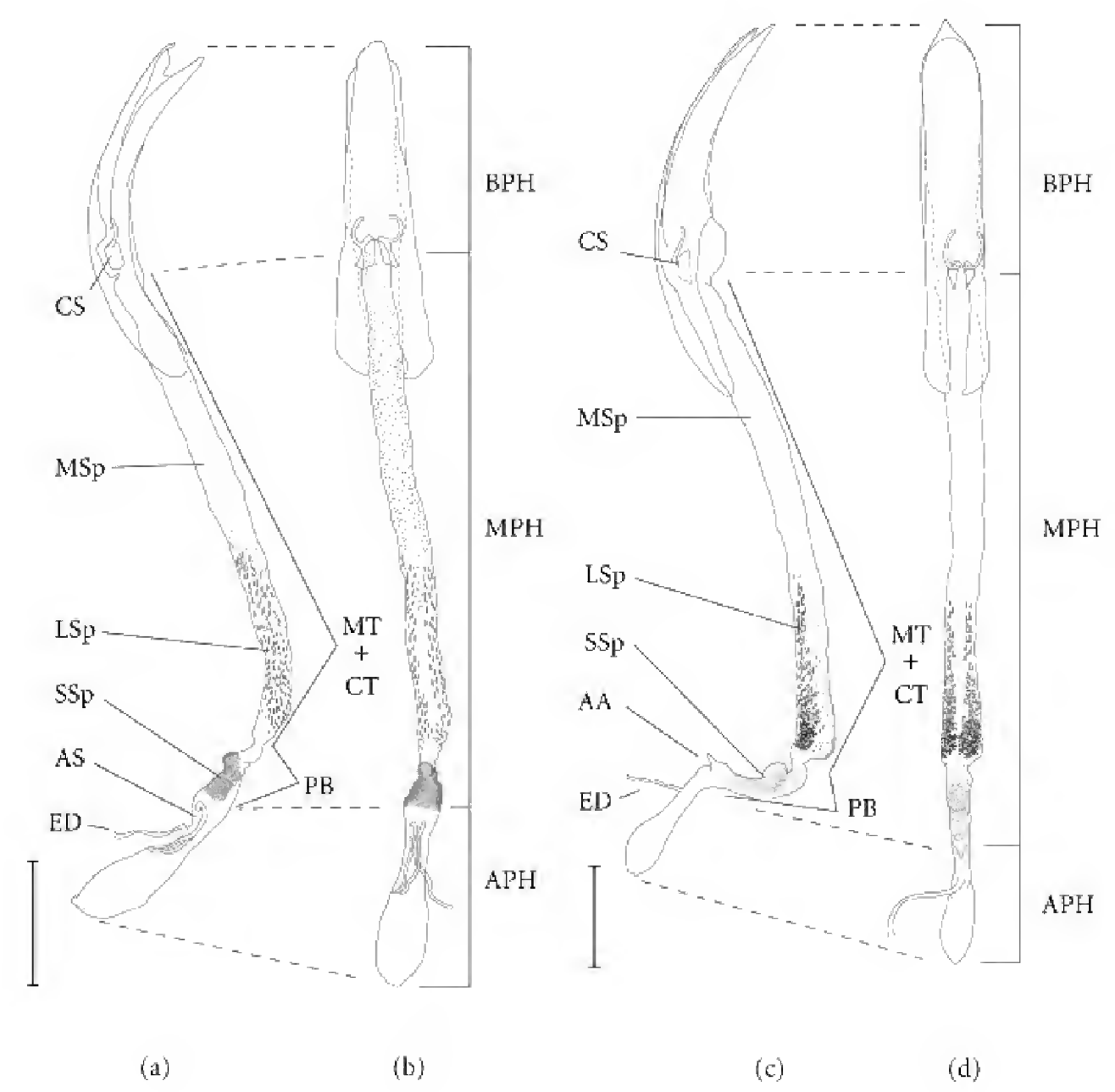

FIGURE 2: Comparison of the type species of *Mesosa* (*Mesosa*) and *Agelasta* (*Dissosira*). (a, b) *M.* (*M.*) *curculionoides*; (c, d) *A.* (*D.*) *catenata*; (a, c). median lobe with endophallus in lateral view; (b, d) ditto in dorsal view. Scale: 1.0 mm. For abbreviations see text.

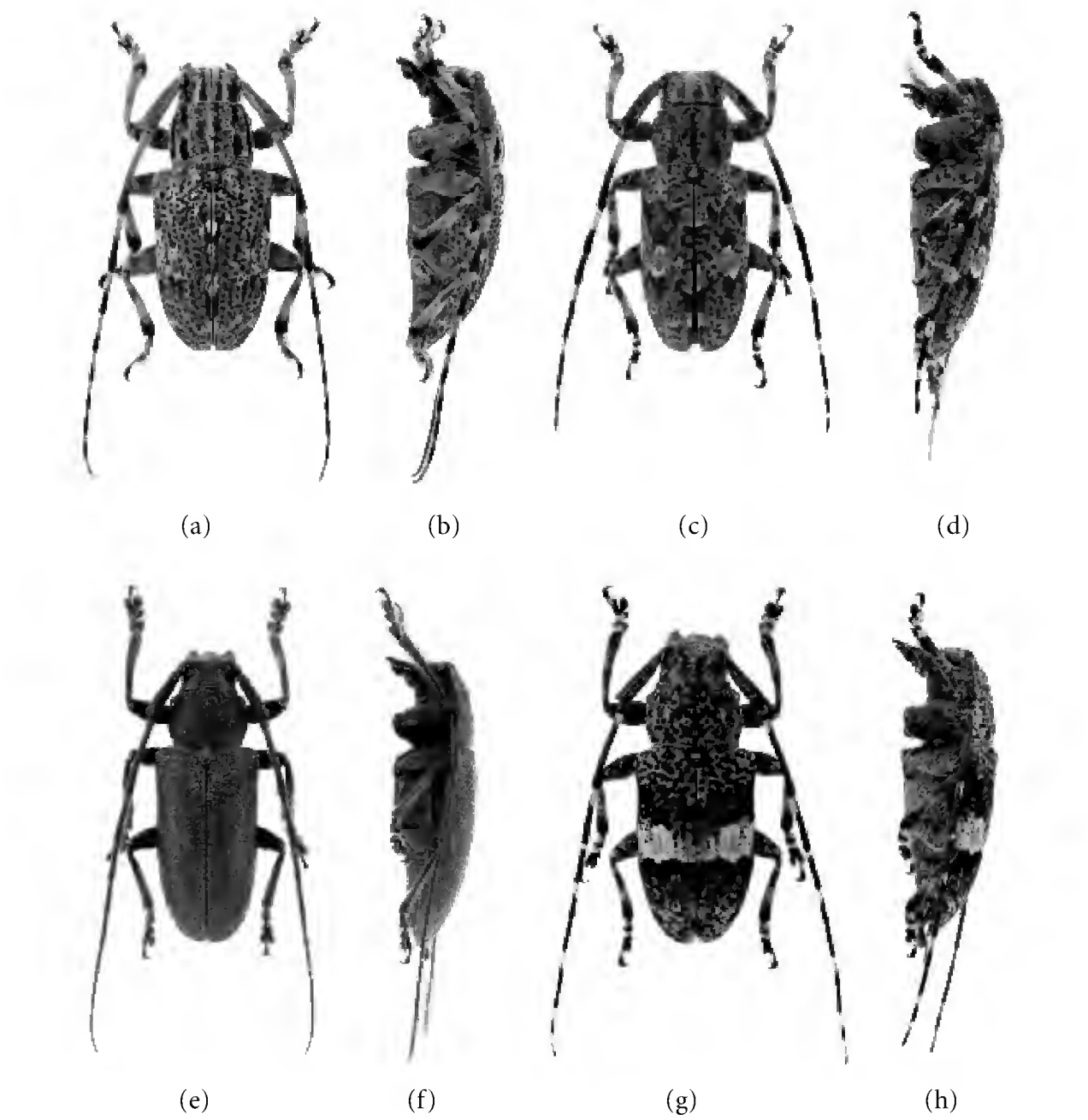

FIGURE 3: Male habitus of *Agelasta* (*Dissosira*) spp. (a, b) *A.* (*D.*) *catenata*; (c, d) *A.* (*D.*) *perplexa*; (e, f) *A.* (*D.*) *rufa*; (g, h) *A.* (*D.*) *konoi*; (a, c, e, g) dorsal view; (b, d, f, h) lateral view.

or whitish brown bands which are marginated with black irregular spots. Prosternal process roundly sloped and not truncate in lateral view.

Male Genitalia ($n = 2$). Tegmen in lateral view slightly curved, slender rhombic, and widest near middle in ventral view. Ringed part slightly expanded laterad at near middle of tegmen, thence arcuately narrowed basad. Lateral lobes about 1/3 of total length of tegmen, with inner sides almost straight and outer sides nearly straight toward apical third, thence slightly narrowed to angularly rounded apex, provided with two kinds of setae of which one is long and thick, concentrated near apex, and another is rather short and thin, arising chiefly from outer sides of apical half.

Median lobe in lateral view gentry curved; apex in ventral view weakly pointed; median strut dehiscent from basal 2/5.

Endophallus almost three times as long as median lobe in lateral view. The relative length of each broad membrane area of endophallus to median lobe are as follows: ML : TLE : BPH : MPH (MT+CT : PB) : APH = 3.7 : 10.0 : 2.2 : 5.9 (5.0 : 0.8) : 2.0. MPH with MT+CT well swollen and projected on ventral side near base. APH well swollen in oval shape, with ligulate shaped AA near apex.

MSp distributed in nearly apical 2/3 of MT+CT. LSp distributed in almost basal 1/3 of MT+CT on dorsal side, thick and short, arranged into two longitudinal irregular lines, unidentate in apical area, thence becoming thicker, denser, and multidentate toward basal area. SSp short, small and unidentate, covered almost of laterodorsal side and basal 1/3 of ventral side of PB. MSp area and LSp area adjacent. LSp area and SSp area close to each other.

Specimens Examined. [China] Syntype (BMNH): 1♀, "China" [printed on green oval label]; "Type" [printed on white label with red circle]; "Mesosa/perplexa/Pasc/N. China" [printed on white label]; "Pascoe/Coll./93–60." [printed on white label]; "Mesosa/perplexa/China Pasc." [printed on white label]; 1♂, She Shan, Shanghai, 9. VII, 2002, Hu and Tang leg. [Taiwan]: 1♂, Shouka forest road, Shizi township, Pingtung county, 4. VII, 2006, S-T. Hisamatsu leg. [Japan]: 3♂♂, 3♀♀, Yanaimachi, Matsuyama City, Ehime Pref., 20. VI, 1997, N. Ohbayashi leg.; 1♂, Shindate, Matsuyama City, Ehime Pref., 2. XI, 2004, J. Yamasako leg.; 1♂, same locality, 10. XI, 2004, J. Yamasako leg.

Distributions. China, Taiwan, Japan.

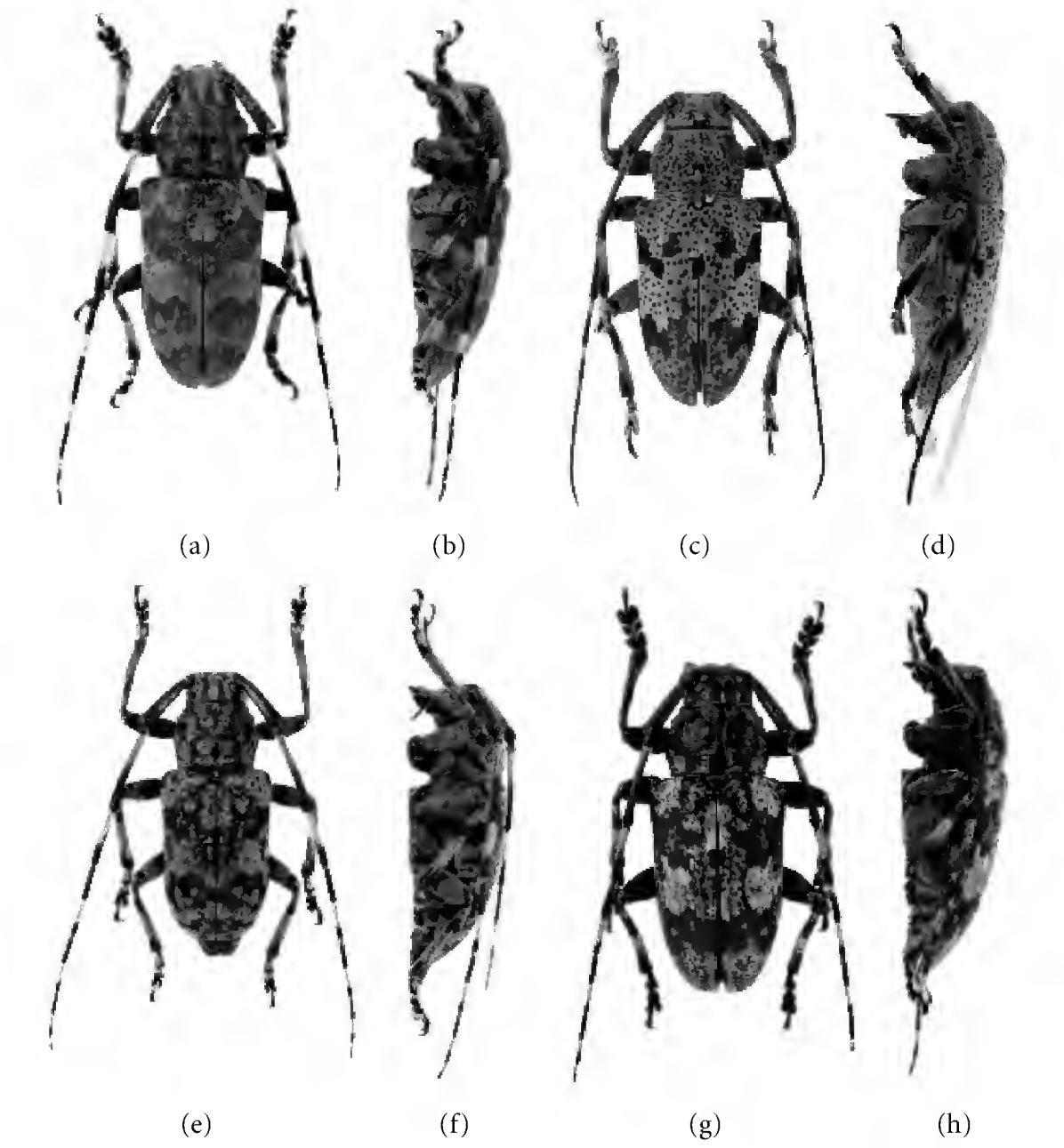

FIGURE 4: Male habitus of *Agelasta* (*Dissosira*) spp. (a, b) *A.* (*D.*) *yonaguni*; (c, d) *A.* (*D.*) *nigrostictica*; (e, f) *A.* (*D.*) *praelongipes*; (g, h) *A.* (*D.*) *kumei*; (a, c, e, g) dorsal view; (b, d, f, h). lateral view.

3.2. Agelasta (Dissosira) columba (Pascoe, 1859), Comb. Nov. (Figure 5(a))

Mesosa columba Pascoe, 1859: 40 [6].

Mesosa (*Mesosa*) *columba*: Breuning, 1939: 402 [2].

Diagnosis. This species is similar to *A. perplexa*, but distinguishable by the following features. Body black, covered with light brown pubescence. Pronotum with two pair of small rounded black maculae on disk. Elytra with several white spots which are sometimes forming transversal band near middle, scattered with some spots of dark brown pubescence which are sometimes forming indistinct transversal band before and after middle of elytra. Prosternal process roundly sloped and not truncate in lateral view.

Specimen Examined. Syntype (BMNH): 1♂, "Ceylon" [printed on light blue circle label]; "Mesosa/columba/Pascoe/type" [printed on white label]; "Type" [printed on white label with red circle].

Distribution. Sri Lanka.

Remarks. No specimen was available for dissection of male genitalia. However, this species distinctly differs from *Mesosa* (*Mesosa*) in the following structures: antennal scape elongate and slightly thickened apically; lower eye lobe relatively large. Besides, the external characteristics of this species are similar to *A. perplexa* and well coincided with *Agelasta* (*Dissosira*). Therefore, we treat this species as a member of this subgenus.

3.3. Agelasta (Dissosira) rufa (Breuning, 1935), Comb. Nov.

(Figures 3(e)–3(f), 6(c), 7(i)–7(l), and 9(c))

Pseudaemocia rufa Breuning, 1935: 269 [9].

Mutatocoptops (*Pseudaemocia*) *rufa*: Breuning, 1939: 506 [2].

Mesosa (*Mesosa*) *rufa*: N. Ohbayashi, 1992: 8 [13].

Diagnosis. Body reddish brown, sparsely covered with yellowish pubescence. Elytra sparsely with spots of yellowish pubescence which are sometimes forming some irregular longitudinal narrow maculae. Prosternal process rounded and not truncate at the apex.

Male Genitalia (n = 2). Tegmen in lateral view slightly curved, rhombic, and widest near middle in ventral view. Ringed part slightly expanded laterad at near middle of tegmen, thence arcuately narrowed basad. Lateral lobes about 1/4 of

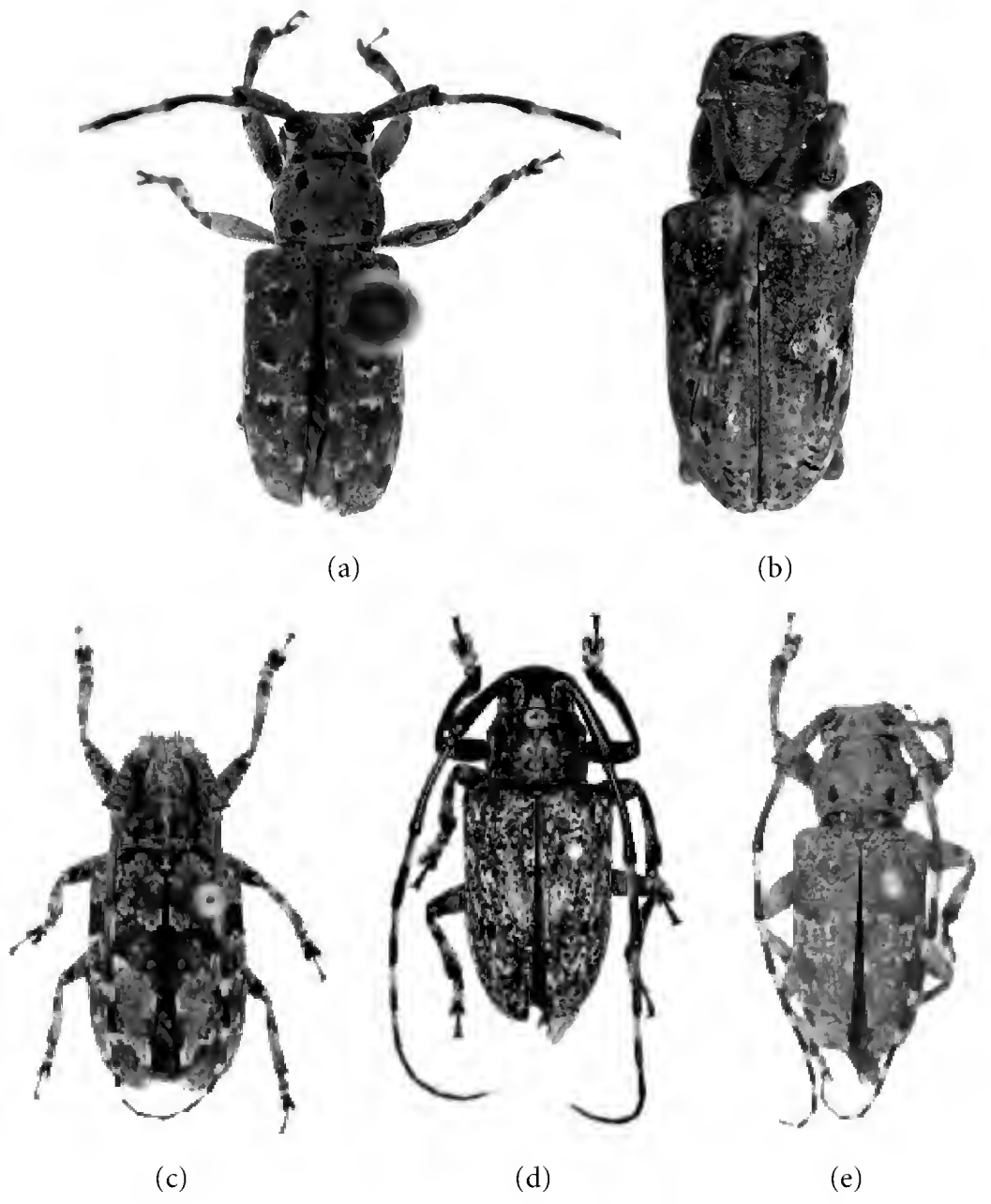

FIGURE 5: Habitus of *Agelasta* (*Dissosira*) spp. in dorsal view. (a) *A.* (*D.*) *columba* (syntype); (b) *A.* (*D.*) *catenatoides* (holotype); (c) *A.* (*D.*) *gardneri* (holotype); (d) *A.* (*D.*) *nigropunctata*; (e) *A.* (*D.*) *siamana* (holotype).

total length of tegmen, with inner sides almost straight and outer sides gently narrowed to rounded apex, provided with two kinds of setae of which one is long and thick, concentrated near apex, and another is rather short and thin, arising chiefly from outer sides of apical half.

Median lobe in lateral view gentry curved; apex in ventral view weakly pointed; median strut dehiscent from basal 1/3.

Endophallus almost 2.5 times as long as median lobe in lateral view. The relative length of each broad membrane area of endophallus to median lobe are as follows: ML : TLE : BPH : MPH (MT+CT : PB) : APH = 4.1 : 10.0 : 2.3 : 5.9 (4.9 : 1.0) : 1.8. MPH with MT+CT well swollen and projected on ventral side near base. APH well swollen in oval shape, with ligulate-shaped AA near apex.

MSp distributed in nearly apical 2/3 of MT+CT. LSp distributed in almost basal 1/3 of MT+CT on dorsal side, thick and short, arranged into two longitudinal irregular lines, unidentate in apical area, thence becoming thicker, denser, and multidentate toward basal area. SSp unidentate, short and small, covered almost of laterodorsal side and basal 1/3 of ventral side of PB. MSp area and LSp area adjacent. LSp area and SSp area slightly separated each other.

Specimens Examined. Holotype (BMNH): 1♀, "Parry's Group/Bonin I./91–25" [printed on white label]; "Type" [printed on white label with red circle]; "Pseudaemocia/rufa mihi/Typ!/det. Breuning" [printed on white label]. Ogasawara Isls., Tokyo Pref., Japan, [Is. Chichijima]: 1♀, 25. X, 1974, M. Iga leg. [Is. Hahajima]: 1♂, 3♀♀, Chibusa-yama, 15. VI, 1992, N. Ohbayashi leg.; 1♂, Okimura, 16. VI, 1992, N. Ohbayashi leg.; 1♂, Mt. Funaki, 16. VI, 1991, T. Ito leg.; 1♀, 17. V, 1984, M. Hasegawa leg.; 1♂, 2♀♀, 15–17. VI, 1985, H. Makihara leg.; 1♂, 2♀♀, Funamidai, 24–27, VI. 1987, M. Nishimura leg.; 1♂, 1♀, Motochi, 25, VI. 1987, M. Nishimura leg. [Is. Mukojima]: 1♂, 19. X, 2002, H. Karube leg.

Distribution. Japan (Ogasawara Isls.).

Remarks. This species is endemic to the Ogasawara Islands, Japan. It was first described based on a female specimen as a unique species of the genus *Pseudaemocia* Breuning, 1935, which was downgraded to a subgenus of *Mutatocoptops* Pic, 1925 by Breuning [2]. Later, Ohbayashi [13] transferred it to *Mesosa* (*Mesosa*) by reason of the resemblance of its larval characters to *M.* (*M.*) *yonaguni*. In spite of its unique appearance of reddish body, very rough punctures on the body and indistinct maculae on the elytra, this species has male genital structures almost in common with *M.* (*M.*) *yonaguni*, and the external features basically coincide with the congeners of

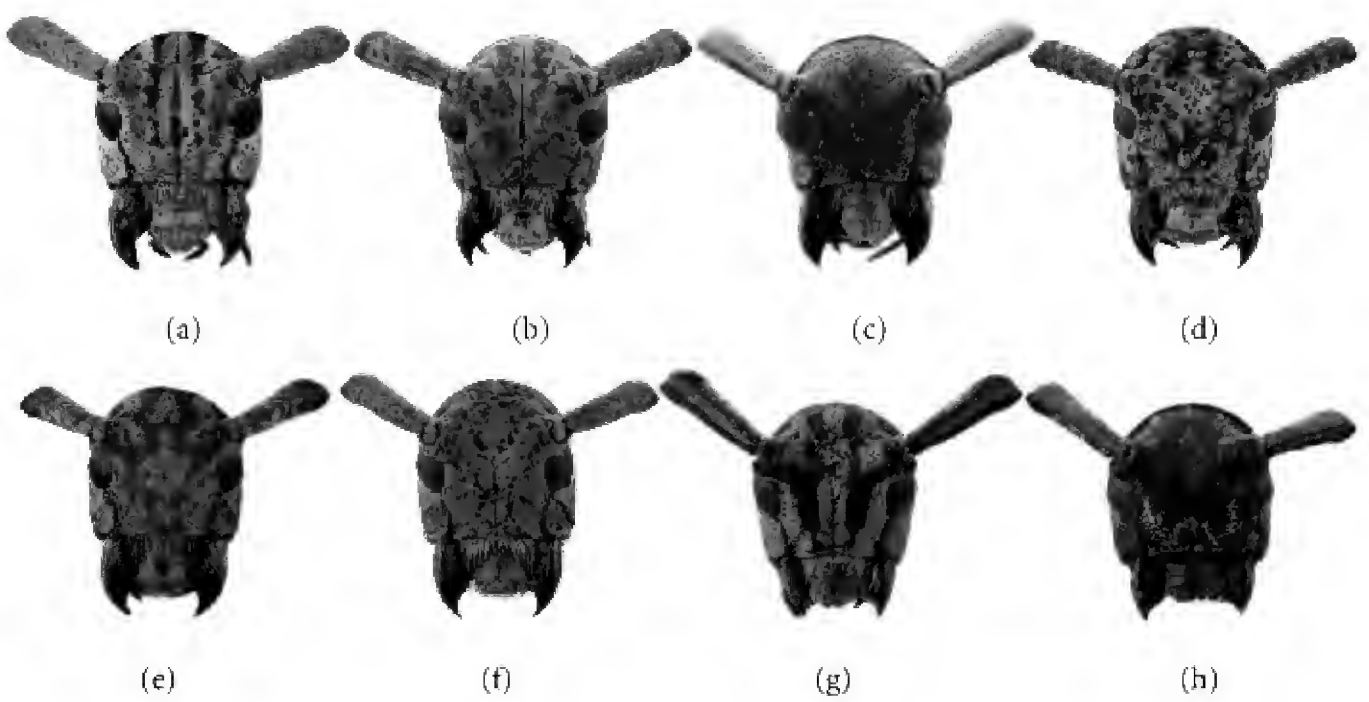

FIGURE 6: Male frontal view of *Agelasta* (*Dissosira*) spp. (a) *A.* (*D.*) *catenata*; (b) *A.* (*D.*) *perplexa*; (c) *A.* (*D.*) *rufa*; (d) *A.* (*D.*) *konoi*; (e) *A.* (*D.*) *yonaguni*; (f) *A.* (*D.*) *nigrostictica*; (g) *A.* (*D.*) *praelongipes*; (h) *A.* (*D.*) *kumei*.

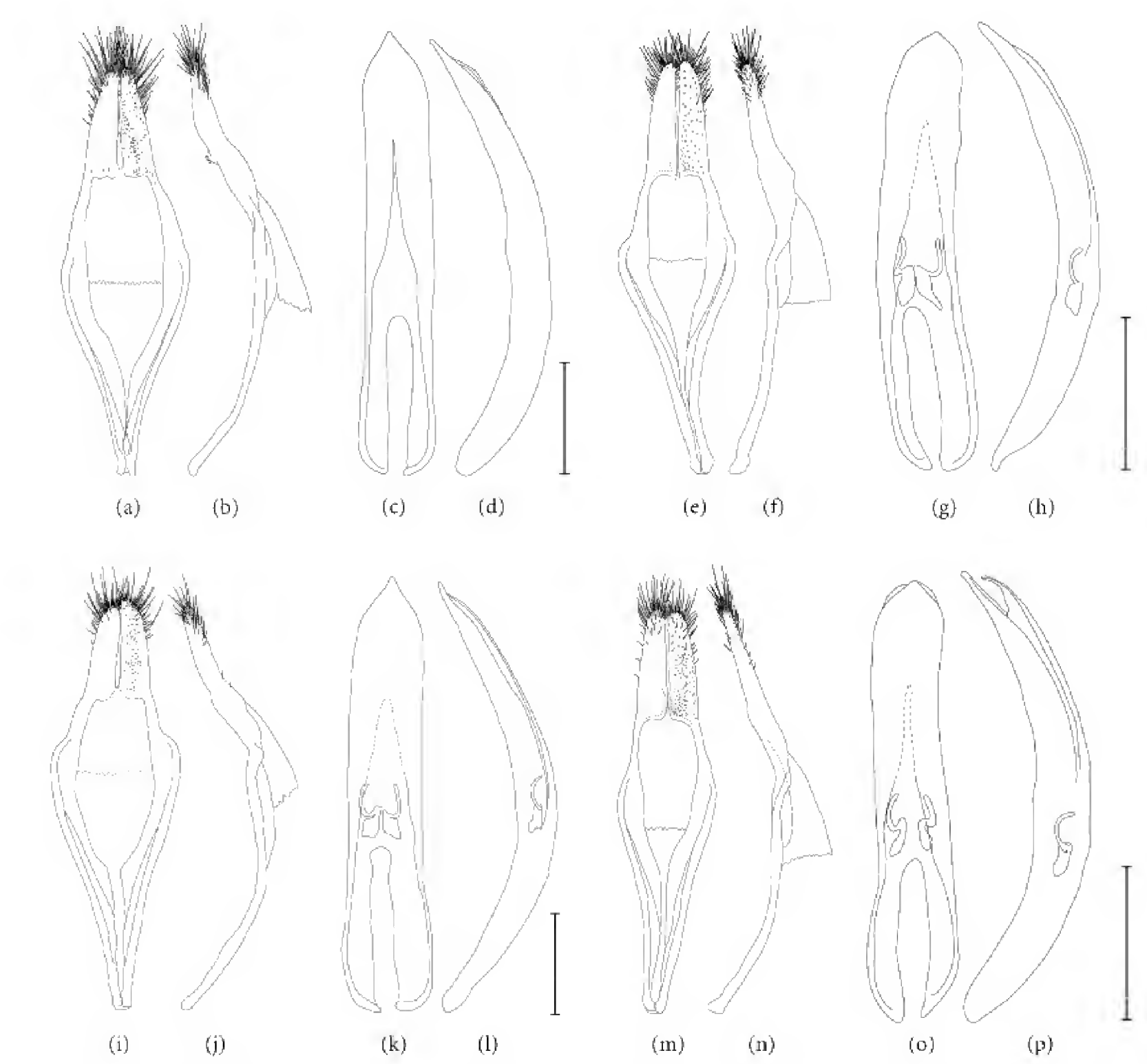

FIGURE 7: The male genital organ of *Agelasta* (*Dissosira*) spp. (a–d) *A.* (*D.*) *catenata*; (e–h) *A.* (*D.*) *perplexa*; (i–l) *A.* (*D.*) *rufa*; (m–p) *A.* (*D.*) *konoi*; (a, e, i, m) tegmen in ventral view; (b, f, j, n) ditto in lateral view; (c, g, k, o) median lobe in ventral view; (d, h, l, p). ditto in lateral view. Scale: 1.0 mm.

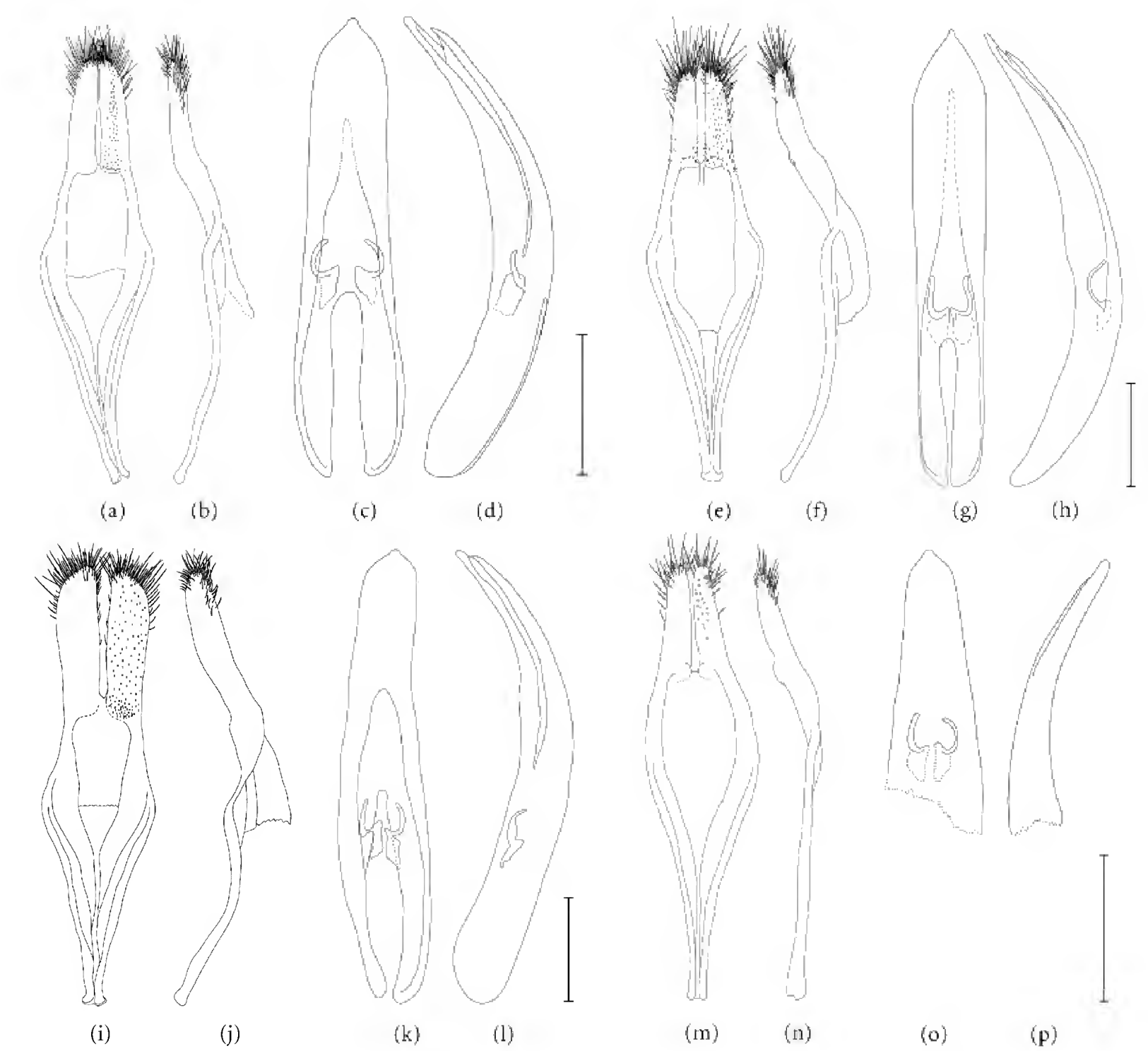

FIGURE 8: The male genital organ of *Agelasta* (*Dissosira*) spp. (a–d) *A.* (*D.*) *yonaguni*; (e–h) *A.* (*D.*) *nigrostictica*; (i–l) *A.* (*D.*) *praelongipes*; (m–p) *A.* (*D.*) *kumei*; (a, e, i, m) tegmen in ventral view; (b, f, j, n) ditto in lateral view; (c, g, k, o) median lobe in ventral view; (d, h, l, p) ditto in lateral view. Scale: 1.0 mm.

Agelasta (*Dissosira*). Therefore, we treat this species as a member of this subgenus.

3.4. Agelasta (Dissosira) catenatoides Yamasako and N. Ohbayashi, Nom. Nov. (Nomen Preoccupied by *Agelasta laosensis* Pic, 1925) (Figure 5(b))

Mesosa laosensis Breuning, 1935: 274 [9].

Mesosa (*Mesosa*) *laosensis*: Breuning, 1939: 402 [2].

Diagnosis. This species is quite similar to *A. catenata*, but distinguishable by body covered with brown pubescence.

Specimen Examined. Photographs of the holotype (MNHN): ♂, "Mesosa/laosensis/mihi Typ/det. Breuning" [printed on white label], "Vieng Kiet/5. oct. 1915/Vitalis" [printed on white label], "type" [printed on yellow label], "laosensis Br." [printed on white label], "TYPE" [printed on red label].

Distribution. Laos.

Remarks. No specimen was available for dissection of the male genitalia. The diagnosis described above is based on some photographs of the holotype. Judging from the photographs, this species is without doubt close to *A.* (*D.*) *catenata* which is the type species of *Agelasta* (*Dissosira*). Therefore, we treat this species as a member of *Agelasta* (*Dissosira*). On the other hand, the species name, *Agelasta laosensis*, is already occupied by Pic [12], and *A. laosensis* (Breuning, 1935 nec Pic, 1925) should be replaced by the secondary homonym. Therefore, we propose a new replacement name, *A.* (*D.*) *catenatoides* nom. nov., for the latter. The specific epithet refers to the resemblance to *A.* (*D.*) *catenata*.

3.5. Agelasta (Dissosira) gardneri (Breuning, 1938), Comb. Nov. (Figure 5(c))

Mesosa gardneri Breuning, 1938: 204 [14].

Mesosa (*Mesosa*) *gardneri*: Breuning, 1939: 402 [2].

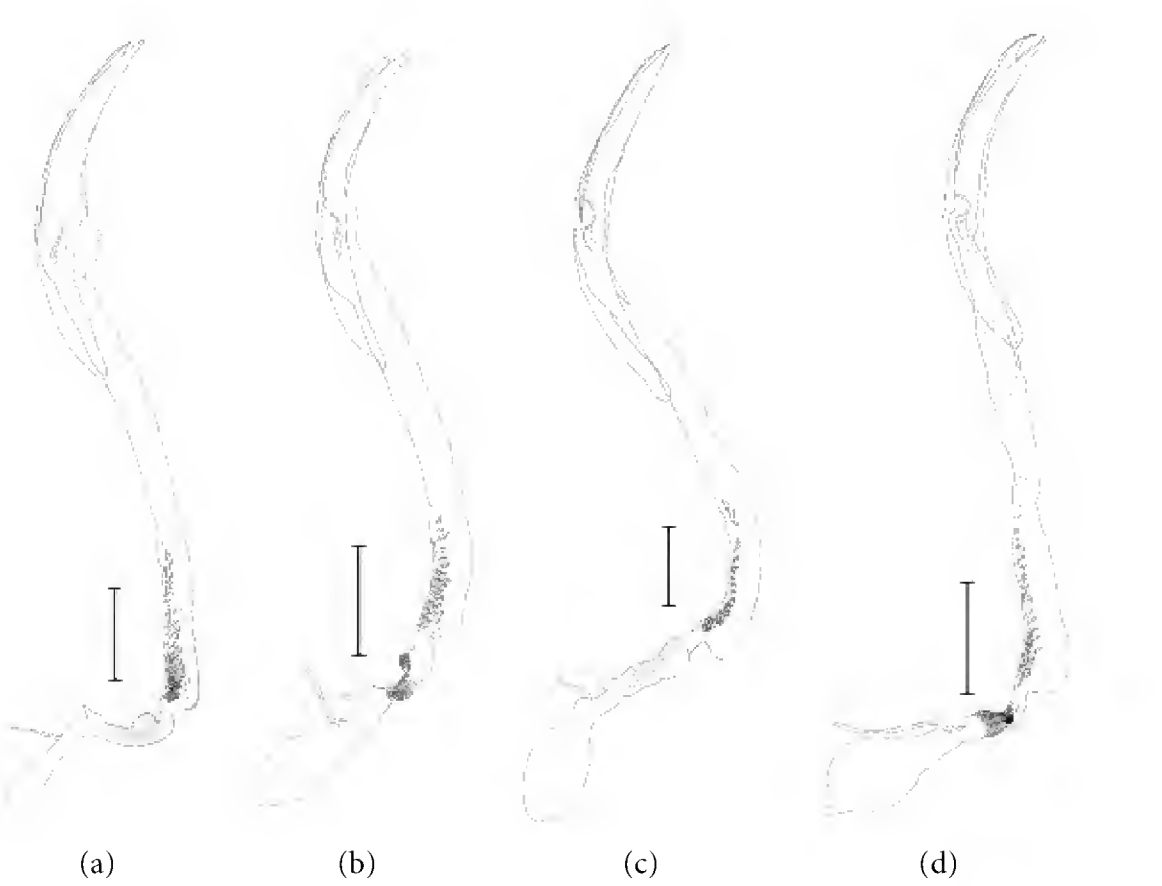

FIGURE 9: The median lobe with endophallus of *Agelasta* (*Dissosira*) spp. in lateral view. (a) *A.* (*D.*) *catenata*; (b) *A.* (*D.*) *perplexa*; (c) *A.* (*D.*) *rufa*; (d) *A.* (*D.*) *konoi*. Scale: 1.0 mm.

Diagnosis. This species is similar to *A. perplexa*, but distinguishable by the following features. Body black, mingled with light and dark brown pubescence. Antennae with each basal part of third to the last segments with white pubescent annulations, which are getting narrower toward the last segment. Elytra with indistinct transversal bands of light brown pubescence near base and after middle, indistinct dark brown transversal bands before middle and near apices, scattered with white spots.

Specimen Examined. Holotype (BMNH): "Lachiwala/Dehra Dun, U. P./F. Ent./17. VII. 1929" [printed on white label], "R. R. D. 892/B. C. R. 284/Cage 712" [printed on white label], "Ex Bauhinia/retusa." [printed on white label], "37" [printed on white label], "Mesosa/gardneri/mihi Type/det. Breuning" [printed on white label], "Type" [printed on white circle label with red margined].

Distribution. North India.

Remarks. No specimen was available for dissection of the male genitalia. However, this species basically shares external characteristics with *A.* (*D.*) *perplexa*. Therefore, we treat this species as a member of *Agelasta* (*Dissosira*).

3.6. Agelasta (Dissosira) nigropunctata (Breuning, 1938), Comb. Nov. (Figure 5(d))

Mesosa nigropunctata Breuning, 1938: 203 [14].

Mesosa (*Mesosa*) *nigropunctata*: Breuning, 1939: 402 [2].

Diagnosis. This species is very similar to *A. catenata*, but distinguishable by the following features. Body black, mingled with black, brown, and white pubescence. Occiput with four narrow longitudinal black bands. Antennae with each basal part of third to 7th segments with white pubescent annulations, which are getting narrower toward 7th segment; the reminders covered with black pubescence. Pronotum with two longitudinal narrow black bands on disk. Elytra irregularly scattered with longitudinal spots of black, brown, and white pubescence. Prosternal process roundly sloped and not truncate in lateral view.

Specimen Examined. 1♂, Mulayit Taung, SE-Burma, 11, 1989 (ZSM, determined by Hüdepohl in 1995).

Distribution. Myanmar.

Remarks. No specimen was available for dissection of the male genitalia. However, this species basically shares external characteristics with *A.* (*D.*) *catenata* which is the type species of *Agelasta* (*Dissosira*). Therefore, we treat this species as a member of *Agelasta* (*Dissosira*).

3.7. Agelasta (Dissosira) konoi (Hayashi, 1956), Comb. Nov.

Remarks. This species is mainly distributed in the northeastern part of the Ryukyu Islands, Japan, and is divided into five subspecies as described below. Here we limited the description to the nominotypical subspecies only, and that of the other subspecies are omitted.

3.7.1. Agelasta (Dissosira) konoi konoi (Hayashi, 1956), Comb. Nov. (Figures 3(g)-3(h), 6(d), 7(m)–7(p), and 9(d))

Mesosa (*Mesosa*) *konoi* Hayashi, 1956: 13, pl. 4, Figure 1 [15].

Mesosa (*Mesosa*) *konoi konoi*: Hayashi, 1962: 33 [16].

Diagnosis. This species is similar to *A. perplexa*, but distinguishable by the following features. Body black, covered with ocher pubescence. Elytra with a yellowish white maculae marginated with black pubescence near middle.

Male Genitalia (n = 2). Tegmen in lateral view slightly curved, slender rhombic, and widest near middle in ventral view. Ringed part slightly expanded laterad near middle of tegmen, thence arcuately narrowed basad. Lateral lobes about 1/4 of total length of tegmen, with inner sides almost straight, and outer sides nearly straight toward apical third, thence slightly narrowed to rounded apex, provided with two kinds of setae of which one is long and thick, concentrated near apex, and another is rather short and thin, arising mainly from laterodorsal sides of apical half.

Median lobe in lateral view gentry curved; apex in ventral view weakly pointed; median strut dehiscent from basal 2/5.

Endophallus almost three times as long as median lobe in lateral view. The relative length of each broad membrane area of endophallus to median lobe is as follows: ML : TLE : BPH : MPH (MT+CT : PB) : APH = 3.5 : 10.0 : 2.1 : 6.1 (5.5 : 0.6) : 1.9. MPH with MT+CT well swollen and projected on ventral side near base. APH well swollen in oval shape, with ligulate-shaped AA near apex.

MSp distributed in nearly apical half of MT+CT. LSp distributed in nearly basal half of MT+CT on dorsal side, thick and short, arranged into two longitudinal irregular lines, unidentate in apical area, thence becoming thicker, denser, and multidentate toward basal area. SSp unidentate, short and small, covered almost of laterodorsal side and basal 1/3 of ventral side of PB. MSp area and LSp area adjacent. LSp area and SSp area close to each other.

Specimens Examined. 1♂, 1♀, Is. Nakanoshima, Tokara Isls., 7. VII, 1960, M. Satô leg.; 3♂♂, 1♀, same locality, 21–22. VII, 1969, M. Sakai leg.; 10♂♂, 10♀♀, same locality, 23. VI, 2003, J. Yamasako leg.

Distribution. Japan (Tokara Isls., Kagoshima Pref.; Is. Izu-Ôshima, Tokyo Pref.).

3.7.2. Agelasta (Dissosira) konoi amamiana (Hayashi, 1962) Comb. Nov.

Mesosa (*Mesosa*) *konoi amamiana* Hayashi, 1962: 13, pl. 3, Figure 11 [17].

Specimens Examined. 1♂, 1♀, Hatsuno, Is. Amami-Ôshima, 10. VII, 1962, N. Ohbayashi leg.; 1♀, same locality, 29. VII, 1962, N. Ohbayashi leg.; 1♂, 1♀, same locality, 13. VI, 1962, M. Satô leg.; 1♂, Shinokawa, Is. Amami-Ôshima, 18. VI, 1997, S. Yoshimichi leg.

Distribution. Japan (Is. Amami-Ôshima, Kagoshima Pref.).

3.7.3. Agelasta (Dissosira) konoi okinoerabuensis (Ohbayashi, 1959), Comb. Nov.

Mesosa (*Mesosa*) *konoi okinoerabuensis* Ohbayashi, 1959: 3 [18].

Specimens Examined. 2♂♂, 1♀, Is. Okinoerabu, 6. VI, 1957, M. Umebayashi leg.; 1♀, same locality, 13. VII, 1963, N. Ohbayashi leg.; 2♂♂, same locality, 27. VI, 1964, M. Nishikawa coll.

Distribution. Japan (Is. Okinoerabu, Kagoshima Pref.).

3.7.4. Agelasta (Dissosira) konoi okinawana (Hayashi, 1960), Comb. Nov.

Mesosa (*Mesosa*) *perplexa okinawana* Hayashi, 1960: 27 [19].

Mesosa (*Mesosa*) *konoi okinawana*: Hayashi, 1962: 13 [17].

Mesosa (*Saimia*) *cervinopicta*: Gressitt, 1951: 220 (part.: Is. Okinawa, Japan) (nec Fairmaire, 1897) [20].

Specimens Examined. 1♂, Takari, Is. Okinawa, 29. VI, 1993, N. Ohbayashi leg.; 1♀, Mt. Yonahadake, Is. Okinawa, Okinawa Pref., Japan, 2. VII, 1993, N. Ohbayashi leg.

Distribution. Japan (Is. Okinawa, Okinawa Pref.).

3.7.5. Agelasta (Dissosira) konoi kumejimana (Kusama and Takakuwa, 1984) Comb. Nov.

Mesosa (*Mesosa*) *konoi kumejimana* Kusama and Takakuwa, 1984: 356 [21].

Specimen Examined. 1♂, Nakadomari, Is. Kumejima, Okinawa Pref., Japan, 25. III–30. VII, 1990, T. Ito leg.

Distribution. Japan (Is. Kumejima, Okinawa Pref.).

3.8. Agelasta (Dissosira) yonaguni (Hayashi, 1962), Comb. Nov.

Remarks. This species is distributed in the southwestern part of the Ryukyu Islands and is divided into four subspecies as described below. Here we limited the description to the nominotypical subspecies only, and that of the other subspecies are omitted.

3.8.1. Agelasta (Dissosira) yonaguni yonaguni (Hayashi, 1962) Comb. Nov. (Figures 4(a)-4(b), 6(e), 8(a)–8(d), and 10(a))

Mesosa (*Mesosa*) *cervinopicta yonaguni* Hayashi, 1962: 5, pl. 1, Figure 5 [22].

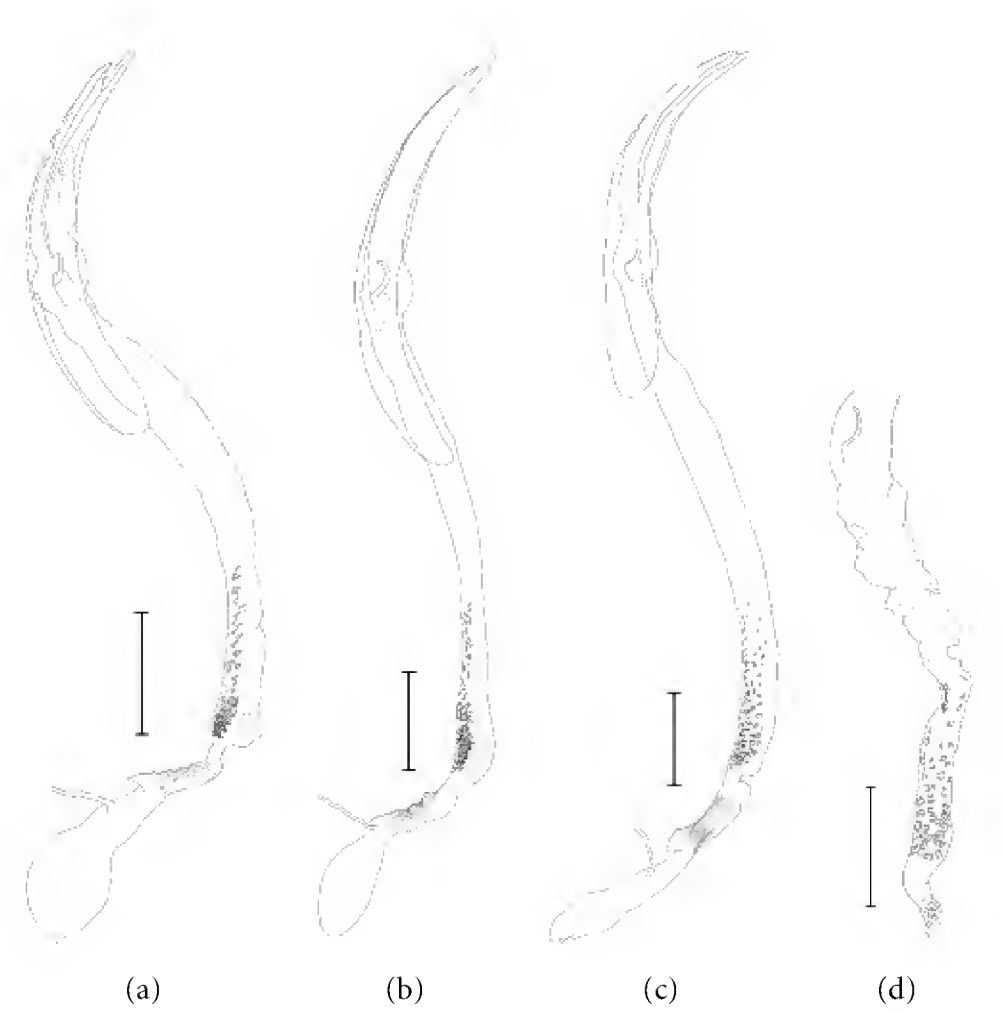

FIGURE 10: The median lobe with endophallus of *Agelasta* (*Dissosira*) spp. in lateral view. (a) *A.* (*D.*) *yonaguni*; (b) *A.* (*D.*) *nigrostictica*; (c) *A.* (*D.*) *praelongipes*; (d) *A.* (*D.*) *kumei*. Scale: 1.0 mm.

Mesosa (*Pachyosa*) *cervinopicta yonaguni*: Samuelson, 1965: 100 [23].

Mesosa (*Mesosa*) *yonaguni*: Kusama and Irie, 1976: 20 [24].

Pachyosa cervinopicta: Miwa, 1935: 37 (Is. Yonaguni, Japan) (nec Fairmaire, 1897) [25].

Mesosa (*Saimia*) *cervinopicta*: Gressitt, 1951: 220 (nec Fairmaire, 1897) [20].

Diagnosis. This species is similar to *A. perplexa*, but distinguishable by the following features. Body black, mingled with yellowish ocher pubescence and black pubescence. Pronotum with disk with some irregular longitudinal black bands. Elytra mingled with yellowish ocher and black patches, of which yellowish ones are forming transversal irregular bands on post humeri, near middle, and near apical fourth. Prosternal process roundly truncated in lateral view.

Male Genitalia ($n = 1$). Tegmen in lateral view slightly curved, slender rhombic, and widest near middle in ventral view. Ringed part slightly expanded laterad near middle of tegmen, thence arcuately narrowed basad. Lateral lobes about 1/4 of total length of tegmen, with inner sides almost straight, and outer sides gently narrowed to angularly rounded apex, provided with two kinds of setae of which one is long and thick, concentrated near apex, and another is rather short and thin, arising chiefly from outer sides of apical half.

Median lobe in lateral view gentry curved; apex in ventral view weakly pointed; median strut dehiscent from basal 1/3.

Endophallus almost three times as long as median lobe in lateral view. The relative length of each broad membrane area of endophallus to median lobe are as follows: ML : TLE : BPH : MPH (MT+CT : PB) : APH = 3.8 : 10.0 : 2.2 : 6.0 (5.1 : 0.9) : 1.8. MPH with MT+CT well swollen and projected on ventral side near base. APH well swollen in oval shape, with ligulate-shaped AA near apex.

MSp distributed in nearly apical 2/3 of MT+CT. LSp distributed in nearly basal 1/3 of MT+CT on dorsal side, thick and short, arranged into two longitudinal irregular lines, unidentate in apical area, thence becoming thicker, denser and multidentate toward basal area. SSp unidentate, short and small, covered laterodorsal side of PB. MSp area and LSp area adjacent. LSp area and SSp area close to each other.

Specimens Examined. 1♂, Sonai, Is. Yonaguni, 10, V, 1963, Y. Arita leg.; 2♀♀, Tendabaru, Is. Yonaguni, 13, V, 1963, Y. Arita leg.; 2♀♀, Tabaru-gawa, Is. Yonaguni, 14, V, 1963, Y. Arita leg.; 5♀♀, Mt. Urabedake, Is. Yonaguni, 6–7, VII, 1969, Y. Hori leg.; 2♀♀, same locality, 11. VII, 1964, N. Ohbayashi leg.; 1♂, Hikawa, Is. Yonaguni, Okinawa Pref, Japan, 16. V, 1963, Y. Arita leg.; 1♂, Mt. Urabedake, Is. Yonaguni, Okinawa Pref, Japan, 20. V, 1989, N. Ohbayashi leg.

Distribution. Japan (Is. Yonaguni, Okinawa Pref.).

3.8.2. Agelasta (Dissosira) yonaguni subkonoi (Hayashi, 1962) Comb. Nov.

Mesosa (*Mesosa*) *subkonoi* Breuning, 1964: 91 [26].

Mesosa (*Mesosa*) *cervinopicta cervinopicta* f. *subkonoi*: Hayashi, 1964: 70, Figure 1 [27].

Mesosa (*Mesosa*) *yonaguni subkonoi*: Kusama and Irie, 1976: 20, Figures 3(a) and 3(b) [24].

Mesosa (*Mesosa*) *perplexa*: Hayashi, 1962: 32 (nec Pascoe, 1858) [16].

Pachyosa cervinopicta: Miwa, 1933: 12 (nec Fairmaire, 1897) [28].

Mesosa (*Saimia*) *cervinopicta*: Gressitt, 1951: 220 (nec Fairmaire, 1897) [20].

Mesosa (*Mesosa*) *cervinopicta cervinopicta*: Hayashi, 1962: 35, pl. 4, Figure 3 (nec Fairmaire, 1897) [15].

Specimens Examined. [Is. Ishigaki, Okinawa Pref., Japan]: 3♂♂, 1♀, Kawarayama, 29. VI, 1964, N. Ohbayashi leg.; Arakawa, 16. VI, 1965, K. Hatta leg.; 2♀♀, Nosoko-dake, 21. VII, 1998, A. Komada leg. [Is. Iriomote, Okinawa Pref., Japan]: 2♂♂, 1♀, Ushikuno-mori, 7. VIII, 1962, Y. Arita and M. Satô leg.; 1♀, same locality, 26. VI, 1965, Y. Hori leg.; 1♂, Sonai, 24. VI, 1965, Y. Hori leg.

Distribution. Japan (Is. Ishigaki, Is. Iriomote and Is. Taramajima, Okinawa Pref.).

3.8.3. Agelasta (Dissosira) yonaguni kashiwaii (Kusama and Takakuwa, 1984), Comb. Nov.

Mesosa (*Mesosa*) *yonaguni kashiwaii* Kusama and Takakuwa, 1984: 11, 358 [21].

Mesosa (*Mesosa*) *cervinopicta* f. *subkonoi*: Hayashi and Nomura, 1964: 67 (Is. Hateruma, Japan) (nec Breuning, 1964) [29].

Mesosa (*Mesosa*) *yonaguni subkonoi*: Kusama and Irie, 1976: 20 (part.: Is. Hateruma) (nec Breuning, 1964) [24].

Specimens Examined. 3♂♂, 1♀, Is. Hateruma, 26. IV, 1975, K. Shimizu leg.

Distribution. Japan (Is. Taketomi, Is. Kohama, Is. Kuroshima and Is. Hateruma, Okinawa Pref.).

3.8.4. Agelasta (Dissosira) yonaguni similaris (Kusama and Takakuwa, 1984), Comb. Nov.

Mesosa (*Mesosa*) *yonaguni similaris* Kusama and Takakuwa, 1984: 11 [21].

Pachyosa cervinopicta: Matsushita, 1933: 344 (nec Fairmaire, 1897) [10].

Mesosa (*Saimia*) *cervinopicta*: Hayashi, 1960: 27 (nec Fairmaire, 1897) [19].

Mesosa (*Pachyosa*) *cervinopicta cervinopicta*: Samuelson, 1965: 99 (nec Fairmaire, 1897) [23].

Mesosa (*Mesosa*) *yonaguni subkonoi*: Kusama and Irie, 1976: 20 (nec Breuning, 1964) [24].

Mesosa (*Mesosa*) *yonaguni semipraelongipes* Kusama and Takakuwa, 1984: 358, errata [21].

Specimens Examined. 1♂, Hirara-shi, Is. Miyako, 14. VII, 2001, N. Ohshige leg.; 1♂, Is. Tarama, 1. VI, 1993, H. Kanazawa leg.

Distribution. Japan (Is. Miyako, Is. Irabu, Is. Ikema, Is. Ôgami and Is. Tarama of Miyako Isls., Okinawa Pref.).

3.9. Agelasta (Dissosira) nigrostictica (Breuning, 1967), Comb. Nov. (Figures 4(c)-4(d), 6(f), 8(e)–8(h), and 10(b))

Mesosa (*Mesosa*) *nigrostictica* Breuning, 1967: 185 [30].

Diagnosis. This species is similar to *A. catenata*, but distinguishable by the following features. Body black or reddish brown, evenly covered with light brown pubescence. Antennae with each basal part of third to sixth or seventh segments with white pubescent annulations, which are getting narrower toward apical segment; the reminders covered with black pubescence. Pronotum with two indistinct longitudinal narrow black bands on disk. Elytra with two pairs of black fragment maculae on lateral side before and after middle. Prosternal process roundly sloped and not truncate in lateral view.

Male Genitalia (n = 1). Tegmen in lateral view slightly curved, rhombic, and widest near middle in ventral view. Ringed part slightly expanded laterad near middle of tegmen, thence arcuately narrowed basad. Lateral lobes about 1/4 of total length of tegmen, with inner sides almost straight, and outer sides nearly straight toward apical third, thence gentry narrowed to rounded apex, provided with two kinds of setae of which one is long and thick, concentrated near apex, and another is rather short and thin, sparsely distributed in ventral side, apical third of laterodorsal sides; each ventral side near base with a transversal obtuse ridge which is haired in mass on the edge of ridge.

Median lobe in lateral view gentry curved; apex in ventral view weakly pointed; median strut dehiscent from basal 2/5.

Endophallus about 2.5 times as long as median lobe in lateral view. The relative length of each broad membrane area of endophallus to median lobe is as follows: ML : TLE : BPH : MPH (MT+CT : PB) : APH = 4.3 : 10.0 : 2.5 : 6.2 (5.3 : 0.9) : 1.2. MPH with MT+CT well swollen and weakly projected on ventral side near base. APH well swollen in oval shape, with ligulate-shaped AA near apex, which is relatively indistinct.

MSp sparsely distributed in nearly apical half of MT+CT. LSp on dorsal side distributed in nearly basal half of MT+CT, thick and short, arranged into two longitudinal irregular lines, unidentate in apical area, thence becoming thicker, denser, and multidentate toward basal area; LSp on ventral side irregularly distributed in nearly basal 1/3 of MT+CT,

short, indistinct and rudimentary unidentate. SSp unidentate, short and small, covered almost of laterodorsal side and basal 1/3 of ventral side of PB. MSp area and LSp area adjacent. LSp area and SSp area close to each other.

Specimens Examined. [Java, Indonesia]: Photographs of the holotype (MNHN): 1♂, "Mesosa/nigrostictica/mihi typ/Breuning dét." [printed on white label], "TYPE" [printed on red label], "MUSÉUM PARIS/1952/COLL R OBERTHUR" [printed on white label margined with black line], "Java/Malang" [printed on white label margined with black line]. [Bali, Indonesia]: 1♂, Bali, Indonesia, 30. V, 1998, Native coll.; 1♂, West Bali, Indonesia, 19–21. XII, 2002, Y. Yokoi leg.; 1♂, Bali, Indonesia, X, 2005, Native leg.

Distribution. Indonesia (Java, Bali).

Remarks. This is first record of this species from Is. Bali. The male genital features above are described based on the specimen from Is. Bali.

3.10. *Agelasta (Dissosira) siamana* (Breuning, 1974), Comb. Nov. (Figure 5(e))

Mesosa (*Mesosa*) *siamana* Breuning, 1974: 73 [31].

Diagnosis. This species is very similar to *A. columba*, but distinguishable by the following features. Body black, covered with light brown pubescence. Antennae with each basal part of third to the last segments with white pubescent annulations, which are getting narrower toward the last segment; the reminders covered with black pubescence. Pronotum with two pair of small longitudinal black maculae on disk. Elytra with indistinct transversal white band near middle, scattered with several brown spots.

Specimen Examined. Photographs of a syntype (MNHN): 1♂, "Mesosa/siamana/mihi typ/Breuning dét." [printed on white label], "TYPE" [printed on red label], "Siam" [printed on white label], "MUSEUM PARIS/COLL. H. W. BATES/1952" [printed on white label margined with black line].

Distribution. Thailand.

3.11. *Agelasta (Dissosira) praelongipes* (Kusama and Irie, 1976), Comb. Nov. (Figures 4(e)-4(f), 6(g), 8(i)–8(l), and 10(c))

Mesosa (*Mesosa*) *praelongipes* Kusama and Irie, 1976: 20, Figures 2(a) and 2(b) [24].

Diagnosis. This species is very similar to *A.* (*D.*) *yonaguni*, but distinguishable by the following features. Prosternal process nearly truncated in lateral view. Fore legs of male distinctly longer than female. Seventh abdominal sternite twice as long as the sixth; pygidium distinctly exposed from the apices of elytra in male. The pubescence on abdominal sternites sparsely arranged or almost disappeared.

Male Genitalia ($n = 2$). Tegmen in lateral view slightly curved, rhombic, and widest near middle in ventral view. Ringed part slightly expanded laterad near middle of tegmen, thence arcuately narrowed basad. Lateral lobes about 1/3 of total length of tegmen, with inner side almost straight, and outer side expanded toward apical 4/5, thence obliquely narrowed to angularly rounded apex, provided with two kinds of setae of which one is long and thick, concentrated near apex, and another is rather short and thin, arising chiefly from outer sides of apical half.

Median lobe in lateral view gentry curved; apex in ventral view weakly pointed; median strut dehiscent from basal half.

Endophallus almost three times as long as median lobe in lateral view. The relative length of each broad membrane area of endophallus to median lobe is as follows: ML : TLE : BPH : MPH (MT+CT : PB) : APH = 3.5 : 10.0 : 2.5 : 6.0 (5.2 : 0.8) : 1.5. MPH with MT+CT well swollen and projected on ventral side near base. APH swollen in elongate oval shape, with ligulate-shaped AA near apex.

MSp distributed in nearly apical 2/3 of MT+CT. LSp on dorsal side distributed in nearly basal 1/3 of MT+CT, thick and short, arranged into two longitudinal irregular lines, unidentate in apical area, thence becoming thicker, denser and multidentate toward basal area; LSp on ventral side irregularly distributed in nearly basal 1/3 of MT+CT, short and rudimentary unidentate. SSp unidentate, short and small, covered basal 3/4 of PB. MSp area and LSp area adjacent. LSp area and SSp area close to each other.

Specimens Examined. 1♂, 1♀, Uinpia, Is. Miyako, 30–31. V, 1975, H. Makihara leg.; 5♂♂, 3♀♀, Aragusuku-kaigan, Is. Miyako, 7. VIII, 1999, T. Mizoguchi leg.; 1♂, 1♀, Is. Kurima, collected the logs at III, 1996 and emerged on VI, 1996, K. Shimizu leg.; 1♂, same locality, IV, 2003, K. Shimizu leg.

Distribution. Japan (South area of Is. Miyako and Is. Kurima of Miyako Isls., Okinawa Pref.).

3.12. *Agelasta (Dissosira) kumei* (Takakuwa, 1991), Comb. Nov. (Figures 4(g)-4(h), 6(h), 8(m)–8(p), and 10(d))

Mesosa (*Mesosa*) *kumei* Takakuwa, 1991: 51, illustration [32].

Diagnosis. This species is similar in the appearance to *A. konoi* or *A. perplexa*, but distinguishable by its large body size. Body black, covered with yellowish light ocher pubescence. Occiput with four longitudinal narrow black bands. Pronotum with three longitudinal black bands on disk. Elytra with three transversal yellowish white bands on behind humeri, near middle, and near apices. Pronotum with three rudimentary tubercles on disk. Prosternal process rounded in lateral view, and not truncate at the apex.

Male Genitalia ($n = 1$, *Partly Broken*). Tegmen in lateral view slightly curved, slender rhombic, and widest near middle in ventral view. Ringed part weakly expanded laterad near middle of tegmen, thence arcuately narrowed to basad. Lateral lobes slightly narrowed toward rounded apex, provided with two kinds of setae of which one is long and thick, concentrated on the apex, and another is rather short, thin, mainly arising from apical half.

Median lobe with apex roundly pointed in ventral view.

Basal half of endophallus is lost. LSp thick and short, arranged into two longitudinal irregular lines on dorsal side of MT+CT, unidentate in apical area, thence becoming thicker, denser, and multidentate toward basal area.

Specimens Examined. 1♀, Is. Lu-dao, Taitung county, Taiwan, 16–20. VI, 1989, Native leg.; 2♀♀, same locality, 5–10. VII, 1989, Native leg.; 1♂, Gangguan, alt. 0–10 m, same island, 5. IV, 2004, T. Kurihara leg.

Distribution. Taiwan (Is. Lu-Dao).

Remarks. The male genitalia of the examined specimen was partly broken, and we could not observe the basal half of median lobe and the basal part of endophallus. However, this species basically shares the external features, the shape of the tegmen of the male genitalia, and the characteristics of LSp of the endophallus with *A. konoi* or *A. yonaguni* and *A. perplexa*. It suggests that this species is closely related to these species. Therefore, we treat this species as a member of *Agelasta* (*Dissosira*).

4. Discussion

Among the species which have been classified into the nominotypical subgenus of *Mesosa*, 12 species distributed in the Oriental region are different from *Mesosa* (*Mesosa*) and have close relationship with *Agelasta* (*Dissosira*).

The genera *Mesosa* and *Agelasta* have mainly been distinguished from each other by the prosternal process, rounded or truncated in lateral view (e.g., Breuning [2]). The shape of the prosternal process is essentially stable in the Mesosini group, and it is worth defining the genera or subgenera by the external features. However, this structure is exceptionally variable and unstable in some groups of the genus *Agelasta*. Therefore, several species included in *Mesosa* have been confused with *Agelasta*, especially *Agelasta* (*Dissosira*), because of this variable structure (Yamasako and Ohbayashi [4]). Yamasako and Ohbayashi [4, 5] had already pointed out that the basic structure of the endophallus is very useful for defining the groups of Mesosini such as the genera or subgenera. Also, it is considered to be useful for analysis of the phylogenetic relationship. Therefore, the generic definition of Mesosini should be decided by the combination of the external and the genital features.

According to this point of view, the genus *Agelasta* is essentially distinguishable from *Mesosa* by the following characteristics: (1) antennal scape elongate, slightly thickened apicad, (2) lower lobes of eyes relatively large, (3) endophallus with LSp on the dorsal side arranged into two irregular longitudinal lines, unidentate in apical area, thence becoming thicker, denser and multidentate toward the basal area, rudimentary unidentate or almost disappeared on the ventral side, almost without AS. These differences suggest that the genera *Agelasta* and *Mesosa* are different phyletic groups.

On the basis of these characteristics, we transferred 12 species to *Agelasta* (*Dissosira*) that were previously classified into *Mesosa* (*Mesosa*) in spite of their rounded prosternal process in lateral view. They should be included in *Agelasta* (*Dissosira*) (sens. str. by Yamasako and Ohbayashi [4]) because they share external features and endophallic structures with the type species of the subgenus, *A.* (*D.*) *catenata*.

Agelasta (*Dissosira*) is widely distributed in the Oriental geographic region except for the Philippines, and its distribution extends northwardly to the Tokara Islands of Japan which is the northern end of the Oriental region.

Appendix

The examined specimen data for the type species of *Mesosa* (*Mesosa*) and *Agelasta* (*Dissosira*) in this study.

Mesosa (Mesosa) curculionoides

Specimens Examined. 1♂, Vestec, Czech, 30. V, 1946, Lenesch leg.; 1♂, 1♀, Leitha-Geb., b. Eisenstadt, 6. VI, 1976, BGLD; 3♂♂, Olympie-Péloponèse, Greece, 2. VII, 1981, A. Le Restif leg.; 1♂, Murauen, b. Mureck, 14. V, 1983, S-STMK; 1♂, Góry, Plock Dist., Poland, 18. V, 2002, M. Szewczyk leg.; 1♀, same locality, 20. V, 2003, M. Szewczyk leg.

Agelasta (Dissosira) catenata

Specimens Examined. 1♂, 1♀, Sayaboury, Laos, 14. VII. 1965, J. A. Rondon leg.; Same locality and collector, 9. VIII. 1965; 1♂, Ban Van Heua, Vientiane, Laos, 15. VIII. 1965 J. A. Rondon leg.; 1♀, Ile de Khong, Laos, 15. IV. 1965 J. A. Rondon leg.

Abbreviations

AA:	Appendix of apical bulb
APH:	Apical phallomer
AS:	Sclerite of apical phallomer
BPH:	Basal phallomer
CS:	Crescent shaped sclerites
CT:	Central trunk
LSp:	Large spicules
ML:	Median lobe
MPH:	Median phallomer
MSp:	Micro spicules
MT:	Medial tube
PB:	Preapical bulb
SSp:	Small spicules
TLE:	Total length of endophallus.

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Research Article

Limited Transmission of the Ectoparasitic Fungus *Hesperomyces virescens* between Lady Beetles

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The ectoparasitic fungus *Hesperomyces virescens* Thaxter (Ascomycota: Laboulbeniales) commonly infects the invasive lady beetle *Harmonia axyridis* (Pallas) and several other aphidophagous lady beetles in North America and Europe. We tested the hypothesis that bodily contact between adults of different lady beetle species supports horizontal transmission of *H. virescens*. We used laboratory assays to determine whether *H. axyridis* or *Olla v-nigrum* (Mulsant) harboring *H. virescens* (i.e., source beetles) transmit the fungus to noninfected target beetles *H. axyridis*, *O. v-nigrum*, *Coccinella septempunctata* L., *Coleomegilla maculata* (De Geer), or *Hippodamia convergens* Guerin-Meneville. Results indicate that intraspecific transmission (i.e., for the source beetles *H. axyridis* and *O. v-nigrum*) was common but interspecific transmission (i.e., from source *H. axyridis* or *O. v-nigrum* to target species) was low. Interspecific transmission occurred at low rates from *H. axyridis* to both *C. septempunctata* and *O. v-nigrum* and from *O. v-nigrum* to both *C. septempunctata* and *H. convergens*. Based upon our laboratory assays of forced pairings/groupings of source and target beetles, we predict that horizontal transmission of *H. virescens* between species of aphidophagous coccinellids is possible but likely rare.

1. Introduction

Laboulbeniales (Ascomycota) are ectoparasitic fungi and nearly all 2,000 described species are obligate parasites that grow on the integument of living arthropods, mostly insects, and usually on the adult stage [1, 2]. Within the ten insect orders that contain host species, about 80% of these parasitic fungi are on beetles (Coleoptera) [2]. Of particular interest are Laboulbeniales that infect Coccinellidae (Coleoptera). Four species of *Hesperomyces* (*H. chilomenis*, *H. coccinelloides*, *H. hyperaspidis*, and *H. virescens*) attack entomophagous Coccinellidae [1, 3, 4]. Of these species, *H. virescens* Thaxter infects more coccinellid species than the other three species [4]. Negative impacts of parasitism by *H. virescens* on lady beetle populations are not well defined, but Kamburov et al. [5] found that infected *Chilocorus bipustulatus* L. adults suffered premature mortality.

Known coccinellid hosts of *H. virescens* include *Adalia bipunctata* (L.), *Brachiacantha quadripunctata* Melsheimer, *Chilocorus stigma* (Say), *Chilocorus bipustulatus* (L.), *Eriopis connexa* Germar, *Cycloneda munda* (Say), *Cycloneda sanguinea* (L.), *Coccinula crotchi* (Lewis), *Coccinula sinensis* Weise, *Coccinella septempunctata* L., *Hippodamia convergens* Guerin-Meneville, *Harmonia axyridis* (Pallas), *Olla v-nigrum* (Mulsant), and *Psyllobora vigintimaculata* (Say) [1, 3–9]. Although *H. virescens* may occur on these species, it may not occur on some other coccinellid species found within the same habitat at the same time. For example, Harwood et al. [8] sampled lady beetles using Malaise traps and recorded *H. virescens* from *B. quadripunctata*, *C. munda*, *H. axyridis*, and *P. vigintimaculata*. The fungus was not on *Coleomegilla maculata* (De Geer) or *Hyperaspis signata* (Olivier) in those samples. Riddick and Cottrell [10] found *H. virescens* infecting *H. axyridis*, *H. convergens*, and *O. v-nigrum* when

beetles were collected using sweep nets. At the same time, *H. virescens* was not on *C. septempunctata*, *C. maculata*, *C. munda*, *Scymnus loewii* Mulsant, or *S. socer* LeConte. Harwood et al. [8] and Riddick and Cottrell [10] reported that the exotic *H. axyridis* had the highest percentage of infected individuals (82.3 and 50.1%, resp.) among the species sampled. Additionally Riddick and Cottrell [10] reported that *H. virescens* infected 33.1% of *O. v-nigrum* adults but only 4.7% of other species of adult lady beetles.

Horizontal transmission between adult Coccinellidae is via direct contact, usually during copulation but also within overwintering aggregations [11–15]. Indirect transmission of Laboulbeniales, that is, beetles infected from ascospores discharged onto a substrate is not likely [2].

Our goal was to use laboratory assays to test the hypothesis that bodily contact between different species of lady beetles provides an avenue for horizontal transmission of *H. virescens*. We paired infected beetles (i.e., source beetles) with noninfected beetles (i.e., target beetles) for varying times to determine whether transmission, within or between species, occurred. Additionally, we examined whether transmission within species occurred via indirect transmission under laboratory conditions.

2. Materials and Methods

2.1. Insects. We used adult beetles from laboratory colonies or field collections in experiments. We established laboratory colonies of *H. axyridis*, *C. maculata*, and *O. v-nigrum* from individual beetles collected at the USDA, ARS, Southeastern Fruit and Tree Nut Research Laboratory, Byron, GA, USA. Colonies were maintained on a diet of pecan aphids (Hemiptera: Aphididae), frozen *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs, and a meat-based diet (Beneficial Insectary, Redding, CA, USA) similarly as described by Cottrell [16]. Adult *H. convergens* and *C. septempunctata* were field-collected and maintained on a similar diet for 2-3 wk before use in experiments. Holding field-collected beetles in the laboratory before using them in assays permitted time to detect any infected individuals.

2.2. Ectoparasitic Fungus. We initiated separate colonies of *H. virescens*-infected *H. axyridis* and *O. v-nigrum* by collecting infected beetles from the field, confirming infection on beetles using a stereomicroscope, and maintaining beetles in the laboratory. We housed groups of infected beetles in containers (19 × 13.5 × 9 cm) and provided food and water as previously described. We perpetuated each of the infected colonies by the periodic addition of noninfected, laboratory-reared adult *H. axyridis* or *O. v-nigrum*. These infected beetles served as *H. virescens* source beetles used in fungus transmission studies. We did not consider beetle age or whether the beetle was field-collected or laboratory-reared when used in assays. Rather, we ascertained that mature thalli were present on the source beetles used in experiments and placed source and target beetles together, in the first transmission experiment (see below), with regard to thalli density on source beetles. Furthermore, we did not determine sex of beetles in order to reduce handling and potential spore dispersal prior to experimentation.

2.3. Transmission Studies

2.3.1. Seven-Day Exposure Experiment. We conducted this experiment using two separate trials, one in January and another in April 2010. For each trial, source *H. axyridis* and *O. v-nigrum* were tested separately against laboratory reared, target *H. axyridis*, *O. v-nigrum*, and *C. maculata*. Before trials began, source beetles were observed using a stereomicroscope, mature thalli were counted and each beetle was designated with high (≥24), moderate (14 to 23), or low (≤13) thalli density (range = 35). In both trials, we placed a source beetle (i.e., *H. axyridis* or *O. v-nigrum*) in a 9 cm diameter Petri dish with a target beetle (i.e., *H. axyridis*, *O. v-nigrum*, or *C. maculata*). Each trial consisted of three replicates of all possible source-target species combinations at a 1 : 1 ratio of source : target. Thus, we used five target beetles of one species per source species in each replicate for a total of 15 targets per trial and 30 targets for the experiment.

In the first trial, *O. v-nigrum* source beetles with similar thalli densities were lacking; therefore, not all five target beetles of each species (i.e., *H. axyridis*, *O. v-nigrum*, and *C. maculata*) were matched with *O. v-nigrum* source beetles harboring similar densities of thalli. However, similar treatments between target species within each replicate were achieved by using three targets each paired with a low thalli density source *O. v-nigrum*, a fourth target paired with a moderate thalli density source beetle and a fifth target paired with a high thalli density source beetle. Additionally during the first trial, all *H. axyridis* source beetles had low thalli densities thus two source beetles were placed with each target beetle (ratio of source : target = 2 : 1) to insure that an outcome showing a lack of transmission was not solely due to low thalli density of a single source beetle.

In the second trial, thalli density varied on both source species but source and targets were paired such that each of the five targets in each replicate was exposed to source beetles with similar thalli density. In both trials, Petri dishes containing source and target beetles were housed in an environmental chamber (25 ± 1°C and 14 : 10 [L : D]h) for seven days and provided food and water. After seven days, we removed source beetle(s) but kept the target beetle in that same Petri dish for one month. During this time, we examined target beetles three times per week under a stereomicroscope for development of mature (or at least nearly mature and identifiable) *H. virescens* thalli. We documented any target beetles containing at least one mature thallus and placed them into 70% ethanol for later confirmation of *H. virescens*.

2.3.2. Tumbled Beetles Experiment. In the previous experiment, pairs of same sex or interspecific source and target beetles may have hindered beetle interaction and affected *H. virescens* transmission. Additionally, thalli distribution on source beetles and the behavior of source beetles (e.g., not attempting to mate) could hinder *H. virescens* transmission. This experiment attempted to negate these factors by forcing

interaction between the source and target. Again, *H. axyridis* and *O. v-nigrum* were the source beetles. The target species were *C. septempunctata*, *C. maculata*, *H. axyridis*, *H. convergens*, and *O. v-nigrum*. We paired source and target beetles in 2-dram vials and then placed the vials on an automated roller (Nostalgia Electrics HRD-565 Hot Dog Roller, Nostalgia Products Group, LLC, http://www.nostalgiaelectrics.com/), as used in insecticide assays to coat the inner walls of vials, and rolled them at 2.6 revolutions/min for 1 h. Paired beetles within the rolling vial were active and tumbling and thus became entwined while attempting to remain upright and maintain their footing on the rotating glass vial (TEC, personal observations). After being tumbled, we placed target beetles into Petri dishes and maintained them in an environmental chamber with food and water for one month. We observed beetles three times per week under a stereomicroscope to detect any mature thallus, documented its presence and preserved infected beetles in 70% ethanol.

2.3.3. Extended Exposure Experiment. We attempted to facilitate transmission of *H. virescens* between source and target beetles by keeping them in close contact for an extended period. We grouped a single source beetle with three individuals of the same target species in a Petri dish and replicated four times ($n = 12$ target beetles per species). This was done using *H. axyridis* and *O. v-nigrum* source beetles with the target species *C. septempunctata*, *C. maculata*, *H. axyridis*, *H. convergens*, and *O. v-nigrum*. These beetles remained together for 6 wk and were fed, watered and observed for development of *H. virescens* thalli. When source and target beetles were of the same species, we easily differentiated the single source beetle and the targets by presence/absence of *H. virescens* and later by thalli density when we detected the first mature *H. virescens* thallus on a target beetle.

2.3.4. Substrate Borne Transmission Experiment. Temporal and spatial overlap of coccinellids in the field might allow for substrate borne transmission as potential hosts contact *H. virescens* ascospores. We placed two source *H. axyridis* adults in a Petri dish with food and water then removed them after 24 hr. Density of thalli on source beetles varied from low to high. Immediately following removal of the source beetles, five target *H. axyridis* beetles were added to the same dish and maintained in that dish, with food and water, for 7 d. This procedure was replicated using five dishes ($n = 10$ source and 25 target *H. axyridis*). After 7 d, we transferred the target beetles into a clean dish (and thereafter on an as-needed basis) and maintained them with food and water for the next three weeks. We used the same experimental design to examine substrate borne transmission when source *H. axyridis* remained in the Petri dish for 120 h. Additionally, we examined the potential of substrate-borne transmission of *H. virescens* by source *O. v-nigrum* to target *O. v-nigrum* using the same experimental design; source beetles were exposed to Petri dishes for 24 and 120 h. For both target species, we examined individual beetles three times per week, during weeks 2–4 of the study, for mature thalli using a stereomicroscope.

Table 1: Previously noninfected target lady beetles infected with the ectoparasitic fungus *H. virescens* when exposed in a Petri dish for 7 d to already infected source *H. axyridis* or *O. v-nigrum* lady beetles.

Source beetle	Target beetle	Proportion infected (±SE)*
H. axyridis	*C. maculata*	0 b
H. axyridis	*H. axyridis*	0.46 ± 0.11 a
H. axyridis	*O. v-nigrum*	0 b
O. v-nigrum	*C. maculata*	0 b
O. v-nigrum	*H. axyridis*	0 b
O. v-nigrum	*O. v-nigrum*	0.87 ± 0.06 a

*Within each source beetle, different letters following the proportion infected indicate a significant difference ($P < 0.05$) between target beetles.

2.4. Statistical Analysis. Within source species, we combined data from both trials of the seven-day exposure experiment. For both the seven-day exposure and tumbled beetles experiments, we used the nonparametric Kruskal-Wallis analysis of variance by ranks to analyze the proportions of infected beetles. We did this separately for when *H. axyridis* or *O. v-nigrum* was the source beetle. We analyzed proportions to take into consideration that some target beetles died before it was conceivable that successful transmission could have been identified and thus these individuals were not included in analyses. When a significant difference between species was found for the proportions infected, the Tukey-Kramer Honestly Significant Difference multiple comparison was used [17, 18].

3. Results

3.1. Seven-Day Exposure Experiment. When *H. axyridis* was the source, the only target species found with mature thalli was *H. axyridis*, and the proportion of infected beetles was significantly higher than no infection observed for *C. maculata* or *O. v-nigrum* ($\chi^2_{0.05,2} = 10.59$; $P = 0.0050$) (Table 1). From the January 2010 trial when source *H. axyridis* had different thalli density, 25, 50, and 25% of the newly infected target beetles had been housed with source beetles rated with low, moderate, and high thalli densities, respectively. Additionally, the average time (±SE) to detect mature thalli on target *H. axyridis* when exposed to source *H. axyridis* was 25.8 ± 1.5 d.

When source *O. v-nigrum* beetles were paired with target *H. axyridis*, *C. maculata*, or *O. v-nigrum*, the only target observed with mature thalli was *O. v-nigrum* and the proportion of infected beetles was significantly greater than for either *C. maculata* or *H. axyridis* ($\chi^2_{0.05,2} = 11.74$; $P = 0.0028$) (Table 1). From both trials, infection of target beetles exposed to *O. v-nigrum* source beetles with low, moderate and high thalli densities was 45, 30, and 25%, respectively. The average time (±SE) to detect mature thalli on target *O. v-nigrum* when exposed to source *O. v-nigrum* was 15.0 ± 0.4 d.

3.2. Tumbled Beetles Experiment. When we paired source *H. axyridis* in a glass vial with the target beetles *C. maculata*, *C. septempunctata*, *H. axyridis*, *H. convergens*, or *O. v-nigrum*

Table 2: Previously noninfected target lady beetles infected with the ectoparasitic fungus *H. virescens* when a pair of target and source lady beetles were placed in a vial and tumbled on a vial roller for 1 h. Source and target beetles were then separated and target beetles observed for mature thalli over the next month.

Source beetle	Target beetle	Proportion infected (±SE)*
H. axyridis	*C. septempunctata*	0.11 ± 0.06 b
H. axyridis	*C. maculata*	0 b
H. axyridis	*H. axyridis*	0.52 ± 0.11 a
H. axyridis	*H. convergens*	0 b
H. axyridis	*O. v-nigrum*	0 b
O. v-nigrum	*C. septempunctata*	0.14 ± 0.10 b
O. v-nigrum	*C. maculata*	0 b
O. v-nigrum	*H. axyridis*	0 b
O. v-nigrum	*H. convergens*	0 b
O. v-nigrum	*O. v-nigrum*	0.61 ± 0.11 a

*Within each source beetle, different letters following the proportion infected indicates a significant difference ($P < 0.05$) between target beetles.

for 1 h and observed the targets for the next month, the only targets observed with mature thalli were *H. axyridis* and *C. septempunctata*. However, horizontal transmission of *H. virescens* was significantly higher between beetles of the same, rather than different, species ($\chi^2_{0.05,4} = 10.28$; $P = 0.0360$) (Table 2). The average number of days before we observed mature thalli on *H. axyridis* and *C. septempunctata* targets was 17.9 ± 0.9 and 21.0 ± 0.0 d, respectively. When *O. v-nigrum* was the source, only *O. v-nigrum* and *C. septempunctata* targets were infected. Transmission of *H. virescens* to *O. v-nigrum* was significantly higher between individuals of the same species than between different species ($\chi^2_{0.05,4} = 10.28$; $P = 0.0360$) (Table 2). As an interesting note, the nonparametric statistics for the analysis of variance by ranks was identical for when *H. axyridis* or *O. v-nigrum* was the source. The average number of days before we observed mature thalli on *O. v-nigrum* and *C. septempunctata* targets was 14.5 ± 0.33 and 22.5 ± 1.5 d, respectively.

3.3. Extended Exposure. Not all beetles (source or target) survived to the end of this experiment. When *H. axyridis* and *O. v-nigrum* source beetles were housed with *H. axyridis* and *O. v-nigrum* target beetles, respectively, most source beetles either survived longer than respective target beetles or past the average time required, as reported in the prior experiments, to detect a mature *H. virescens* thallus on a target beetle (Table 3). As such, insufficient time to transmit the fungus was not of concern. We found a mature thallus on most *H. axyridis* targets (i.e., 83%) after 19 ± 1 d of contact with *H. axyridis* source beetles. Two noninfected target *H. axyridis* did not survive this long and likely died before a thallus could mature and be recorded (Table 3). Average survival time of other noninfected target species, except *O. v-nigrum*, exposed to source *H. axyridis* was longer than 19 d (Table 3). We did not find mature thalli on *C. septempunctata*, *C. maculata*, and *H. convergens* after confinement with source *H. axyridis*. Only one *O. v-nigrum* target, confined with a source *H. axyridis*, harbored a mature thallus at 21 d (a longer period of thallus development than previously noted for target *O. v-nigrum* infected by source *O. v-nigrum*). Three other target *O. v-nigrum* surviving longer than 21 d with the source *H. axyridis* beetles were not infected.

Table 3: Average days (±SE) that *H. virescens* source lady beetles and target lady beetles (that were not infected during the experiment) survived when one source lady beetle and three target lady beetles were housed together in Petri dishes for 44 days.

Source beetle	Target beetle	Average days (± SE) source survived	Average days (± SE) noninfected target survived
H. axyridis	*C. septempunctata*	21 ± 8	39 ± 4
H. axyridis	*C. maculata*	32 ± 7	38 ± 3
H. axyridis	*H. axyridis*	N/A[a]	9 ± 4[b]
H. axyridis	*H. convergens*	23 ± 2	30 ± 5
H. axyridis	*O. v-nigrum*	29 ± 9	16 ± 5
O. v-nigrum	*C. septempunctata*	20 ± 2	44 ± 0
O. v-nigrum	*C. maculata*	20 ± 7	33 ± 5
O. v-nigrum	*H. axyridis*	17 ± 4	34 ± 4
O. v-nigrum	*H. convergens*	22 ± 7	35 ± 4
O. v-nigrum	*O. v-nigrum*	5[c]	7 ± 1[d]

[a]All source beetles either survived longer than target beetles or past the time when a mature thallus was detected on infected target beetles.
[b]Two beetles survived for 5 or 13 d. A mature thallus was detected on all other target beetles between 15 and 21 d.
[c]One beetle survived only 5 days and others either survived longer than target beetles or survived past the time when infection was detected on target beetles.
[d]Most beetles (75%) died before mature thalli were likely to have been observed. Two of three that survived longer than 7 d were observed infected.

We found two target *O. v-nigrum* infected after 13 ± 0 d when housed with source *O. v-nigrum* and these two infected targets represent 100% of available *O. v-nigrum*. The other *O. v-nigrum* targets (i.e., 83%) did not survive long enough for *H. virescens* thalli to mature (Table 3). The only other target found infected by source *O. v-nigrum* was *H. convergens*. A mature thallus was found on three (i.e., 30%) *H. convergens* after 17 ± 2 d (excluding two beetles that survived for only 13 d). Noninfected targets (i.e., *C. septempunctata*, *C. maculata,* and *H. axyridis*) survived longer than the time required before detection of a mature thallus on target *O. v-nigrum* or *H. convergens* (Table 3).

3.4. Substrate-Borne Transmission. We found no evidence of substrate-borne transmission of *H. virescens* (as suggested by a mature thallus on a target beetle) between adults of the same species, for either *H. axyridis* or *O. v-nigrum*. Mortality of target *H. axyridis* (mean ± SE) that remained alive for at least 15 d was 8 ± 8 and 12 ± 8% whether source beetles were exposed to Petri dishes for 24 or 120 h, respectively; similarly, mortality of target *O. v-nigrum* was and 8 ± 5 and 16 ± 10%, respectively.

4. Discussion

Transmission of *H. virescens* from a source to a target beetle was successful when a mature (or nearly mature) *H. virescens* thallus was on the target beetle [19]. In the absence of a mature thallus on a target beetle, we made no observations regarding whether ascospores transferred from source to target beetles. As such, we do not comment on whether absence of transmission resulted from transferred ascospores that germinated on the target beetle but failed to develop to maturity. Many physical, chemical, and biological factors affect adhesion of fungal spores to surfaces with subsequent attachment to and germination [20], any or all of which may have affected successful transmission in this study.

Direct transmission of Laboulbeniales, generally via sexual contact and within overwintering aggregations, is likely the primary mode of dispersal within the Coccinellidae [6, 11, 12, 14]. Overall, substrate-borne transmission of Laboulbeniales is rare considering that the ascospore is short lived [2]. We did not attempt to group beetles to determine evidence of transmission via sexual contact. Rather, our study grouped beetles in situations unlikely to occur naturally but very likely to result in considerable contact between source and target beetles. Under these conditions, we observed transmission (as denoted by target beetles with mature *H. virescens* thalli) between source and target beetles when both occupied the same container at the same time. In contrast, we did not observe transmission when target beetles occupied a container after removal of source beetles. Results presented here support the hypothesis that direct bodily contact between coccinellid hosts is necessary for transmission of *H. virescens* [2] and other Laboulbeniales [19, 21].

Transmission of *H. virescens* between coccinellids of the same species was more common than between different species in this study. Note that the tumbling experiment forced all paired source and target beetles, regardless of sex or species, to make considerable contact and allowed for horizontal transmission of *H. virescens* spores. Despite this, transmission between coccinellids of the same species still dominated. In the tumbling experiment, we only documented transmission between different species with target *C. septempunctata* exposed to *O. v-nigrum* or *H. axyridis* source beetles. Tumbling that led to the discharge of spores (onto the glass vial), which were transmitted to hosts is unlikely but cannot be ruled out from the results provided here. In addition to *C. septempunctata*, the only other instances of transmission between species was with target *H. convergens* exposed to source *O. v-nigrum* and target *O. v-nigrum* exposed to source *H. axyridis*.

In general, a higher rate of successful pathogen transmission within the same species is not surprising. Even though some entomopathogenic fungi such as *Beauveria bassiana* (Balsamo) and *Metarhizium anisopliae* (Metschnikoff) can infect a broad range of host insect species, isolates within each of these species can have a high degree of host specificity [22]. In fact, Cottrell and Shapiro-Ilan [23] found relatively high host specificity of *B. bassiana* isolates from source *O. v-nigrum* when tested against target *O. v-nigrum* and target *H. axyridis*. A similar scenario appears likely for *H. virescens* infecting different species of Coccinellidae. Isolates/strains of *H. virescens* may exist under field conditions and only infect closely related Coccinellidae or even a single species. At present, isolates/strains of *H. virescens* are unknown. Perhaps *H. virescens* that has had several passes on the same species is more virulent toward that species than to other species, similarly as Steinhaus [24] described increasing virulence of an entomopathogen.

In this study, the time required to detect a mature thallus on a target was less when the source and target were both *O. v-nigrum* than when the source and target were both *H. axyridis*. The separation in time to detect mature thalli on these two species is not explained simply by our schedule of observing specimens for mature thalli three times per week as opposed to daily. Even when we paired source and targets for one hour or seven days, detection of mature thalli occurred earlier when transmission was from *O. v-nigrum* to *O. v-nigrum* than from *H. axyridis* to *H. axyridis*. If there are no *H. virescens* isolates/strains infecting *O. v-nigrum* and *H. axyridis*, this time differential could be explained by nutritional quality of the two hosts and/or that more time is required for the ascospore to germinate and for the "foot" and haustoria to develop on *H. axyridis*. Although *H. virescens* may be pathogenic to numerous species of Coccinellidae, virulence against some species may be attenuated depending upon the number of passes it has gone through on other species, for example, *H. axyridis*. This could also explain why more time was required to detect a mature thallus on target *C. septempunctata*, infected from a source *O. v-nigrum*, than for *O. v-nigrum* intraspecific transmission.

As stated previously, our assay conditions used forced pairings/groupings not likely to occur in the field, yet we documented limited transmission between species. Direct contact between coccinellids of different species can occur in the field as promiscuous males attempt to mate with females of other coccinellid species [25, 26] (EWR, personal observation). Copulation (or mating attempts) between different coccinellid species has been observed in field cage tests among the phytophagous lady beetles *Henosepilachna yasutomii* Katakura and *H. niponica* Lewis [27]. Additionally, copulation attempts by a male *C. maculata* with an unidentified beetle species (Coleoptera: Cleridae) has been observed in the field (TEC, personal observation). Although we did not attempt to document copulation when source and target lady beetles were in Petri dishes for 7 d, we only observed transmission of *H. virescens* within the same host species. When source and target beetles were confined for an extended interval, mortality of source and target beetles was problematic in some groupings. Nonetheless, the results were similar as previously observed, that is, transmission of fungus occurred within the same host species with the exception of one target *O. v-nigrum* becoming infected from source *H. axyridis* and three *H. convergens* becoming infected from source *O. v-nigrum*. Infection through contact with substrate borne ascospores is rare among Laboulbeniales because the ascospore is short lived [2]. We did not find evidence of substrate-borne transmission between coccinellids in this study.

It is not clear why some coccinellid species sampled by Harwood et al. [8] and Riddick and Cottrell [10] had a relatively low prevalence of *H. virescens* infection. Their sampling methods, species abundance, or host specificity of *H. virescens* could have been influential. Further transmission studies on other species of Coccinellidae could provide insight regarding host specificity of *H. virescens*.

Interestingly, *H. virescens* represents one of the first parasites to infect *H. axyridis* in North America. Another parasite found on *H. axyridis* in North America is the ectoparasitic podapolipid mite *Coccipolipus hippodamiae* (McDaniel and Morrill) [28]. After *H. axyridis* established and quickly dispersed across North America, natural enemies may now be adapting to it. What impact *H. virescens* has on the dynamics of any coccinellid population is yet to be determined given that it is reported to cause anywhere from little impact to premature mortality [5, 6, 29].

In conclusion, it is likely that high host specificity and an apparent need for substantial periods of close contact between potential hosts will limit transmission of *H. virescens* by *H. axyridis* and *O. v-nigrum* with other coccinellids in the field [8, 10].

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Research Article

A System for Harvesting Eggs from the Pink-Spotted Lady Beetle

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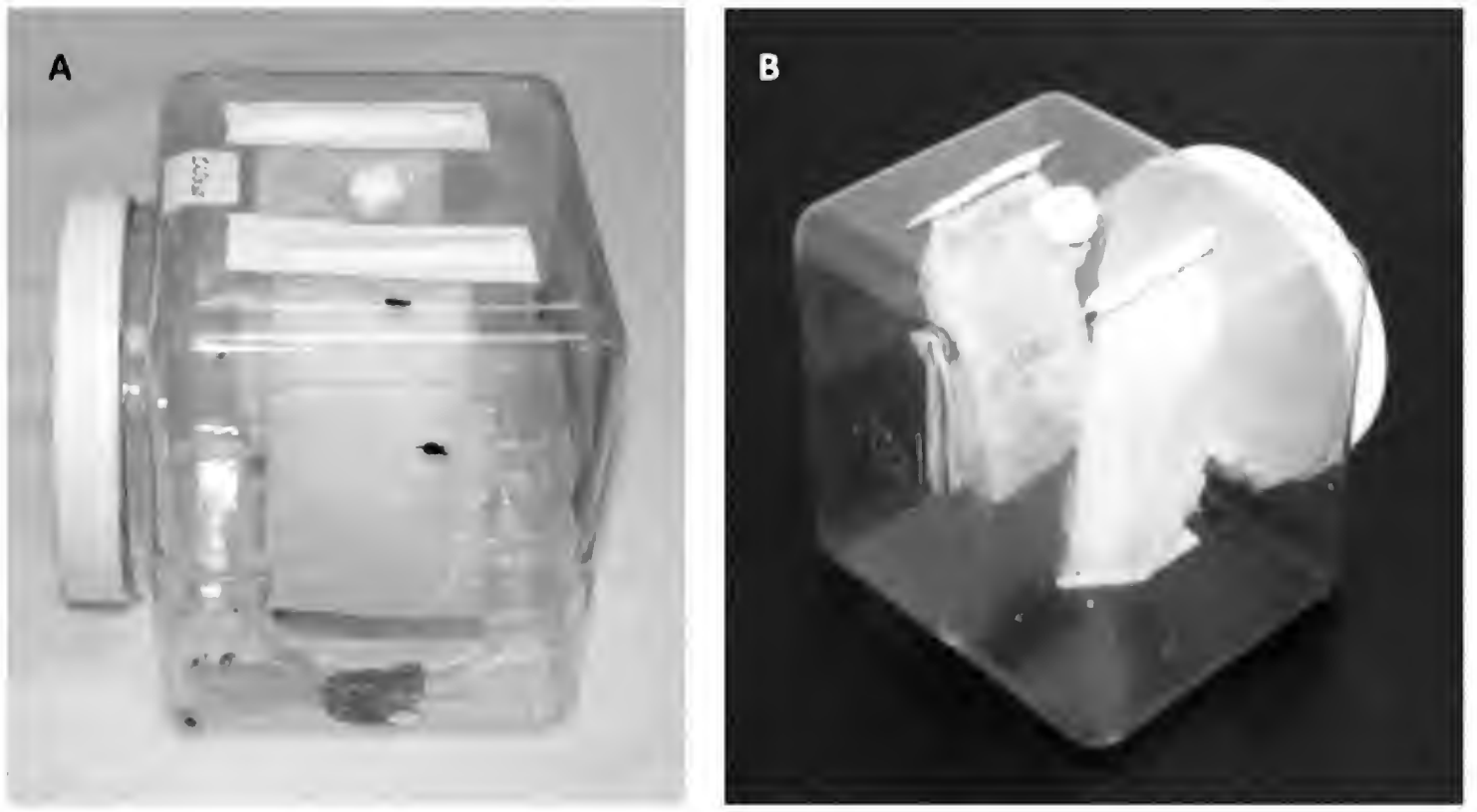
We describe a system for harvesting eggs from a predatory insect, the pink-spotted lady beetle, *Coleomegilla maculata* De Geer (Coleoptera: Coccinellidae). Adult beetles placed in square, transparent containers that included oviposition substrates hanging from the top of the cage deposited eggs on the materials provided. We harvested eggs from these substrates in quantities sufficient for either destructive sampling or synchronous development of larvae. We evaluated effects of crowding inside cages; effects of a chemical attractant on oviposition behavior; egg cannibalism. Females preferred a textured surface rather than a smooth, waxy one for laying eggs. Crowding inhibited oviposition of beetles. Presence of a chemical attractant (methyl salicylate) did not significantly improve oviposition. This paper describes an inexpensive system for harvesting eggs from *C. maculata*. Refinement of this system should improve oviposition and reduce cannibalism.

1. Introduction

The pink-spotted lady beetle, *Coleomegilla maculata* De Geer (Coleoptera: Coccinellidae), is a generalist predator native to North America [1]. In nature, it feeds on a wide range of soft-bodied insects and mites, and plant products (e.g., pollen, bean leaf tissue [2]) in managed and unmanaged landscapes [3–6]. In its native range, *C. maculata* does not overwinter in houses or become a nuisance pest. *C. maculata* is amenable to rearing, which makes it a good prospect for commercial production and genetics studies. Although several diets for *C. maculata* have been described for specific purposes [2, 7–13] and used in risk assessment studies [14], there is no single-standardized artificial food available for this species. Furthermore, cage systems for rearing *C. maculata* on a large scale are virtually unknown.

Our goal in this study was to develop a technique to harvest eggs of this predatory species in quantities sufficient for RNA/DNA extraction or large-scale production of insects for release studies or augmentative biological control. The oviposition behavior of *C. maculata* in a natural environment is well studied [6]. Females prefer ovipositing on plants with epidermal hairs (trichomes) on leaf surfaces rather than plants with smooth surfaces, devoid of trichomes [15, 16]. In laboratory conditions, individual females oviposit on smooth surfaces such as containers of food or water [7] or on the smooth surface of a Petri dish or other enclosures [8]. Removal of these eggs from the dish surface is possible but time consuming and often damages the eggs, resulting in poor-quality samples for downstream processes such as nucleic acid extractions (personal observation). Thus, one goal for an artificial system was to identify an oviposition substrate that could encourage oviposition and facilitate successful harvesting of eggs. Methyl salicylate (MeSA) is a common herbivore-induced plant volatile that attracts beneficial insects in many crops [17, 18]. It has been shown to attract at least one lady beetle [19]; however, the potential to stimulate or promote oviposition is unknown.

Cannibalism often occurs in the field and in the laboratory [8], which suggests that this behavior is an adaptive strategy for this species [20]. Thus, eggs are a challenge to obtain because both larvae and adults cannibalize them when reared under crowded conditions (personal observation). An additional goal of the research was to identify conditions that decreased cannibalism. We describe a cage system for harvesting lady beetle eggs for ecological and genetics experiments.



(a) (b)

FIGURE 1: Enclosures for collecting *Coleomegilla maculata* eggs: cage for adults including food, water, insects, and hanging oviposition substrates (a) and cage for performing oviposition substrate choice assay (b).

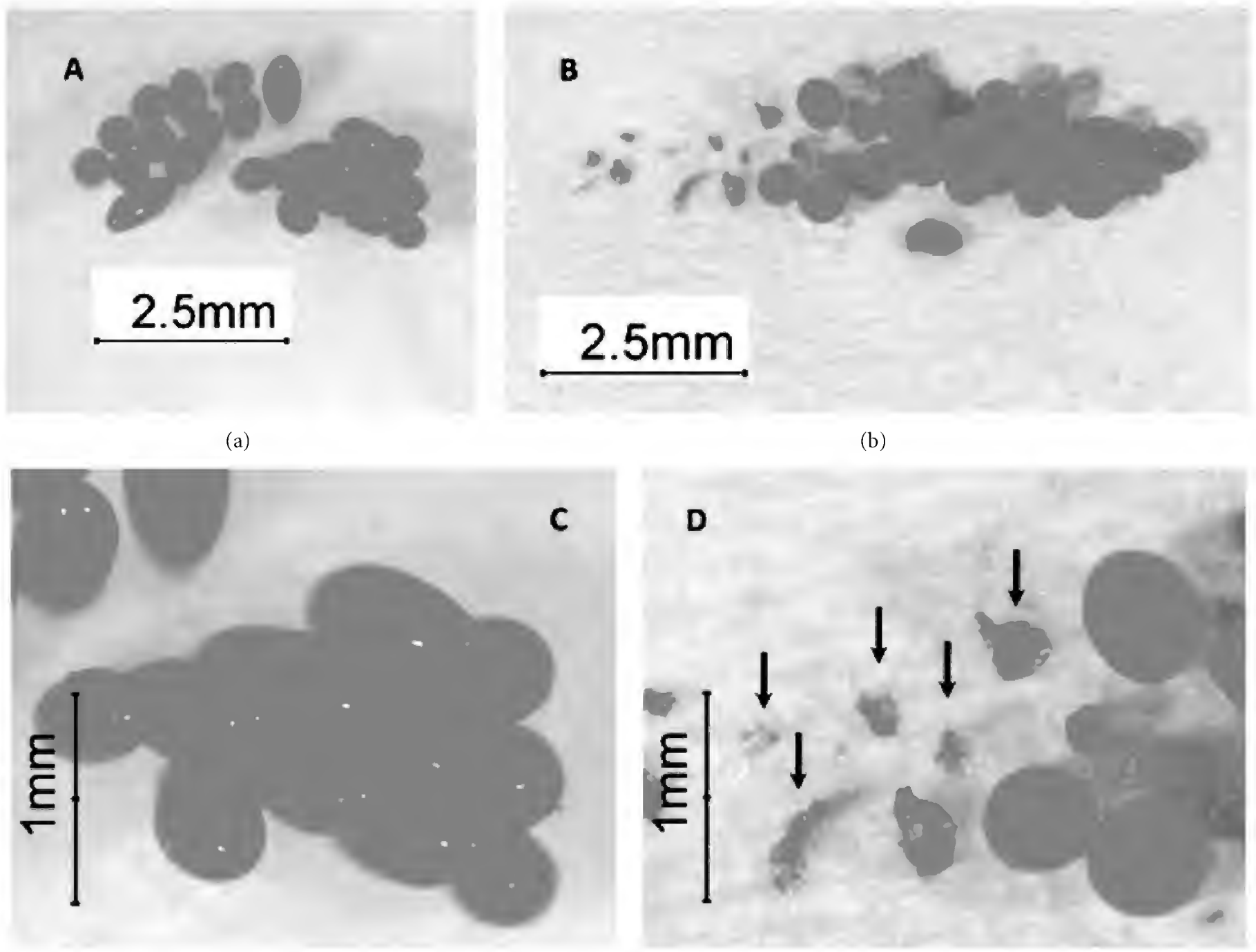
2. Materials and Methods

2.1. Insects. We established a laboratory colony of *C. maculata* from adults in continuous culture from Beltsville, Maryland, USA and Brookings, South Dakota, USA. Predators were cultured initially in standard disposable Petri dishes (100 × 15 mm), until populations demanded larger containers. Adult and larval stages were fed a combination of foods including bee pollen (Y. S. Organic Bee Farms, Sheridan, IL, USA), Brewer's yeast, honey, powdered sugar, and *Daphnia* (Hikari Bio-Pure, Hayward, CA, USA), and live eggs of plant bugs (*Lygus lineolaris* Palisot de Beauvois or *Lygus hesperus* Knight) in excess. We provided a free source of water on cotton balls in microcentrifuge tubes (1.5 mL capped with cotton) at the base of each cage. We maintained insects in an environmental chamber with a 16L: 8D lighting schedule at 23°C and 55%RH during the day and 20.5°C and 52%RH during the night.

2.2. Cages. Adult cages were clear plastic containers made by Rez-Tech Company (Kent, OH, USA). The dimensions of the cage were 14 cm × 14 cm × 15 cm, capacity 2,000 mL. We cut two slits (10 cm long) and a single 2 cm (diam) hole into one side of the container; oviposition substrates were in the slits (Figures 1(a), 1(b)), at a hanging position. Oviposition substrates were at the top of the cages, at some distance from food sources (at the base of the cage). Our hypothesis was that females would be less likely to cannibalize conspecific eggs if they deposited them on substrates that offered some seclusion away from food sources. To harvest eggs, we simply removed oviposition substrates, examined them, and replaced them with new ones. Females deposited their eggs in distinct masses (batches) on these substrates. We plugged the 2 cm diameter hole with cotton when not transferring beetles into cages.

2.3. Influence of Substrate Type on Oviposition. Materials tested as oviposition substrates were Kimwipes, providing a textured surface, and the paper backing of Parafilm strips, providing a smooth surface. We used five subsample cages, containing between 25 and 50 adults of similar age, at least 1 wk after adults eclosed. Male-to-female sex ratio was approximately 1 : 1 in all cages. We replaced insects that escaped or died. Each cage contained a set of textured and a set of smooth oviposition substrates of equal surface area (Figure 1(b)) and thus provided equal choice for oviposition. Each set consisted of eight individual sheets. We collected eggs each day (Monday–Friday) for 17 total collection dates. We recorded number of egg masses, number of eggs intact, and those cannibalized. Cannibalized eggs had remnants of eggs left on the substrate (Figure 2). Each day of egg collection represented a single sample, and the five cages provided replication of the treatments per day. We recorded number of egg masses and total eggs produced in each cage. We used total eggs collected from oviposition substrates per day for data analysis.

2.4. Influence of Crowding on Oviposition and Incidence of Cannibalism. To determine the influence of crowding on oviposition, we manipulated the number of adults in cages. Adult specimens were reproductively active (10–25 days old) and placed in cages as follows. In the first test, cages contained 100 females with 100 males, 50 females with 50 males, 25 females with 25 males, or 10 females with 10 males for a total of 4 cages. In the second test, a single cage contained 20 females with 20 males, 20 females without males, 10 females with 10 males, or 10 females without males for a total of 4 cages. Because of the large number of males and females manipulated in this experiment (in 8 total cages), it was not practical to use the cage as the source of replication. Using Kimwipes tissue as oviposition substrate



(a) (b)

(c) (d)

FIGURE 2: Intact (noncannibalized) eggs of *C. maculata* in a mass (a, c) and cannibalized eggs in a mass (b, d). Arrows indicate remnants of cannibalized eggs on substrate (d).

based on results from the study described above, we collected eggs from oviposition substrates from each of these cages for 10 consecutive days. Two sets of hanging oviposition substrate were used in each cage (see Figure 1(a)). We recorded number of egg masses and total eggs produced in each cage. We also estimated number of eggs cannibalized as previously described. We used total eggs collected from oviposition substrates per day for data analysis.

2.5. Influence of an Attractant on Oviposition. An arena was constructed from clear plastic containers of two sizes, the size mentioned above and a central chamber approximately twice as large, 14 cm × 14 cm × 30 cm, capacity 4000 mL, connected by clear plastic tubes. We provided food and water in the central chamber, while an oviposition substrate was in each of the two lateral chambers (Figure 3). We released 40 male and 40 female adults of reproductive age into the central chamber. Just prior to attachment of the oviposition substrate in one of the lateral chambers, we pipetted 2 μL of pure methyl salicylate (Alfa Aesar, Ward Hill, MA, USA) onto the central upper portion of the set of substrate leaflets. This treatment produced a human-detectable scent. We counted insects directly after chamber attachment and again at 4, 20, 24, and 28 hr, and a final count and egg collection at 44 hr. We determined gender of beetles in both chambers after 44 hr. To control for the possibility of methyl salicylate incorporation into the plastic, containers were washed thoroughly after each experiment with warm soapy water, then rinsed twice with bleach, and then rinsed with 70% ethanol followed by another rinse with distilled water. The containers were then allowed to dry in sunlight for 24 hours. We replicated the experiment five times with new insects used in each replicate. Each replicate was set up in a glasshouse at noon, with the same arena orientation to avoid any influence of daylight on movement of insects into lateral chambers. After final assembly and initial tally of insect movement (within 2 min), the arena was relocated to an environmental chamber as described above. We used the total number of insects in control or test lateral chambers and the number of intact eggs on oviposition substrates at the end of the experiment for data analysis.

2.6. Data Analysis. Experiments were set up following a completely randomized design. We used the paired t-test

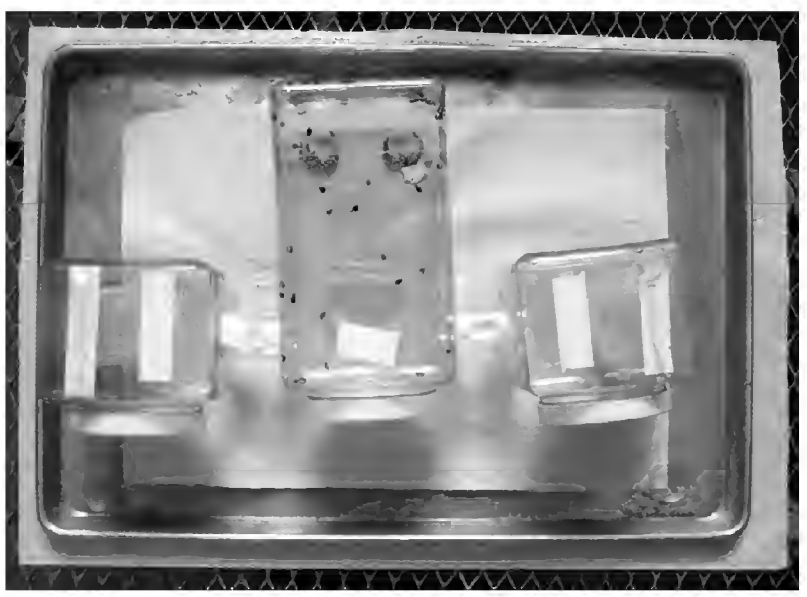


Figure 3: Three-chambered arena designed to measure beetle movement and oviposition in response to odors from methyl salicylate (MeSA). Beetles are visible in the central chamber.

to evaluate substrate type (textured versus smooth) on oviposition and cannibalism. We used one-way repeated measures analysis of variance (RM-ANOVA) using the Holm-Sidak method to evaluate the significance of crowding on oviposition and cannibalism. We also used a paired t-test to evaluate the influence of an attractant on insect movement and oviposition. Absolute data were square root transformed prior to analysis. Means were significantly different if $P < 0.05$. We used SigmaStat 3.0.1 (Systat Software Inc., Richmond, CA, USA) for data analysis. All data presented herein represent nontransformed values.

3. Results

3.1. Influence of Substrate Type on Oviposition. Over the course of the experiment, we collected 120 and 79 egg masses from textured and smooth substrates, respectively. The mean ± SEM number of eggs on textured and smooth substrates per cage per day was 19.0 ± 2.9 and 12.1 ± 2.5 eggs, respectively. Significantly more eggs were on textured substrate ($t = 2.2$, df = 16, $P = 0.046$).

We observed cannibalism of eggs on both oviposition substrates. The mean ± SEM number of eggs cannibalized on textured and smooth substrates per cage per day was not significant (3.7 ± 0.9 and 3.0 ± 0.8 eggs, resp.) ($t = 1.0$, df = 15, $P = 0.32$).

3.2. Influence of Crowding on Oviposition and Incidence of Cannibalism. We found that crowding of *C. maculata* adults (with males) in cages affected oviposition and egg cannibalism (Table 1). More eggs were laid by 10 females (with males) than by 50 or 100 females (with males) in experimental cages per day ($F = 6.6$, df = 3, 27; $P = 0.002$). The number of eggs laid by 25 females (with males) did not differ from other treatments. More eggs were eaten (cannibalized) by adults in cages with 25 females (with males) than with 100 females (with males). Cannibalism of eggs in the other treatments, 10 females (with males) and 50 females (with males), did not reveal any significant differences from the other treatments. Crowding of *C. maculata* females (with or without males) at adult densities tested did not significantly affect oviposition rate or egg cannibalism (Table 2). No differences were detected between treatments consisting of 10 females with or without males and 20 females with or without males ($F = 0.16$, df = 3, 27; $P = 0.9$).

Table 1: Effects of crowding (of *C. maculata* females with males) on the mean ± SEM number of eggs laid and cannibalized per cage per day.

Treatment	Eggs laid	Eggs cannibalized
10 females + males	24.3 ± 5.8^{a}	2.6 ± 1.3^{ab}
25 females + males	10.4 ± 4.5^{ab}	3.2 ± 1.4^{a}
50 females + males	6.5 ± 3.9^{b}	0.6 ± 0.6^{ab}
100 females + males	1.7 ± 1.6^{b}	0^{b}

Means ± SEM followed by a different letter in a column are significantly different ($P < 0.05$).

Table 2: Effects of crowding (of *C. maculata* females with or without males) on the mean ± SEM number of eggs laid and cannibalized per cage per day.

Treatment	Eggs laid	Eggs cannibalized
10 females + males	9.2 ± 2.8^{a}	3.3 ± 1.6^{a}
10 females	10.3 ± 5.1^{a}	0.1 ± 0.1^{a}
20 females + males	9.7 ± 3.4^{a}	3.1 ± 2.0^{a}
20 females	9.3 ± 4.3^{a}	0.5 ± 0.5^{a}

Means ± SEM followed by the same letter in a column are not significantly different ($P > 0.05$).

3.3. Influence of Attractant on Insect Movement and Oviposition. The attractant (methyl salicylate) affected the number of *C. maculata* adults moving in the arena (Table 3). More insects were in the test chamber than in the control chamber ($t = 3.3$, df = 5, $P = 0.02$). Males and females were moving into both chambers, as illustrated in Figure 4. The number of eggs laid in test and control chambers did not differ significantly ($t = 0.47$, df = 4, $P = 0.66$).

4. Discussion

The results described indicate that an easily accessible and inexpensive material can serve as an oviposition substrate for high volumes of egg collection. The use of hanging strips of tissue with a moderately textured surface provides a means of collecting multiple egg masses from groups of insects, while allowing assortive mating within the population. The hanging substrate provided refugia for resting, mating, and oviposition and was relatively easy to handle. Yellow or orange egg masses were easily visible on the white substrate and could be clipped from the leaves and transferred to another container or harvested for downstream processing.

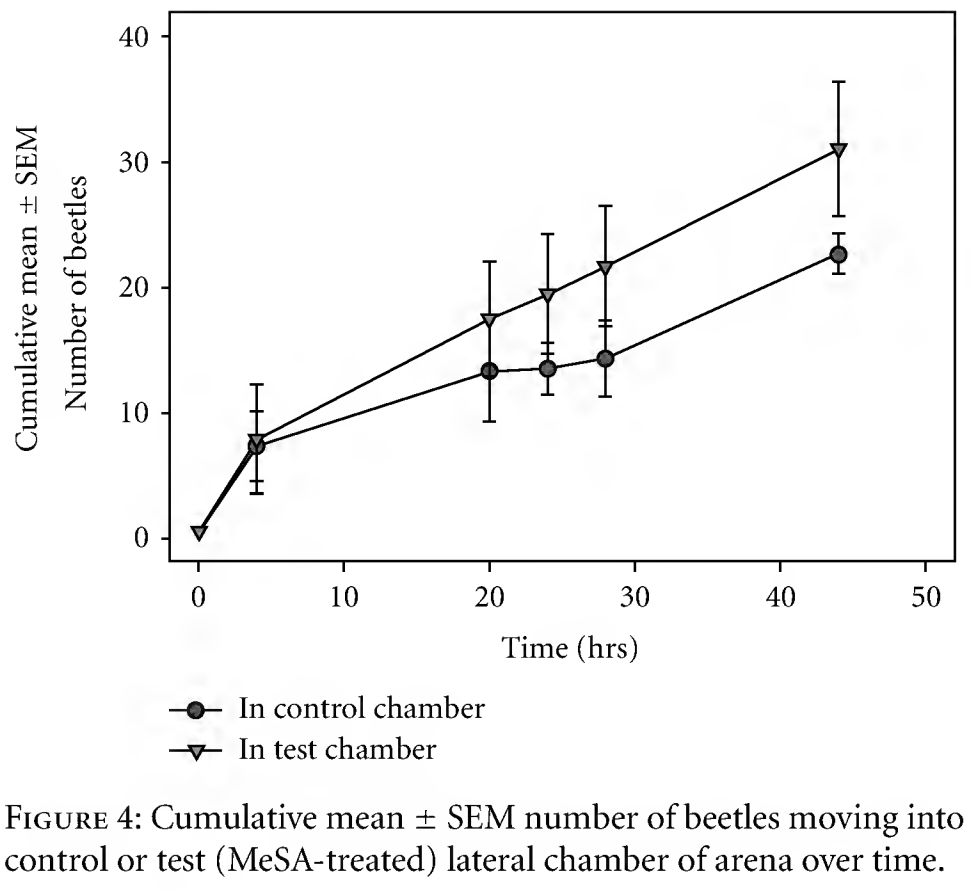


FIGURE 4: Cumulative mean ± SEM number of beetles moving into control or test (MeSA-treated) lateral chamber of arena over time.

TABLE 3: Effects of attractant (methyl salicylate) on the mean ± SEM number of *C. maculata* adults and eggs in control and test chambers of arena.

Treatment	Adults per arena	Gender	Eggs laid
Control chamber	11.8 ± 3.0^{a}	11 males: 12 females	55.2 ± 27.1^{a}
Test chamber	16.3 ± 4.4^{b}	12 males: 19 females	58.2 ± 26.0^{a}

Means ± SEM followed by a different letter in a column are significantly different ($P < 0.05$).

We describe an oviposition choice assay for laboratory culture. *C. maculata* prefers to oviposit on leaves with trichomes rather than on smooth leaves in the field [16]. Another study of this predator utilized sulfite paper as an oviposition substrate [11]. Our study indicates that in laboratory culture, a smooth paper substrate is also less preferable. More egg masses and total eggs were deposited on the textured surface compared to the smooth surface. Repeated mating may be important for cultures of *C. maculata*, and mating in this species has been shown to be density dependent and enhanced by male isolation [21]. Our crowding experiments suggest that high population densities of *C. maculata* restrict mating and oviposition. While some density of insects below 50/2000 mL was shown to be more optimal for egg production than 100 or 200/2000 mL, our tests do not identify a precise number or sex ratio per container to maximize egg production or minimize cannibalism. Additional replicated experiments may clarify this. It should be noted that the decrease in egg production in cages containing 100 or 200 individuals could represent cannibalism that was not detected. While every effort was made to visualize remnants of eggs that would indicate cannibalized egg masses, it is possible that eggs produced in those cages were completely consumed. Further research will help clarify this.

Regrettably, we did not extend our study to assess the influence of oviposition substrate and predator density on hatch rate and fertility of eggs. An examination of these life-history parameters is important to developing a mass rearing system, and we should consider them in a followup study.

Egg cannibalism by *C. maculata* is common in the laboratory and in the field [22, 23]. Neonates of this species feed on conspecific eggs and apparently benefit from this behavior [23]. Some studies on this species suggest that larvae should be provided individual containment to curb cannibalism [8, 11]. Because neonates remain on their egg mass for a period of time (24–48 hr) after hatch, daily or other periodic collection of eggs will reduce egg plus neonate interactions as well as egg plus adult interactions. Our results did not identify a density or sex ratio that reduced cannibalism. Cannibalism was not reduced significantly on either of the substrates tested. It has been suggested that pubescence on leaves may interfere with foraging, thus plants with glandular trichomes "provide ovipositional refuges from cannibalism" [16]. Additional surfaces such as fabrics may provide oviposition substrates that reduce such losses. Our collections were daily, and research has shown that oviposition by *C. maculata* is periodic, with eggs laid primarily afternoon and before midnight [16]. Egg collection might be optimized by either varying timing or frequency of collections or by restricting substrate availability. The cage system described here is ideal for these experiments.

We attempted to answer the question of how to stimulate oviposition from groups of insects using the arena method and oviposition substrates. Ovipositional stimulants have been tested for this species. For example, extracts from wood [24] and extracts of *Juniperus virginiana* L. plants [25] were found to stimulate oviposition from individual females. Methyl salicylate is not an oviposition stimulant for *C. maculata* based on our preliminary results. Future research should evaluate a variety of other compounds at a range of concentrations.

Results presented here demonstrate a system that produces substantial quantities of insect biomass at defined stages of development. The cage system described yielded the desired quantity of eggs; a set of six cages with ten females per cage can supply 100–200 eggs per day on a continuous basis (results not shown). RNA extraction for gene expression requires specimens at identical developmental stage. Eggs and newly hatched larvae are the smallest stage of these insects, at roughly 200 μg per individual. Because standard extraction kits utilize tissue samples 20–40 mg, a minimum quantity of 100 individuals per extraction sample is necessary. Furthermore, because it is more convenient to process samples of at least two at a time, a minimum quantity of 200 individuals is desirable. Researchers need to be able to culture candidate species in large quantities for large-scale genetic sequencing or on small scales for individual pair mating and selective inbreeding. Facilitated by studies described here, we have collected nucleic acids from every life stage of *C. maculata* and selected isofemale strains. Further refinement of this system should improve oviposition and reduce cannibalism. Then we can potentially scale up this system for commercial mass rearing.

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Research Article

Laboratory Rearing of *Laricobius nigrinus* (Coleoptera: Derodontidae): A Predator of the Hemlock Woolly Adelgid (Hemiptera: Adelgidae)

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Coleopteran species are biological control agents of numerous invasive pests. *Laricobius nigrinus* (Coleoptera: Derodontidae), a predaceous, univoltine species, spends the summer aestivating but is active for the rest of the year. *Laricobius nigrinus* possesses many essential attributes for effective biological control of the hemlock woolly adelgid (Hemiptera: Adelgidae). The predator must be reared in large numbers for field releases. We describe some of the studies that led to the successful procedures currently used for mass rearing *L. nigrinus*.

1. Introduction

Laricobius nigrinus Fender (Coleoptera: Derodontidae) is a potential biological control agent of hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), an exotic pest that attacks and kills hemlock trees (*Tsuga canadensis* L. (Carr.) and *T. caroliniana* Engelm.) in the eastern United States. Since its first release in 2003 [1], *L. nigrinus* has been established in the plant hardiness zones 7a, 6b, and 6a in the USA [2].

The ability to mass rear a biological control agent is fundamental in any classical biological control program. Delays in the program are often related to difficulties in laboratory rearing [3]. Biological control of HWA was hindered by poor success in mass rearing promising predators. Efficient rearing methods for producing large numbers of *L. nigrinus* is critical for it to be a viable biological control agent of HWA. HWA infests eastern hemlock in over 40% of its geographic range [4] and continues to spread [5], causing extensive damage and mortality of *Tsuga* spp. in the eastern USA [6, 7].

Laboratory rearing of *L. nigrinus* at Virginia Tech was initially constrained by high mortality rates and a lack of knowledge as to which life stages incur significant mortality because *L. nigrinus* has an obscure and complicated lifecycle. Additionally, adults have no observable sexual dimorphism [8]. They emerge from the soil in the fall and feed on developing HWA nymphs throughout the winter [9]. In early spring, eggs are laid in adelgid ovisacs where the larvae hatch and develop through four instars feeding on HWA eggs [10]. Mature larvae drop to the soil, each forms a pupal cell, pupates, and enters aestivation as an adult for the summer.

1.1. The Sequential Development of a Rearing Procedure from 2000 to 2010. Initially, adults of predators were reared on HWA-infested twigs maintained in flat-bottom Plexiglass oviposition cages containing a layer of peat moss. Fourth instars, upon reaching maturity, dropped down to the layer of peat moss on the floor of the cage, where they pupated and developed into adults.

Two primary objectives that resulted from our initial rearing efforts were to

(i) Identify the life stages of *L. nigrinus* that incur high developmental mortality in the laboratory,

(ii) Improve production at each life stage where survival was low.

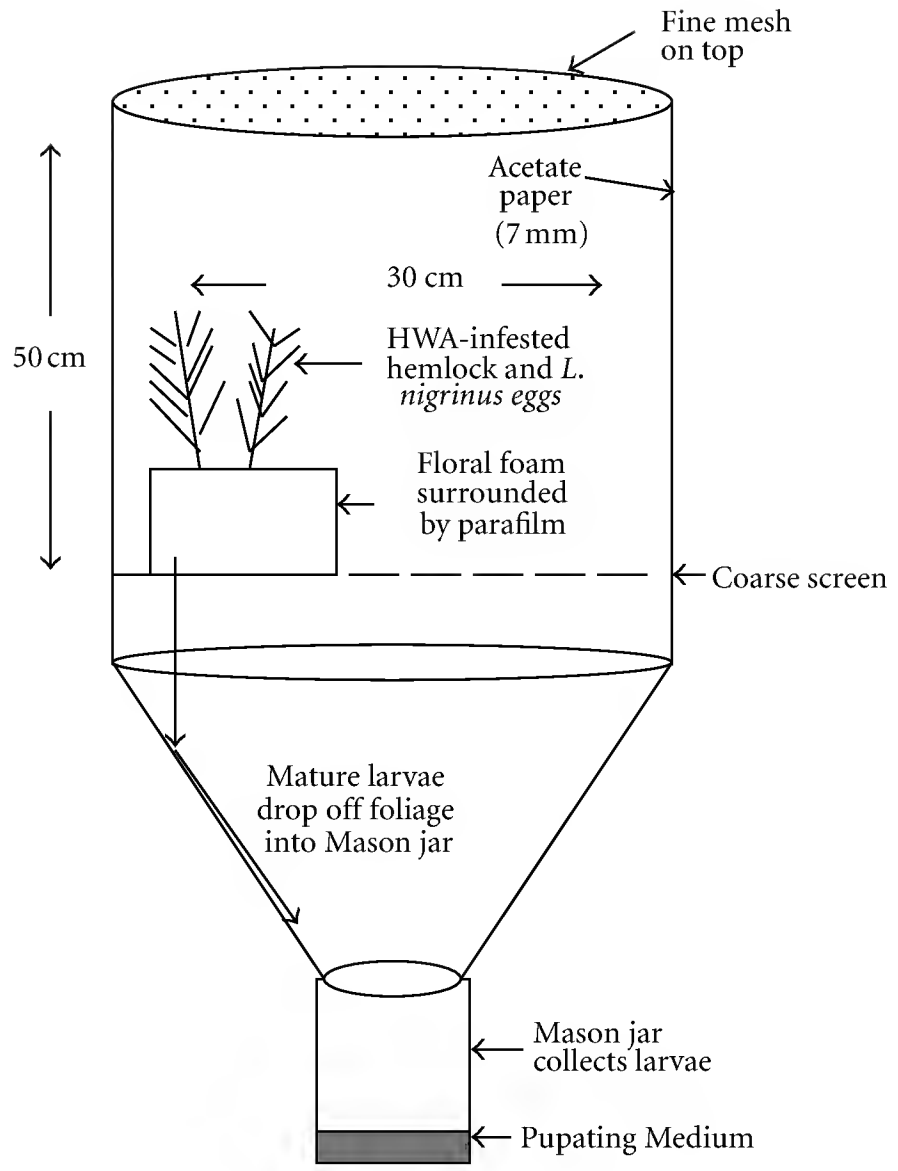

FIGURE 1: Diagram of the form and function of the *Laricobius nigrinus* larval rearing cage.

2. Methods and Materials

Studies were partitioned into two periods. Efforts focused on determining/enhancing survival of each life stage from 2000 to 2004. Emphasis on improving production numbers of *L. nigrinus* was the focus from 2005 to 2010.

2.1. Determining the Survival of Each Life Stage: 2000–2004. A rearing cage was designed to intercept mature larvae as they dropped from the hemlock foliage (Figure 1). The top section of the cage is an open-ended 30 cm diameter Dura-Lar acetate cylinder (0.018 cm thick) (Grafix, Cleveland, OH) with the top end covered with PeCap polyester mesh (0.14 mm^2) (Sefar America Inc., Kansas City, MO). The base of the cage is a nonswirl galvanized steel funnel (McMaster-Carr Co., Atlanta, GA) with a top diameter of 30 cm in which the acetate cylinder is placed. Hardware cloth ($5 \times 5\ mm^2$ mesh size) is cut to fit the inside of the funnels. The hardware cloth is placed inside the funnel base as a coarse screen, resting where the funnel constricts. HWA-infested hemlock twigs with *L. nigrinus* eggs are inserted into water-saturated (Oasis Deluxe) floral foam blocks ($8 \times 10 \times 3\ cm^3$ bricks). Each block is wrapped in Parafilm M (Fisher Scientific, Hampton NH) to retain moisture and prevent prepupae in search of a pupation site from entering the blocks.

Floral foam blocks containing hemlock branches with *L. nigrinus* eggs are placed on the hardware cloth within the funnel cages. When eggs hatch, the larvae develop in the hemlock foliage, feeding on HWA eggs within the funnel cages. Mature larvae drop from the foliage and accumulate in the (Kerr) 8 oz. mason jars (Jarden Home Brands, Muncie, IN) attached beneath the funnel. The jars are spray-painted black and the covers cut open. The jar cover rims are attached to the bottom of the funnels with glue; this way mason jars can be easily removed and reattached. Two teaspoons of steam-sterilized peat moss and 2 pieces of moistened filter paper (Whatman No. 1: 35 mm diam.) are placed in the mason jars three weeks after adding *L. nigrinus* eggs to the cage. Larvae having ingested a sufficient amount of HWA eggs drop from the hemlock foliage in search of a pupation site. The Mason jars are checked daily and mature larvae counted.

Funnel cages were set up in custom-built racks and held in concrete block rooms maintained at 13° ± 2°C with fluorescent lights on timers programmed to provide a photoperiod that mimicked natural conditions (increasing day length from 12 to 14 h light over spring). A detailed description of the recommended rearing procedures during these months is provided by Lamb et al. [11].

2.2. Enhancing Survival for Each Life Stage

2.2.1. Feeding Phase: Adults and Developing Larvae from October to June

(1) Adult Survival at 4° and 13°C after Emergence from Aestivation. Early emergence of adults had been a persistent issue, because of limited available HWA prey, resulting in high mortality. Techniques to minimize early emergence of aestivating *L. nigrinus* adults were developed by Lamb et al. [12], but some beetles continue to emerge before HWA break aestivation, making survival of early emerging beetles a continuing issue.

In 2002, in anticipation of early predator emergence, eggs laid by sistentes were stored in a refrigerator at 4°C from April to August. As adults emerged from aestivation before HWA, 70 of them were transferred to feeding containers with hemlock branches infested with aestivating HWA nymphs and one cold-stored branch with developing progredientes. This was not an ideal number of preys for the 70 adult beetles, but additional food was not available for the early emerging adults. There was sufficient HWA prey for only eight containers. All adults that emerged early (before HWA break aestivation) were fed every 12 days throughout September with HWA. One half of the containers was stored in environmental chambers at 4°C 12 : 12 (L : D) h, and the other half in a cold room at 13°C 12 : 12 (L : D) h. The number of adults surviving at each feeding period was recorded.

(2) Adult Sex Ratio within the Colony. Feeding containers were randomly selected from the colony, and individual adults were placed in separate 384 mL, clear plastic containers with a heavily HWA-infested hemlock twig (20–30 cm total linear length). After three days, each twig was examined for eggs with a microscope. Adults that did not lay eggs were

returned to separate containers with fresh host material and reexamined after another three days. Adults that still did not oviposit were considered to be males or unfertile females. In March and April 2003, adults from 21 containers were dissected and sexed ($n = 699$).

(3) Effect of Predator Egg Density on Survivorship to Mature Larvae. A randomized block design experiment was set up with five densities of *L. nigrinus* eggs (10, 20, 30, 40, or 50) in funnel cages with an adequate amount of HWA prey. The five densities formed a block and two blocks were arranged in 10 funnel cages. There were two replicates with a total of 20 cages. The number of larvae that reached maturity and dropped to the mason jar below each funnel was counted. A one-way analysis of variance was used to determine whether egg density influenced larval survival ($n = 20$).

(4) Survival of "Immature" Larvae. Immature larvae frequently drop early into collecting jars. They are usually smaller, darker in color, and less mobile than mature larvae and often still have white wool attached to their dorsal side. These larvae will search for prey if transferred back to a hemlock branch rather than drop off the branch as mature ones do. Immature larvae found in mason jars throughout the spring of 2002 and 2003 were transferred to fresh hemlock branches with HWA ovisacs and placed in one of nine "immature" funnel cages. The number of these larvae reaching maturity from each funnel cage was recorded and the overall survival rate calculated.

The mature larvae collected from the "immature" funnel cages were transferred to corresponding soil containers. The number of adults emerging from these containers was recorded and the emergence rate of adults that had left the hemlock foliage prematurely as immature larvae was calculated.

2.2.2. Nonfeeding Phase. In nature, mature larvae burrow into the soil immediately upon dropping from the hemlock foliage. In the lab, these larvae are transferred from the mason jars below the funnel cages to containers for pupation, eclosion, and adult aestivation. These containers have two layers of filter paper as a base lining to prevent pooling when the pupation medium is moistened with methyl paraben solution (0.42 g/250 mL distilled water) throughout the summer to inhibit fungal growth. To each pupation container is added at least 5 cm of pupating medium consisting of an equal mixture of peat moss (Premiere Horticulture Inc., Quakertown, PA), sphagnum moss (Mosser Lee Long Fiber, Westsel Inc., Harrisonburg, VA), and sand (Quikrete Play Sand, The Quikrete Product Line, Atlanta, GA). Peat moss is sifted through hardware cloth ($3 \times 3\,mm^2$), and sphagnum moss is ground in an industrial blender and sifted through hardware cloth. The mixture is moistened and steam-sterilized twice for 12 h, separated by 24 h at room temperature, and placed in plastic pupation containers that have at least one polyester mesh-covered hole for ventilation. Larvae burrow into the pupation medium, create a cell within the soil, assume a c-shaped position in the cell, and develop into pupae in approximately 14 days.

Pupation containers are kept at 15° ± 2°C and 12 : 12 (L : D) photoperiod for optimal pupal development [13]. Each container is maintained at ~30% saturation, receiving ~5–8 squirts of methyl paraben solution weekly. Pupation lasts approximately 14 days, and the newly eclosed adults remain under the soil surface, in aestival diapause, throughout the summer. This new generation of adults begins emerging from the soil in early fall. The pupation containers are checked daily for emerging adults over a period of several months (August–December). Emerging adults are transferred to adult containers.

(1) Prepupal Survival, Pupal Sex Ratio, and Adult Emergence from Aestivation. As sex cannot be determined in the adult stage because genitalia retract into the body after eclosion [14], sex ratio of progeny was obtained by microscopic examination of the external genitalia characters of pupae [15]. In spring 2003, six soil containers were randomly selected from the colony three weeks after larvae entered the soil. Using a paintbrush, the soil was sifted and each pupa sexed and transferred to a corresponding male or female container with fresh pupation medium. These containers were maintained with the rest of the colony in the cold room (15° ± 2°C and 14 : 10 (L : D) photoperiod, watered weekly) throughout the summer. Adults emerging from each container in the fall were recorded daily.

In spring 2004, 28 soil containers were selected to assess pupal survival and sex ratio. During this year, there was considerably more mold development in soil containers than in previous years even though the same methods were used for preparing the pupation medium. Soil containers with high levels of mold (present on entire surface and throughout the pupation medium), medium (present on entire surface only) and low mold contamination (present on less than half the surface) were selected three weeks after larvae had entered the soil. For each container, the soil was sifted through and surviving pupae sexed and transferred to corresponding male and female containers with fresh pupation medium. These containers were maintained with the rest of the colony at 15° ± 2°C, 12 : 12 (L : D) photoperiod until adult eclosion, 19° ± 2°C, 16 : 8 (L : D) h until 27 September 2004, and then decreased to 13° ± 2°C, 10 : 14 (L : D) photoperiod until emergence from the pupation medium [12]. Each container was watered weekly throughout the study period and the adults emerging from each container in the fall were recorded.

(2) Effect of Abiotic Factors on Pupal Survival/Adult Emergence from Aestivation

(a) Soil Moisture and Disturbance. Ten mature larvae were placed in each of 48 clear polystryene containers (950 mL) that has an 8 cm diameter ventilation hole in the lid and 2 layers of filter paper moistened with methyl paraben solution. Pupation medium was added to a height of 2 cm

in each container (3 : 2 mixture of potting soil : peat moss) (Miracle-Gro, Scotts Company, Marysville, OH) maintained at one of three moisture levels (% saturation): high (35–45%), medium (20–25%), or low (5–10%). A Lincoln soil moisture meter (Forestry Suppliers Inc., Jackson, MS) was used to measure the relative soil moisture level in control containers (set up at same time with no larvae added) every other day throughout the study. Moisture levels were maintained by adding the same amount of water to test containers as the control containers.

For each moisture level, half of the containers was randomly selected and disturbed by sifting the pupation medium and counting the number of live individuals. The soil was sifted twice, three and six weeks following larval entry into the soil to determine survival to the pupal stage. Surviving individuals were put back in the soil in the container, and the total number of adults emerging from each container in the fall was recorded daily. This experiment was set up as a randomized complete block design with larval cohort serving as blocks (8). The effects of moisture and disturbance on pupal survival and adult emergence were determined with a 2-factor ANOVA using proc glm in SAS; means were separated using Fishers LSD ($n = 48$).

(b) Pupation Medium and Moisture Level. Four types of media were maintained at three moisture levels (12 treatments) during *L. nigrinus* pupation and aestivation to determine optimal conditions for survivorship at the pupal and adult stages. The experiment was set up as a generalized randomized block design with eight replicates in each of four blocks with larval cohorts serving as the blocks. The four soil types varied in concentrations of ground sphagnum moss, peat moss, and sand (3 : 0 : 1, 2 : 1 : 1, 1 : 2 : 1, 0 : 3 : 1 (sphagnum : peat : sand)).

For each block, 96 plastic containers (384 mL) with ventilated lids (5 cm diam.) were set up with two layers of moistened filter paper and 4 cm of pupation medium. Five mature larvae were added to each container. This process was repeated four times with a total of 20 genetically diverse larvae in each container. A third of each soil type was maintained at one of the following moisture levels: 30, 45, and 60% saturation. A control container representing each of the 12 treatment combinations was set up at the same time as each block. Moisture level of control containers was measured each week using a Lincoln soil moisture meter. The same amount of methyl paraben solution (same weight) was added to each treatment and control container.

To estimate survival through pupation, one container from each treatment was randomly selected eight weeks after larval entry into the soil and the media were scooped out of containers and combed thoroughly for recently eclosed adults under the microscope. Survivorship and approximate depth were recorded for each recovered adult. Total number of adults and time of emergence for each container were recorded daily from July 22 to December 11, 2002. The proportion of adults emerging and average time of emergence were compared across treatments using a 2-factor ANOVA in SAS, and means were separated using Fishers LSD ($P < 0.05$).

(c) Optimal Density per Pupation Container. Three levels of larval density per container were tested using a generalized randomized block design with larval cohort serving as the block. For each block, 13 plastic containers (950 mL low density polyethylene) were set up with 5 cm of pupation medium (2 : 2 : 1 sphagnum : peat : sand) and two layers of filter paper. Four replicates of five, 10, or 15 mature larvae were added to each container; the 13th container served as a moisture control. This was repeated six times to produce densities of 120, 240, and 360 individuals/larval density. In all, five blocks were set up on five consecutive days in May. Pupation medium was maintained weekly at 45% saturation by monitoring and manipulating the moisture level of the control containers using the Lincoln soil moisture meter and distilled water. Equal volumes of methyl paraben solution were added to the test containers as in the control containers using a balance scale. Adult emergence and duration of aestivation were determined for each container and compared across treatments using a 1-way ANOVA in SAS ($P < 0.05$).

(d) Assessing Importance of Sterilized Soil. Sterilized soil showed increasing levels of mold in 2004 and 2005. An experiment to test the effects of soil type (soil mix versus forest soil) and sterilization using an autoclave was initiated in May 2006. Thirty mature *L. niginus* larvae were placed in each square container with either soil mix or forest soil that had been autoclaved or left unsterilized. Five containers were used for each treatment ($n = 5$). Soil moisture was maintained at 20% to avoid excessive mold. Containers were kept at 15°C until beetles had entered the adult stage and then maintained at 19°C. Percentage emergence data were analyzed as a one-way CRD ANOVA using SAS with arcsine-square root transformation to stabilize variances.

2.3. Rearing Procedures to Increase Production Numbers of L. nigrinus: 2005–2010. The experimental studies from 2000 to 2004 improved rearing practices for 2005 to 2010. This included selection of the appropriate temperature, sex ratio, predator egg density, size of rearing containers, pupation medium, density of number of beetles, and abiotic factors that influence survival to adult emergence. Inclusion of field-collected beetles in the founding colony is important. Beginning in 2005, collections of adults from field populations in western USA were carried out annually and included into the rearing colony to provide hybrid vigor.

3. Results

3.1. Survival of Each Life Stage: 2000–2004. The numbers of larvae that reached maturity, pupating, and aestivating adults emerging were obtained for the first time in 2001. The colony began with 350 field-collected adults that produced 7,500 larvae, of which 69% pupated. Adults in aestivation and immediately following emergence from aestivation suffered high mortality, with 200 adults surviving in the fall (Table 1). In 2002, 1,000 field-collected adults added to the founding colony improved larval production that was much higher

Table 1: Survival of *Laricobius nigrinus* (Coleoptera: Derodontidae) at different life stages from laboratory rearing efforts between 2000 and 2004.

Life stage	Total number of individuals and mortality rate (%) per life stage				
	2000	2001	2002	2003	2004
Reproductive adults (starting colony)	200[F]	350[F]	100[L] 1,000[F]	3,000[L]	7,000[L] 660[F]
Mature larvae drop from foliage	N/A	7,500 (28%)	37000 (~30%)	30,000 (30+%)	27,000 (30+%)
Pupae	N/A	5,175 (31%)	25,900 (27%)	12,300 (41%)	7,000[Mf] + 2,200 [M] (43%), (94%)
Adults emerging from aestivation	30 (85%)	1,867 (36%)	21,000 (19%)	13,000 (6%)	8,000 (13%)
Adults surviving as HWA breaks aestivation in October	8 (74%)	200 (89%)	3,700 (83%)	12,000 (8%)	8,000 (0%)

[F] Adults collected in the field from western hemlock trees in Victoria, British Columbia.
[L] Adults reared in the laboratory at Virginia Tech.
[Mf] In soil containers that were free of mold contamination.
[M] In soil containers contaminated with mold.

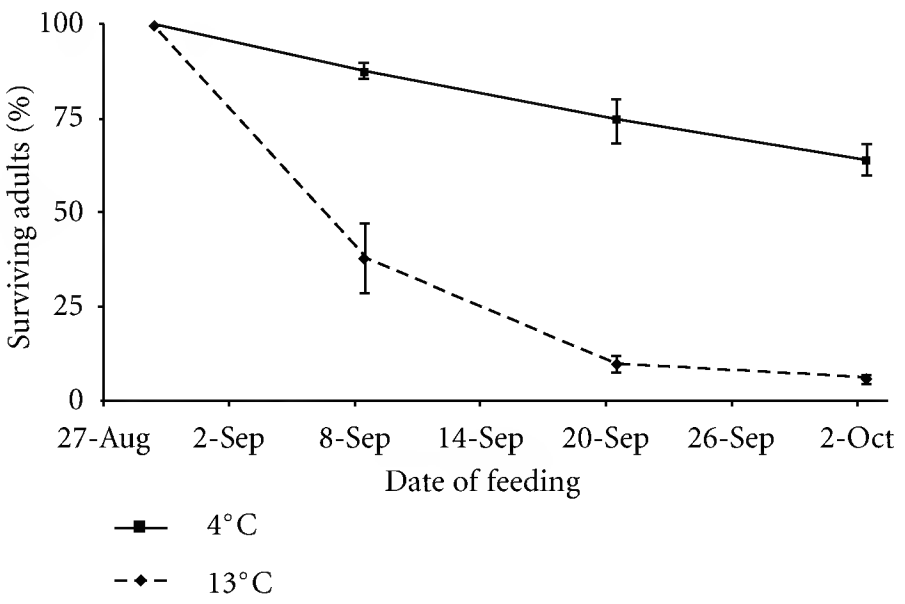

Figure 2: Mean percentage (±S.E.) of the original adults surviving at each feeding period maintained at 13° and 4°C in the weeks following emergence in 2003.

than in 2003 where only lab-reared beetles were used. Pupal survival was greater as well in 2002 compared with 2003 and 2004. Considerable pupal mortality in 2004 was likely attributed to contamination of the soil by mold.

3.2. Developing Rearing Procedures for Each Life Stage

3.2.1. Feeding Phase

(1) Adult Survival at 4° and 13°C after Emergence from Aestivation. Adult survival was higher and more consistent following emergence from aestivation when held at 4°C than at 13°C (Figure 2). Mortality rate was high during the first 12 days when over 60% of adults stored at 13°C and 13% at 4°C died. On October 2, after three feedings, 5.7% of the adults held at 13°C and 64.2% of those at 4°C were still alive.

(2) Adult Sex Ratio within the Colony. Of the 699 adults sexed, 458 were ovipositing females. Mean female-to-male ratio ($\overline{X} \pm$ S.D.) within a container during the peak oviposition period in March/April was 1.91 (±0.18) : 1.

(3) Effect of Predator Egg Density on Larval Survival. Density of eggs per funnel cage (up to 50 individuals per cage) did not affect larval survival ($F_{4,19} = 0.90$, $P = 0.490$). Mean percentage of eggs ($\overline{X} \pm$ S.D.) that hatched and newly eclosed first instar reaching larval maturity was 73.7 ± 15.4%, indicating that higher densities of eggs can be used in funnels to maximize larval production.

(4) Survival of "Immature" Larvae. There were 6,116 immature larvae recovered in mason jars throughout the spring of 2002. Of these, 3,486 (57%) reached maturity after being transferred back to funnel cages with prey and completed larval development.

In the spring of 2003, 8,002 immature larvae were collected from the mason jars and transferred back to funnel cages to complete development. Of these, 3,905 larvae (48.8%) completed development, entering the soil for pupation and aestivation. In fall, 1,843 of these individuals emerged from aestivation, representing 23% of immature larvae reaching adulthood.

3.2.2. Nonfeeding Phase

(1) Prepupal Survival, Pupal Sex Ratio, and Timing of Adult Emergence from Aestivation. In fall 2003, the mean percentage of larvae ($\overline{X} \pm$ S.D.) that developed into pupae per soil container was 58.7 ± 18.2%. Of the 183 pupae sexed, 95 were female and 88 were male, with sex ratio ($\overline{X} \pm$ S.D.) of 1.08 : 1 ± 0.51 : 1 F : M per adult container. Females and males had similar survival throughout aestivation (64.2 and 64.7%, resp.); however, males emerged earlier than females (Figure 3(a)).

In 2004, of the 933 pupae sexed, 515 were female and 418 were male. Ratio of female to male pupae per container was 1.19 : 1 ± 0.74 : 1. Survival of males through aestivation (40.9%) was higher than females (35.7%), but the time at which they emerged was better synchronized than the previous year (Figure 3(b)). Different emergence between years is attributed to a change in storage conditions; in 2004, adults were maintained at a higher temperature (19°C) throughout the summer, based on findings reported in [12].

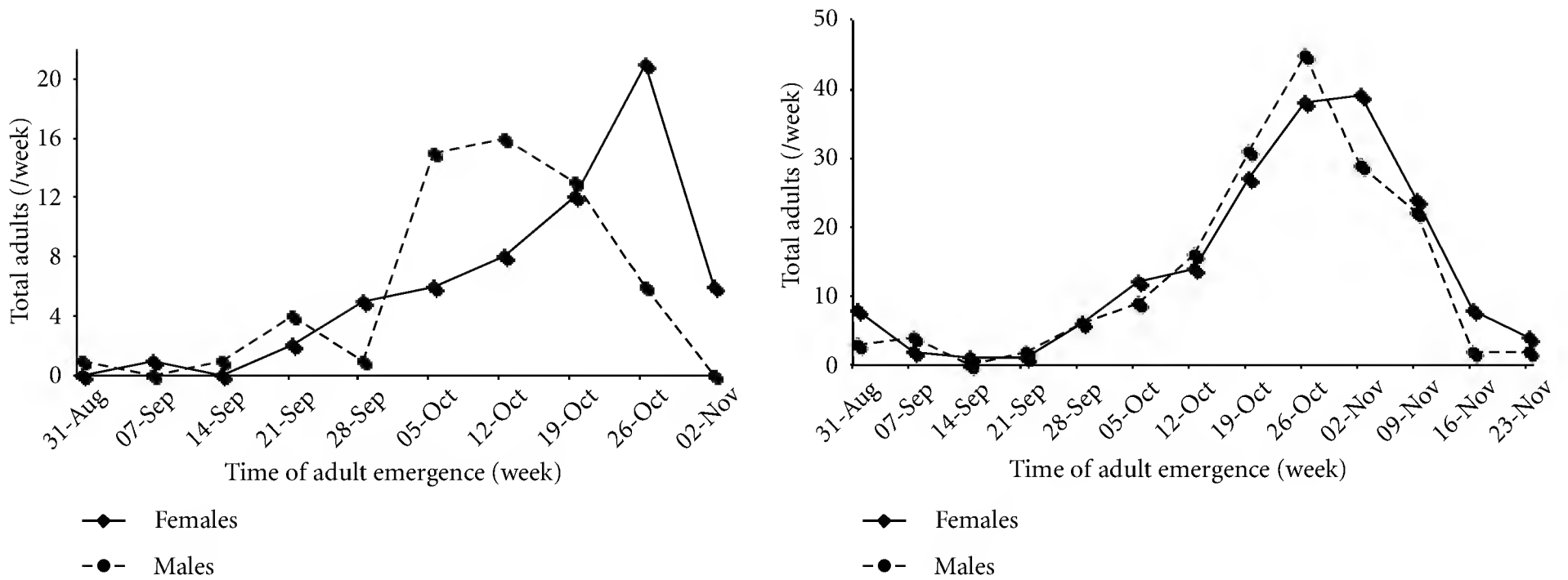

FIGURE 3: Weekly total number of female (solid line) and male (broken line) adults emerging from aestivation in fall 2003 (a) and 2004 (b). Note difference in scale: 2003 from 238 pupae sexed; 2004 from 933 pupae sexed.

(2) Effect of Abiotic Factors on Pupal Survival/Adult Emergence from Aestivation

(a) Soil Moisture and Disturbance on Pupal Survival. Moisture level did not affect the development of pupae ($F_{(1, 23)} = 0.03$, $P = 0.9709$). The mean percentage of larvae ($\overline{X} \pm$ S.E.) developing into pupae at all moisture levels was 69.1 ± 2.3%. However, the number of adults emerging from aestivation in the fall was affected by moisture level ($F_{(2, 47)} = 6.02$, $P = 0.0050$) and by disturbance of pupae ($F_{(1, 47)} = 4.08$, $P < 0.0498$). More adults emerged from containers with 40% or 20% moisture than at 5% (Figure 4). Disturbance of the soil to recover individuals lowered adult emergence by 10%. Mean emergence ($\overline{X} \pm$ S.E.) of undisturbed pupae emerging as adults was 49 ± 2.3% compared with 39 ± 3.0% for disturbed pupae.

(b) Pupation Medium and Moisture Level. Mean pupal survival was 72.1 ± 3.5% ($\overline{X} \pm$ S.E., $n = 20$). It was not affected by the composition of medium ($F_{(2, 6)} = 1.19$, $P = 0.348$) nor the moisture level within the range of 30–60% ($F_{(3, 6)} = 0.329$, $P = 0.804$). However, the number of adults emerging from aestivation was lower for individuals held in pure peat moss than those in pure sphagnum moss or the 1 : 2 sphagnum:peat mixture (Figure 5). The latter mixture had the highest mean emergence (61.5±0.8%) and the lowest was from containers with pure peat moss (52.8 ± 1.1%). Soil moisture did affect the percentage of adults emerging from aestivation (Figure 4, 2nd experiment). Beetles stored in soil with 30% moisture level emerged in greater numbers than those held at higher moisture levels.

The timing of emergence from diapause was not affected by soil type ($F_{(3, 284)} = 0.30$, $P = 0.822$). Adults remained in the ground for 123.9 ± 0.25 days. Moisture level affected

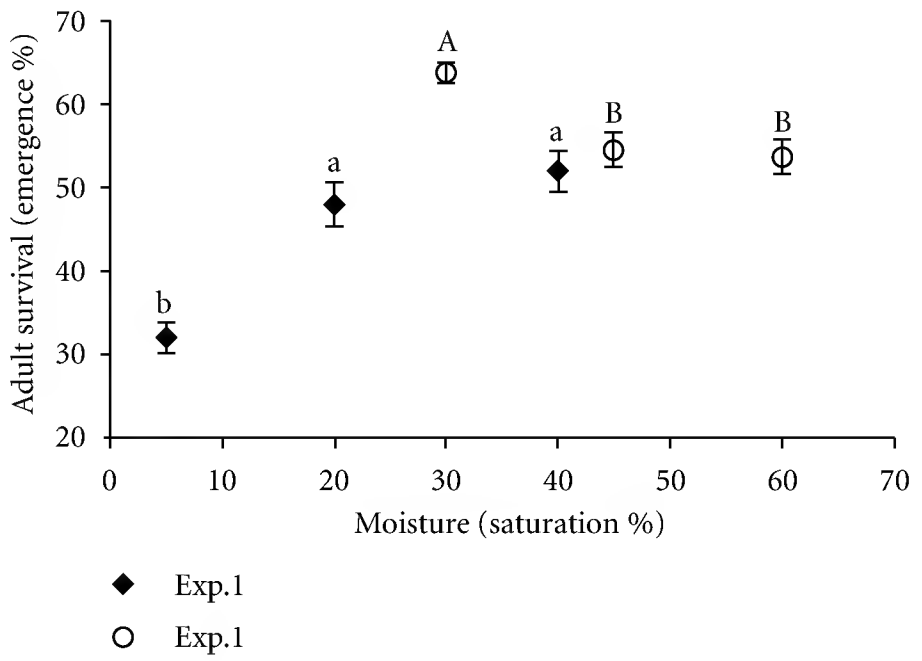

FIGURE 4: Mean percentage (±S.E.) of adults emerging when maintained at 5, 20, and 40% soil saturation in Exp. 1 and at 30, 45, and 60% soil saturation in Exp. 2. Means with different lower case letters are significantly different (Exp. 1, $n = 24$) and means with different upper case letters are significantly different (Exp. 2, $n = 372$).

time of emergence as adults in soil maintained at 30 or 45% saturation emerged before those held at 60% saturation ($F_{(11, 284)} = 22.51$, $P < 0.0001$). However, mean duration of aestivation of adults stored at 60% moisture level (126.4 ± 0.24) was only four days longer than the duration at lower moisture levels (122.6 ± 0.25).

(c) Optimal Density per Pupation Container. The density of adults per container (30, 60, and 90) did not influence the percentage of adults emerging from aestivation (32.2 ± 4.57%) ($F_{(4,53)} = 1.73$, $P = 0.1865$) or the duration of aestivation (142.7 ± 0.6 days) ($F_{(4, 53)} = 0.02$, $P = 0.9836$).

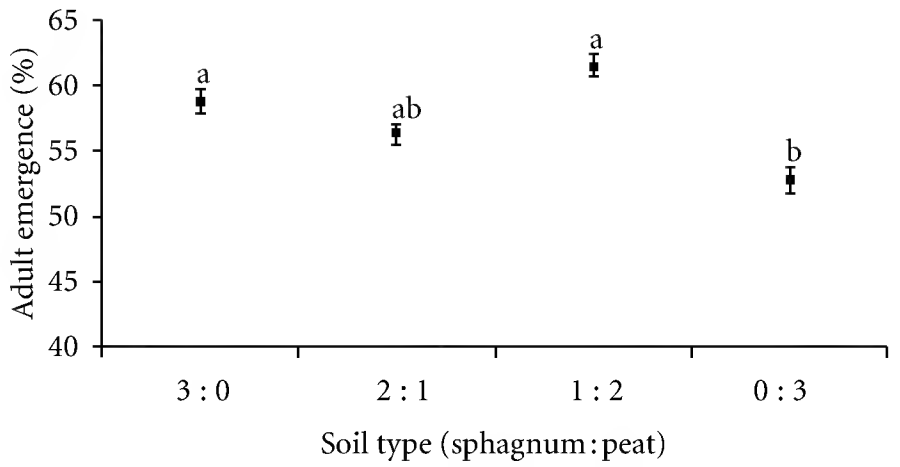

FIGURE 5: Mean percentage (±S.E.) of adults emerging from aestivation when stored in four different ratios of sphagnum : peat during pupation and aestivation. Means with different letters are statistically different ($F_{(3, 284)} = 4.10$, $P = 0.0072$).

(d) Assessing Importance of Sterilized Soil. In fall of 2006 containers with unsterilized soil mix had higher mean emergence than the other treatments (67.3 ± 4%) (Table 2). Emergence from containers with unsterilized forest soil exceeded all those containers with autoclaved soil (39.3 ± 3%).

3.3. Rearing Procedures to Increase Production Numbers of L. nigrinus: 2005–2010. Production of *L. nigrinus* adults that successfully emerged after aestivation improved after 2006 (Table 3). In 2005 and 2006, the numbers of larvae produced dropped from about 31,000 in 2002–2004 (Table 2) to 17,042 (Table 3). Emerging adults dropped more drastically from 10,000 (Table 2) to 2,725 beetles (Table 3). Mold contamination of the soil medium was likely the main cause. The sterilizing study helped us determine that sterilizing the soil contributed to the onset of mold problems. By not sterilizing the soil, the mold problems disappeared. It was likely that beneficial microorganisms were being removed during sterilization allowing saprophytic fungi to flourish, but we did not investigate this further.

Beginning in 2007, we finally reached a stable point in rearing production. From 2007 to 2010, we produced an average of 19,036 adults per year (Table 3). There is still considerable variation from year to year due to environmental conditions. In 2009 our production dropped, due mostly to severe winter kill of HWA from low temperatures in February that year throughout the mid-Atlantic states, resulting in poor food availability for developing larvae. In contrast, HWA populations recovered in 2010. Food availability was adequate, and we obtained a remarkably high level of emergence, where 71% of the larvae survived pupation and aestivation.

4. Discussion

The creation of the funnel cage contributed greatly to the assessment of survivorship as well as providing a functional way to rear larvae. *L. nigrinus* larvae developed within the funnel without additional maintenance, such as adding foliage or searching for lost larvae under the microscope, as in previous years. Collection of *L. nigrinus* prepupae dropping from the foliage enabled us to determine egg and larval survival and allowed for the calculation of survivorship through *L. nigrinus* pupation and aestivation. A third benefit of using this cage is that, during spring, there is a consistent accessible supply of *L. nigrinus* larvae dropping from the foliage, allowing experiments to be set up with less effort and coordination since larvae do not have to be reared individually.

There are some challenges to using the funnel cages. Mature larvae must be transferred within 36 h of dropping, before they create a pupal cell and become immobile. Checking the jars daily for mature larvae is labor intensive and costly. Without adequate prey within the funnel cages, large numbers of immature larvae that end up in the jars must be transferred back to hemlock branches containing HWA eggs for them to survive. Also, high numbers of immature larvae increase the time required to check the funnels jars.

Survival is low when adults emerge from aestivation before October. Maintaining them at 4°C increases their survival. Since aestivating 1st instar HWA sistentes are not suitable food for postemergence adult beetles, HWA eggs laid by sistentes in the previous spring should be stored at 4°C throughout the summer to provide early emerging adults with developing progredientes nymphs. The increase in larval production in 2002 is attributed to the almost exclusive use of field beetles for the starting colony. These were larger and apparently oviposited more eggs than lab-reared adults. Sex ratio of pupae in mold-free soil was close to 1 : 1. Emergence of males occurred earlier than females when held at constant 15° ± 2°C, 14 : 10 (L : D) photoperiod. This may explain the high female : male ratio observed in ovipositing females. When maintained at a high temperature (19°C) after adult eclosion and lowered to 13° ± 2°C, 12 : 12 (L : D) photoperiod in late September to stimulate emergence, the emergence period of adults was much shorter than the emergence period of adults the previous year [12].

Moisture level and soil type influence the number of adults that emerge. Emergence is the highest at moisture levels of 30–40% saturation although a wide range of moisture levels in the soil is tolerated. This is advantageous because precipitation varies from year to year. Adults have the highest emergence from a mixture of sphagnum and peat mosses. Although these factors affect emergence, larval cohort often accounts for much of the variation observed in emergence, suggesting there are unexplored factors involved.

The time at which larvae enter the soil ranges over a period of 12–15 weeks and appears to influence the number of adults emerging from aestivation. The same pattern is observed each year in the colony; larvae maturing early in the spring have a higher rate of adult emergence than those maturing later in the year. This pattern may be explained by variation in nutritional value of HWA over a season. Larvae maturing early in the season feed on eggs laid by sistentes and those maturing later in the season feed on eggs laid by progredientes. By late spring, two generations of HWA have fed on the hemlock branches, which may be depleted in resources, possibly affecting the nutrition of HWA. The

Table 2: Total number of *Laricobius nigrinus* larvae, emerged adults, and percent emergence in each treatment.

Container	Soil type	Autoclaved	n	Total larvae	Emerged adults	% Emergence ± SE[A]
Square	Mix	Yes	5	150	30	20.0 ± 4% c
Square	Mix	No	5	150	101	67.3 ± 4% a
Square	Forest	Yes	5	150	5	3.3 ± 1% d
Square	Forest	No	5	150	59	39.3 ± 3% b

[A]Means followed by a different letter are significantly different from one another (Tukeys, $P \leq 0.05$).

Table 3: The number of *Laricobius nigrinus* in each life stage at the Virginia Tech Insectary from 2005 to 2010.

Year	Founding colony (reproductive adults)	Mature larvae	Adult : larvae	Emerging adults (after aestivation)
2005	713[a]	19,593	1 : 27	3,430
2006	1,067[a]	14,492	1 : 14	2,019
2007	1,231[b]	40,978	1 : 33	15,294
2008	1,200[a]	46,028	1 : 38	20,601
2009	1,030[a] + 200[c]	32,382	1 : 26	13,283
2010	870[a] + 200[c]	38,690	1 : 36	27,504

[a]Wild-caught adults from Washington, USA.
[b] Wild-caught adults from Washington, USA and Kentland Farm, Virginia, USA.
[c]Adult F_1 generation reared in the insectary at Virginia Tech.

nutritive chemical composition of host plants can affect the quality of phytophagous hosts and has been known to affect their predators [16, 17], particularly *S. tsugae* [18], a predator of HWA.

The experiments conducted between 2000 and 2004 led toward more consistent production of larvae and lower mortality at each individual stage. Consequently, the number of beetles produced is now more predictable and appears to be mostly a function of food quality (prey). In general, rearing beetle predators on a natural diet is enormously difficult when prey is not consistently available [19]. Artificial diets have been developed and tested but usually result in significantly greater mortality during development of the predator [20, 21]. Artificial diets can be used to augment predator rearing [22] and are currently being investigated for *L. nigrinus* [23], but much work is still needed. Therefore we continue to rely on the collection of preys from abundant populations on healthy host trees.

Developing a reliable mass rearing procedure was a critical objective addressed by these experiments. The results from the experiments carried out from 2000 to 2004 led to a detailed description of *L. nigrinus* rearing procedures documented in [11]. These have lead to procedures being followed by rearing labs at Virginia Tech, Clemson University, and the University of Tennessee. The success in colony rearing to date has resulted in the release of more than 100,000 adult *L. nigrinus* at 267 locations in 13 states in the eastern USA [24]. The techniques developed here are also applicable to rearing other *Laricobius* species being considered for release [25, 26].

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Research Article

Reproductive Biology, Mating Behavior, and Vibratory Communication of the Brown-Winged Stink Bug, *Edessa meditabunda* (Fabr.) (Heteroptera: Pentatomidae)

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We describe different aspects of the reproductive biology, mating behavior, and vibratory communication of the pentatomid *Edessa meditabunda* (Fabr.). This species shows lower copulation frequency and reproductive potential with longer sexual maturation period compared to other species of pentatomids. Females with multiple mating show increased fecundity when compared with single-mated females and both increased fecundity and reduced longevity when compared with virgin females. Courtship and mating behavior and vibratory signals are typical and similar to what was observed in other species of pentatomids, except that males started the courtship. These results constitute the first paper on biology, behavior, and vibratory communication among species of the subfamily Edessinae.

1. Introduction

The brown-winged stink bug, *Edessa meditabunda* (Fabr.) (Heteroptera: Pentatomidae), has been reported as a minor component of the stink bug species complex that is economically important pest in many crops mainly of families Solanaceae and Leguminosae [1]. In Brazil, *E. meditabunda* is present in the central-west and southern states [2–4]. Although *E. meditabunda* has been reported on a large number of plant families [5–7], soybean plants (*Glycine max* L.) seem to be a preferred host for adult feeding and reproduction [2].

The life cycle of *E. meditabunda* has been reported by several authors [8–11], however, with no detailed information about the reproductive and sexual behavior of the species.

Reproductive behavior of pentatomids is mediated by communication signals of different modalities among which sex pheromones are involved in female-male encounters, courtship, and matting [12–16]. Sex pheromones of stink bugs show high variability in the chemical structure, but in some cases, as in *Nezara viridula* (L.) and *Chinavia* spp. the pheromones consist of isomers of the same compound (trans and cis (*Z*)-bisabolene epoxide) in different ratios [16]. Communication with vibratory signals transmitted through the plant is used among others for mate location and recognition [17–20]. Communication with species-specific substrate-borne vibratory signals as the key element of mating behavior has been demonstrated in different species of Heteroptera, for example, in *Nezara viridula* [21], *Acrosternum hilare* (Say) [22], *Lygocoris pabulinus* (L.) [23], *Chinavia impiciticornis* (Stål) (= *Acrosternum impicticorne*), *Euschistus heros* (Fabr.), *Piezodorus guildinii* (Westwood), *Thyanta perditor* (Fabr.) (Heteroptera: Pentatomidae) [17], and many other species [19].

No data on sex pheromones or communication signals of any other modality and general mating behavior among species of Edessinae have been described. Therefore, we investigated different aspects of *E. meditabunda* reproductive biology, mating behavior, and communication signals.

2. Material and Methods

2.1. Insect Rearing. Adults of *E. meditabunda* were collected from soybean fields in Brasilia, DF, Brazil, and maintained in a colony in the Semiochemicals Laboratory at Embrapa Genetic Resources and Biotechnology (Brasilia, DF). The insects were reared in plastic cages (8 L) and fed on bean pods (*Phaseolus vulgaris*), soybean pods, and stalks of "boldo-brasileiro," *Plectranthus barbatus* (Lamiaceae). Stalks of *boldo* were placed in 7.5 cm high × 7.5 cm diameter plastic containers, half-filled with vermiculite (sterilized at 120°C for one hour), and sprayed with tap water to simulate a wet soil conditions to the plants and provide humidity.

The containers were placed inside the rearing cages (one vial/cage), which were kept in a climatic room at 26 ± 1°C, 60 ± 10% RH, and photophase of 14 L : 10 D. Twice a week the containers were sprayed with tap water to keep the vermiculite moist and the plants replaced every week. Most of the eggs were oviposited on leaves of *boldo*, and, after oviposition the eggs mass attached to the leaves were transferred to new rearing cages. Egg masses were examined daily for hatching and molting, and nymphs were fed in the same way as adults. After completing the immature development (<24 h after the final molt), adults were collected and managed as described above for the experiments and for colony maintenance.

2.2. Sexual Maturity and Longevity of Males and Females. To determine the sexual maturation age and longevity for each sex separately, one virgin male (1 to 2 days after the final molt) was placed together with three 15-day-old females in plastic cup (500 mL) and fed as previously described. Subsequently, one virgin female (1 to 2 days after the final molt) was placed together with three 15-day-old males. The groups ($N = 10$ for each adult combination) were observed daily every 30 min between 13:00 and 18:30 h that was the time interval when major frequency of copulation was previously observed. When a female or a male of each group (3 females or 3 males) dies, it was replaced by a new insect at the same age.

2.3. Mating Frequency Experiments

(1) Multiple-Mated Females. One virgin female and one virgin male (<24 h after final moult) were placed into plastic pots of 500 mL ($N = 20$) to record the mating sequence. The insects were observed daily every 30 min during the period of higher frequency of copula (determined in previous observations) during the photophase (from 8:30 to 18:30 hours). The couples were allowed to mate throughout their lifetime. For this group of females, only those observed in copula at least two times ($N = 18$) during their life span were considered for data analysis.

(2) Single-Mated Females. One virgin female and one virgin male (<24 h after final moult) ($N = 20$) were placed into plastic pots of 500 mL ($N = 20$) and observed every 30 min for the first mating (from 8:30 to 18:30 hours). Once mating had finished males were removed and females were observed daily until they died. Only females observed in copula ($N = 15$) were included in data analyses.

(3) Virgin Females. Single virgin females (<24 h after final moult) were isolated in plastic pots of 500 mL ($N = 20$) and observed for egg production and longevity.

Insects of the different treatments were fed as described above. Reproductive parameters (number of egg mass/female, number of eggs/mass, number of eggs/female, and number of nymphs/number of eggs (fertility)) were recorded for each tested female. The duration of copula was estimated from couples that start and finish the mating during the observation period (from 8:30 to 18:30 hours). We measured the time to start oviposition (i.e., the preoviposition period), the intervals between the first mating and first oviposition, the time between consecutive ovipositions, the total reproductive period (time between first and last oviposition), and the females longevity.

2.4. Courtship Behavior and Vibratory Communication. The behavioral sequences of courtship and mating in *E. meditabunda* were observed simultaneously with the records of male and female vibratory signals (Figure 3). Insects were separated by sex in the first 24 hours after the final moult and maintained in different rooms until they reach the sexual maturation. A pair of virgins and sexual mature male and female (15–20 days old) was placed on the membrane of a 10 cm diameter low-midrange loudspeaker (40–6000 Hz frequency response, 8′Ω impedance; RadioShack, Taiwan). An acrylic box (9 cm diameter × 4 cm high) was placed over the speaker without contacting the loudspeaker membrane to prevent the insects moving away from the membrane surface. The loudspeaker was placed into a sound-insulated room to decrease environmental noise. All observations were conducted between 13:00 and 18:30 when most of the mating activities have been previously observed. If the insects did not display any courtship behavior within 20 minutes of the observations, they were classified as failed courtship. In this experiment, 62 couples were observed. To describe the courtship and mating behavior, the previously determined behavior categories in pentatomids were used [24, 25] (Table 1). The sequence of behavioral categories was registered in each observed couple.

The vibratory signals captured by the loudspeaker were amplified by a home-made operational amplifier TL081CN, digitized (Aardvark-Direct Pro 24/96 (Aardvark Computer Systems, Washington, USA), and stored on a computer using Cool Edit Pro software (Syntrillium Software 2001—Fort Wayne, Indiana, USA). Signals were followed in real time with headphones and recorded until the insects stopped singing.

Vibratory signals were analyzed by the Sound Forge 4.5 software (Sonic Foundry http://www.sonicfoundry.com/). A pulse was defined as a unitary homogeneous parcel of vibration of finite duration [26]; pulse trains as repeatable and temporally distinct groups of pulses and a song as a sequence of pulses and/or pulse trains with distinct beginning and end.

Table 1: Courtship sequence performed by *Edessa medidabunda* described in the ethogram.

Code of the behavior	Description of the behaviors
MFRG	Males and females at rest and grooming
MS1	Males spontaneously emit song type 1
MS2	Males spontaneously emit song type 2
MstS1	Males interrupt the emission of MS1
Mw	Males walk around the arena
FS1	Females emit song 1 in response to MS2
D	MS2/FS1 duet
MApFf	Male approaches the female from the front
MApFb	Male approaches the female from the back
MAnFAn	Male antennates female's antennae
MAnFt + p	Male antennates female's thorax and moves to the posterior side of her abdomen
Mb180° + RT	Male puts the head behind the female abdominal tip and butt her abdomen until she adopts the copulatory position At the time turning 180° from the female posterior, the insects are oriented end to end in copulatory position
Pr + 180°	Male rotates its pygophore 180° so that it is inverted
PM	Pair mate
MRF	Male rejects female and walks away from her
FRM	Female rejects male and runs away from male
NM	Pairs not mate

Spectra were described by the dominant, first harmonic, and other subdominant frequency peaks, by the spectral width 20 dB below the amplitude of dominant frequency value and by frequency modulation described as downward or upward-orientated frequency sweeps quantified by the frequency difference per signal duration (Hz/s). Songs were classified, according to their order of appearance in a duetting couple [22].

2.5. Statistical Analyses. Reproductive parameters were analyzed by generalized linear models (GLM) and deviance analyses (ANODEV). The models have a factor for treatments and Gaussian distribution of errors for time variables, Poisson distribution of errors for fecundity parameters (number of egg masses/female number of eggs/mass and number of eggs/female), and binomial distribution of errors for fertility (number of nymphs/number of eggs). Contrast analyses were used to multiple comparisons of means. To test the relation between successive mating and cumulate fecundity, a linear model was used, with the cumulate fecundity (cumulate numbers of eggs/female) as dependent variable.

Data from observations of all courtship sequences were used to create a first-order Markovian behavioral transition matrix of total frequency of transitions (i.e., moving from one behavioral step to the next). The repetition of a single behavior (self-transition) was not included in the records to avoid the possible influence in the relative weight of transitions between behaviors. Transition probabilities were calculated from the observed frequency of a transition between two events divided by the total number of occurrences of the first event [27]. The expected values of the matrix cells were found using the iterative proportional fitting method of [28], and the statistical significance of the individual transitions were evaluated using a log-likelihood ratio test (G test) and the results presented graphically in the ethogram. Data are shown as means ± SD, together with the number of signals analyzed (N) and the numbers of individuals (n).

3. Results

The age at which females reached the sexual maturity (18.08 ± 1.26 days) was estimated from the first copulation of virgin females maintained with old males. It was significantly different from the age at which males reached the sexual maturity (15.92 ± 0.86) (estimated from first copulation of virgin males maintained with old females) (ANODEV χ^2_1 = 30.15, $P < 0.001$).

Multiple mating with the same male increased female's fecundity of *E. meditabunda* (ANODEV χ^2_2 = 51.95 $P < 0.001$) but not its longevity (Table 2). The reproductive period also increased ($\chi^2_2 = 651.1$ $P < 0.001$) with the increase of the number of copulations (Table 2). For multiple-mated females, we observed 3.92 ± 0.79 copula (N = 20) with duration of 222.55 ± 60.02 min (N = 11) and intervals between copulations of 6.35 ± 3.8 days. The pre-oviposition period of virgin- and single-mated females was significantly longer when compared with multiple-mated females (ANODEV $\chi^2_2 = 81.54$ $P < 0.001$) (Table 2). However, the interval between the first mating and first oviposition was similar for both groups of mated females and females with multiple mating showed shorter intervals between consecutive ovipositions (Table 2). Females with multiple mating laid a higher number of egg mass than did virgin and once-mated females (ANODEV χ^2_2 = 86.51 $P < 0.001$)

Table 2: Effect of mating frequency on the reproductive biology and longevity of *Edessa meditabunda* females in the laboratory.

	Preoviposition period (days)	Days between 1° mating and 1° oviposition	Days between consecutive oviposition	Number of egg mass/female	Number of eggs/mass	Total eggs/female	Reproductive period (days)	Fertility[b] (%)	Female longevity (days)
Multiple-mated females	18.50 ± 2.06[a] ($N = 20$)	1.35 ± 0.48[a] ($N = 20$)	6.26 ± 3.02[a] ($N = 95$)	5.70 ± 1.17[a] ($N = 20$)	13.50 ± 0.79[a] ($N = 115$)	77.65 ± 16.11[a] ($N = 20$)	30.45 ± 10.22[a] ($N = 20$)	96.11 ± 9.70[a] ($N = 115$)	56.65 ± 7.51[a] ($N = 20$)
Single-mated females	21.27 ± 1.53[a] ($N = 15$)	1.53 ± 0.74[a] ($N = 15$)	9.21 ± 2.67[b] ($N = 14$)	2.07 ± 0.70[b] ($N = 15$)	13. 32 ± 1.88[a] ($N = 31$)	26.87 ± 9.30[b] ($N = 15$)	9.14 ± 5.17[b] ($N = 15$)	95.16 ± 13.47[a] ($N = 31$)	57.87 ± 6.32[a] ($N = 15$)
Virgin females	21.25 ± 2.76[a] ($N = 9$)			0.70 ± 0.77[c] ($N = 17$)	9.45 ± 2.58[b] ($N = 11$)	5.78 ± 6.44[c] ($N = 18$)	1.22 ± 0.44[c] ($N = 19$)		64.50 ± 9.53[b] ($N = 18$)

Letters in each column followed by the same letter are not significantly different (ANODEV and contrast analyses $P > 0.05$).

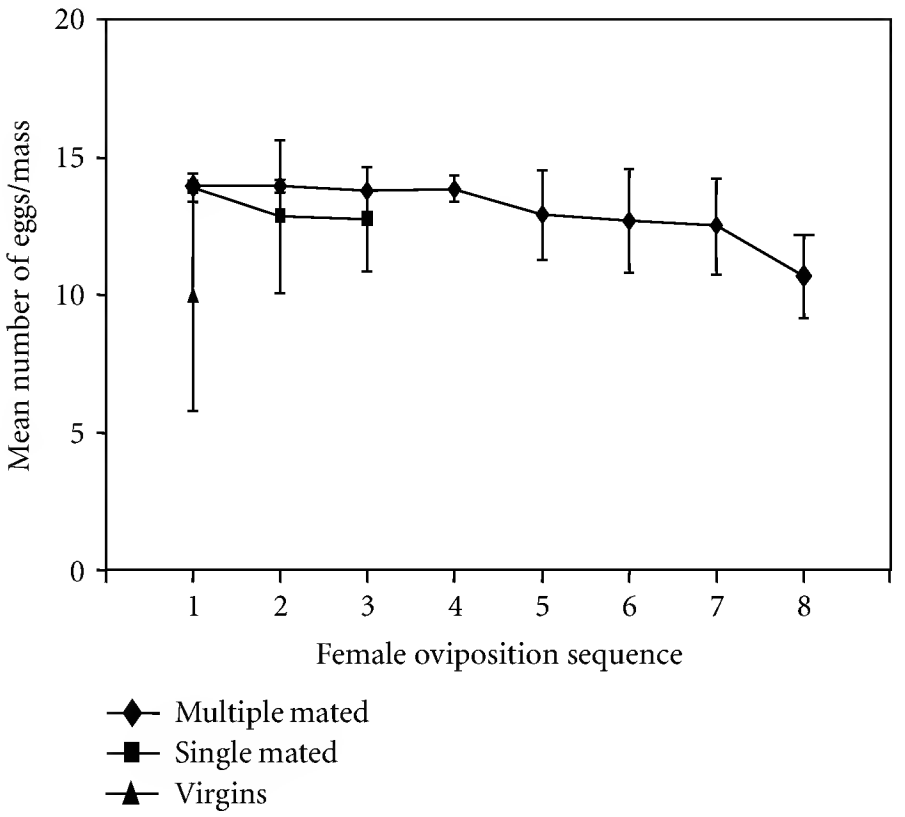

FIGURE 1: Mean number of eggs oviposited by *Edessa meditabunda* females in successive ovipositions. Multiple-mated females: females maintained with males during all their reproductive life. Single-mated females: females maintained with males until complete one mating. Virgins: females maintained isolated of males during all their reproductive life. Vertical lines indicate the standard deviation of the means.

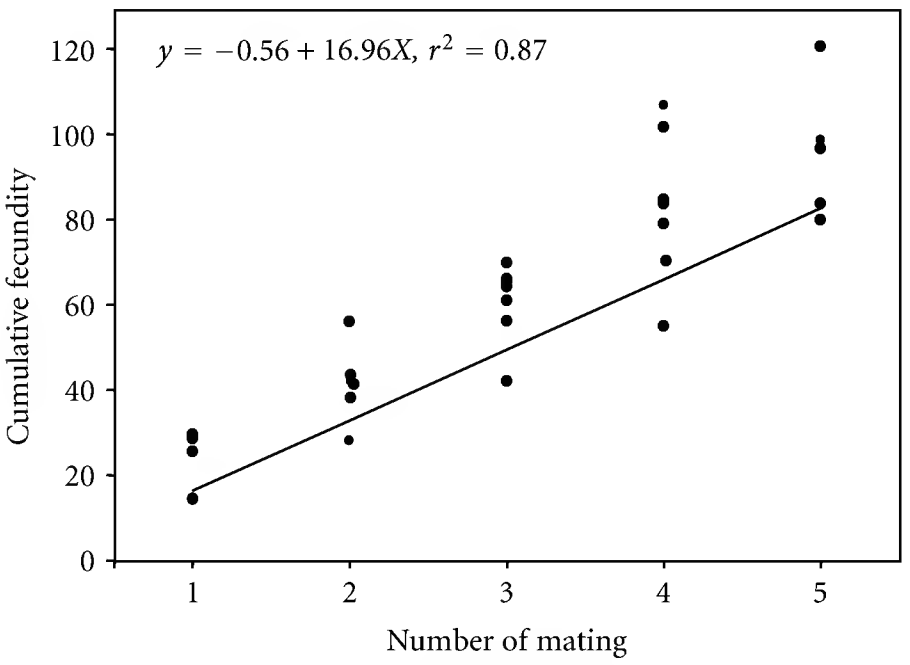

FIGURE 2: Relation between number of mating and cumulative fecundity (cumulated number of eggs/female after successive mating). A linear model was adjusted to the variables. Points indicate the cumulative number of eggs of each individual observed and line the values estimated from the adjusted model.

(Table 2). However, there was no significant difference in the number of eggs per mass in multiple- or single-mated females (Table 2) but a significant difference in the number of eggs/mass from these groups of females in comparison with the lower number determined in virgin females (Table 2).

Most eggs were laid at night in two rows of approximately seven eggs per row, generally, under the surface of *bold* leaves and never on the smooth surface of the cages. Despite the increased fecundity of multiple-mated females there was no significant difference in the fertility (number

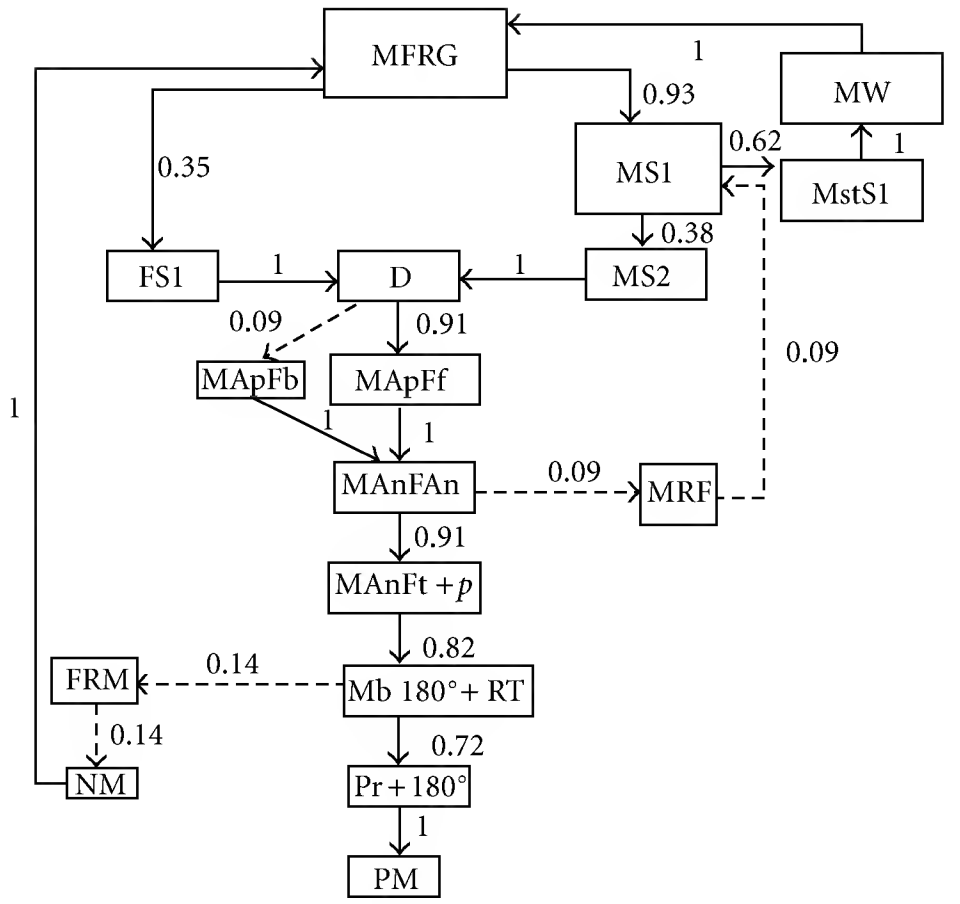

FIGURE 3: Sequence of courtship and mating behavior of *Edessa meditabunda*. Values represent the probability of transitions between behaviors. Solid-line arrows indicate the significant transitions ($P < 0.05$) and dashed-line arrows not significant transitions ($P > 0.05$). Boxes representing behavioral categories are in relative size to the frequency-observed behaviors from 62 pairs (male and female) observed. Codes for behavioral categories are listed in Table 1.

nymphs/number of eggs) of the single- or multiple-mated females (Table 2). Thus, multiple mating was required for fecundity but not for egg viability.

Despite of higher fecundity, the number of egg/mass was constant and no effect of female age was observed for multiple- or single-mated females; the number of eggs/mass did not show significant differences in consecutive ovipositions in these groups of females (ANODEV $\chi^2_7 = 4.08$ $P = 0.77$ for multiple-mated females and $\chi^2_2 = 0.64$ $P = 0.72$ for single-mated females) (Figure 1). In the same way and for multiple-mated females, the cumulative fecundity (mean number of cumulative eggs oviposited by females) after one to five mating shows a significant linear increase ($F_{1-67} = 443.6$ $P < 0.001$ $r^2 = 0.87$) (Figure 2).

At close range (i.e., below 10 cm between mates), successful copulation followed the usual behavioral steps described until now in most stink bugs as resting, grooming (i.e., rubbing the antennae, thorax, or abdomen with a par of leg), approaching, antennation during male-female interaction, abdominal vibration, genitalia contact, and copulation [15]. Courtship was initiated by male approaching the female by emission of vibrational signals before any physical contact, indicating that at close range vibratory signals are involved in the first encounter. The courtship steps are characterized and coded in the Table 2, and their transitional probabilities are shown in the ethogram (Figure 4).

Vibratory communication started with the emission of the first male song (MS1) (Figure 4(a)). This song was produced when a male was alone in the arena, in the presence of a

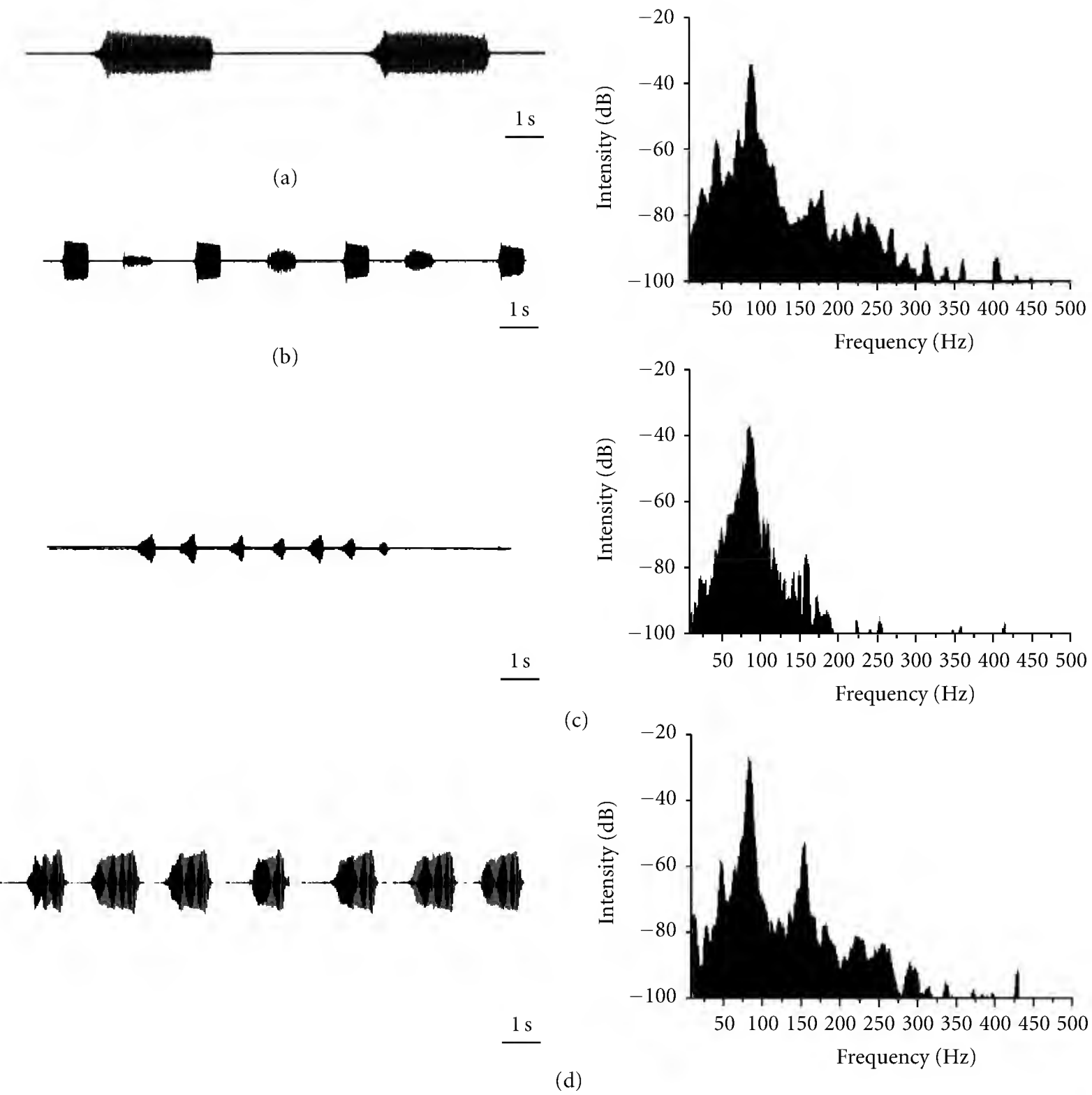

FIGURE 4: Oscillogram and frequency spectrum of one pulse of *Edessa meditabunda* vibratory songs: (a) male song 1 (MS1); (b) male rivalry song (MR); (c) male song 2 (MS2); (d) female song 1 (FS1).

female or as response to a female song. During courtship, the MS1 may be changed to MS2 or interrupt it for some minutes (Figure 4(c)). In response to MS2 females emitted their first song (FS1), and, after the emission of some pulses, the couple started to exchange FS1 and MS2 first as intercalated and later with superimposed pulses (Figure 4). After some seconds of duet song emission, the male approached the female and started to antennate her body and antennae. The female responded with a slow antennation of male's head and antennae. Subsequently, the male antennated the posterior end of female's abdomen. During this mutual stimulatory antennation phase, both proceeded with the FS1 and MS2 song emission. In the final courtship step, both mates stopped singing, and the male lifted the female with the head to get the copula position, then turned its body 180° to female's back and its phygophore, so that male and female in copula faced each other in opposite direction, and finally the copulation occurred (Figure 4). We recorded no vibratory emissions of copulated male and female. The male rival song (MR) was emitted when two males were placed with a female in the arena, these males emitted pulses similar (same temporal and spectral parameters) to MS1 pulses in an alternated a-b-a-b-a-b fashion. The pulses of one of the males were emitted with higher amplitude, until the other was silenced (Figure 4(a)).

Vibratory signals showed typical temporal and spectral characteristics described until now for most Pentatominae species (Figure 4, Table 3). The male song MS1 was a typical calling song, with relatively long pulses (~1 sec in duration) that may be repeated for several minutes. The dominant frequency and band width were also in the typical range of pentatomid vibratory signals (Table 3). The second song, MS2, was composed by a pulse trains of 5 to 11 short pulses with similar frequency characteristics as those of the MS1 (Table 3). Females emitted only one song type (Figure 4(d)) with specific temporal parameters and similar dominant frequency and band width as males' songs (Table 3). FS1 showed a clear frequency modulation with the dominant

Table 3: Temporal and spectral characteristics of male and female songs of *Edessa meditabunda*. Mean ± SD are show with the number of signals test (N) and the number of insect tested (n).

Song	Duration (ms)	Repetition time (ms)	Dominant frequency (Hz)	Bandwidth −20 dB (Hz)
MS1	1666,74 ± 147,38 (76/11)	4590,09 ± 1834,26 (65/11)	73,75 ± 2,60 (76/11)	20,08 ± 4,77 (76/11)
MS2	175,71 ± 83,91 (75/12)	305,97 ± 210,92 (64/12)	80,27 ± 5,68 (75/12)	57,87 ± 19,39 (75/12)
FS1	433,04 ± 78,81 (80/11)	677,19 ± 104,48 (80/11)	76,93 ± 5,82 (80/11)	17,73 ± 7,29 (80/11)

MS1: male song 1, MS2: male song 2, FS1: females song 1.

frequency decreasing throughout the duration of each pulse (Table 3). The duet emission of MS2 and FS1 shows a clear courtship function initiating all the sequential behaviors that lead to copula.

4. Discussion

Results presented here are the first report on the *E. meditabunda* reproductive biology, behavior, and vibratory communication. Adults of *E. meditabunda* showed lower copulation frequencies, reproductive potential, and longer sexual maturation period if compared with other pentatomid species reared under similar [29, 30] or different [25, 31, 32] laboratory conditions. The preoviposition period (time between emergence and first egg mass) for females with one or multiple matting was also longer when compared with other pentatomids [29, 32].

Repeated mating increased female fecundity in *E. meditabunda*, but it was costly in terms of reduced longevity compared with virgin females. Studies with some pentatomids reported similar correlation as a result of mating frequency [29, 32–34]. Decreased longevity and increased reproduction associated with multiple mating have been also reported for other insect species, like *Coccinella septempunctata* (L.) and *Propylea dissecta* (Mulsant) (Coleoptera: Coccinellidae) [35]. This seems to be the general negative cost of multiple mating [36].

Arnqvist and Nilsson [36] suggested that, in many species, especially among insects, exists an optimal remating rate for females so that one or a few matings are necessary to increase the offspring production. In such a way, additional matings are not necessary. Results of works with Heteroptera showed that in some species exists an optimal number of mating [31, 32, 37, 38] but in others not [39, 40]. In *E. meditabunda*, multiple-mated females have increased fecundity but did not show any fecundity peak along successive mating.

The fecundity of multiple-mated *E. meditabunda* could be considered low when compared with previous studies [11] or with data on other stink bug species [30, 31]. The low fecundity could be a characteristic of the central Brazil populations and may be related to the reproductive biology of the insect, since both adults showed long sexual maturation period and females a short period of oviposition. This may be the one of the reasons for the low population densities of *E. meditabunda* observed in the field.

At close range, the main behavioral steps of courtship in *E. meditabunda* did not differ from those observed for other species of pentatomids [17, 23–25, 31], except that, in all couples that emitted vibratory signals, males started the courtship by approaching females, antennate them, and emit vibratory signals. The sequence of steps was highly stereotyped, suggesting that once a male starts the courtship, the subsequent steps will most likely follow. Copulation was successful when the female remained stationary after the first contact. High percentage of courted females (53.06%) refuses copulation and run away from males during the antennation phase of courtship behavior. A similar failed courtship behavior was observed in *Murgantia histrionica* [25]. This fact could be a characteristic of the male selection behavior of females during courtship in some species of stink bugs or an effect of the artificial arenas used in the experiments.

The temporal and spectral patterns of vibratory signals of *E. meditabunda* were similar to the characteristics described previously for species of Pentatominae. However, some differences were found in the emission of signals and in the songs repertoire. In most Pentatominae species, two or three different male and female songs have been described [17–19] with calling and courtship songs of different temporal and spectral characteristics. In *E. meditabunda*, the repertoire of signals appears to be less complex with just one female and two male songs. The MS-2 has been emitted in the calling and courtship phase of the reproductive behavior. In most until now investigated Pentatominae species vibratory communication starts by female songs that trigger males to sing and move towards her [17–19]. The absence of a female song initiating the vibratory communication in *E. meditabunda* could be a characteristic of vibratory communications in Edessinae or could be related to the chemical communication in this species.

As sex pheromones of *E. meditabunda* was not identified, vibratory communication could have a central role to the sexual behavior in this species and males could use the vibratory signals to attract females.

Further observations on a plant are needed to confirm behavioral data and the role of communication signals of other modality described in this study for couples mating on a loudspeaker membrane. A possible influence of the size of arena on the vibratory communication cannot be discarded. Because the communication on stink bugs normally start on plants at distances of several cm (sometimes reaching 1 m or more) [18, 19], the reduced dimensions of the arenas used in our experiments (9 cm) could influence or inhibit the emission of some signals. In addition, the male calling song seems to act also as a rivalry song when a second male is present as reported by Shestakov [41] for Asopinae bugs.

Results here presented describe the mating biology, behavior, and vibratory signals of *E. meditabunda*. Multiple

mating showed to be advantageous for *E. meditabunda* females. During courtship, *E. meditabunda* communicates with signals produced by abdominal vibration. Songs are similar to those of other stink bugs studied with the exception that the courtship is initiated by males rather than by females as reported for other stink bugs.

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Research Article

Taxonomic Studies on the Genus *Athesapeuta* (Coleoptera: Curculionidae: Baridinae) from India with Description of Three New Species

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Six species of genus *Athesapeuta* Faust (Coleoptera: Curculionidae: Baridinae) from India and the adjacent countries are included, of which three new species, namley *meghalayensis* sp. nov., *richardi* sp. nov., and *spinulatus* sp. nov., are described. An annotated checklist of known species along with their synonymy and distribution is given. Descriptions are supplemented with details of genitalia and elytral vestiture; a key to the species studied is provided.

1. Introduction

Athesapeuta belongs to the tribe Madarini which at present is considered under the subfamily Baridinae of the family Curculionidae [1]. The majority of its species are known from the Oriental region, of which eight are from India. Except for the studies by Faust [2, 3] and Marshall [4], taxonomic studies on the Indian fauna are inadequate, scattered, and need to be updated and supplemented with descriptions of genitalia, morphometrics, and terminology. In particular, elytral vestiture and female genitalia which are emerging as important characters have not been included in descriptive work. The present study addresses these gaps for the currently recognized species and adds three new species.

2. Materials and Methods

Voucher specimens and type material are deposited with the National Pusa Collection (NPC) of the Division of Entomology, Indian Agricultural Research Institute, New Delhi. Materials from the National Pusa Collection (NPC) of the Division of Entomology, Indian Agricultural Research Institute, New Delhi; Forest Research Institute (FRI), Dehra Dun; Zoological survey of India (ZSI), Kolkotta; Panjab University (PU), Chandigarh, were included. Paratypes were studied for all the species described, and they are compared with original literature and photographs. All taxonomic characters, except the elytral vestiture and genitalia, were studied in intact specimens. Specimens were processed following Supare et al. [5], and genitalia and elytral vestiture were studied following Ramamurthy and Ghai [6]. The terminology of Supare et al. [5], Thompson [7], Poorani and Ramamurthy [8], O′Brien and Pakaluk [9], Wanat [10], and Davis [11] was followed for the description of female and male genitalia. General taxonomic characters and genitalia were studied with Leica M205FA stereozoom microscope, and elytral vestiture was studied with Leica DM1000 phase contrast microscope. Photographs were captured using the software Leica application Suite ver. 2.8.2 on a Leica DFC290 camera. Illustrations were made using a drawing tube fitted with a camera lucida and scales of magnification provided in the illustrations. Total length given in the descriptions is excluding rostrum, and the standard length is from anterior margin of pronotum to end of pygidium. The measurements given in the descriptions are mean and standard error except for new species, wherein measurements are of holotype.

3. Genus: *Athesapeuta* Faust

Athesapeuta Faust 1894 [2]. Type species: *Baridius subsignatus* Motschulsky, 1866 [12] = *Athesapeuta motschulskyi* Voss, 1958 [13]; gender: Female. (By "Original Designation").

Description. General colour shiny black; body rhomboidal, subcylindrical, or oblongovate, much longer than broad. Head not separated from rostrum by a deep transverse incision, frons narrower than base of rostrum, 1.5–4x as broad as long. Rostrum long, cylindrical, straight at least in basal half, separated from head by a sharp sulcus between eyes; more or less curved with mandibles adducent type, dentate internally or conical with straight cutting edge, 0.79–0.98x as long as head and pronotum combined (Figure 1(a)); eyes 1.28–2.12x as wide as long. Antennae inserted at 1.1–1.8x of length from base of rostrum; scape 6.25–10x as long as broad; funicle widening distally; seventh funicle not fitting closely to club (Figure 1(b)). Prothorax generally shallowly bisinuate at posterior dorsal margin, anterior dorsal margin truncate, not produced over head when viewed in profile, as broad as to 1.28x as broad as long. Scutellum generally trapezoidal, as broad as to 1.5x as broad as long. Elytra oblong or ovate, smooth; 0.5–0.92x as wide as prothorax; separately arcuate at base; deeply striate, striae 10 complete; separately rounded at apices, with narrow membrane and paler fringe along apical margin, without any trace of subapical calli (Figure 1(c)). Legs with femora clavate (Figure 1(d)), unarmed, not sulcate beneath; tibiae with longitudinally confluent punctures, sulcate, not tuberculate (Figure 1(e)); claws almost parallel to each other, more or less divergent, narrowly separated (Figure 1(f)). Sternum with a furrow on prosternum; procoxa 1.53–2.5x as wide as intercoxal process; hind margin of prosternum produced in middle; mesosternum transversely impressed, intercoxal process 0.9–1.25x as broad as a coxa; metasternum elongate, 1.5–1.66x as long as mesocoxa; pro-, meso-, and metasterna not interrupted in continuity by mesosternum (Figure 1(g)). Abdomen with first and second ventrites not connate; pygidium fully or broadly exposed, directed posterioventrally, visible dorsally, without transverse carina along hind margin of elytra in general; lateral part of fifth ventrite visible dorsally on each side of pygidium. Female genitalia with spermatheca more or less sclerotised at distal arm, distal arm shorter than proximal arm; spiculum ventrale 1.5–2.5x as long as basal plate; apical end with hairs. Male genitalia with median lobe moderately sclerotised; apophyses 0.25–1.06x as long as median lobe.

4. Annotated Checklist

Faust [2]described this genus, and Voss [13] synonymised *Baridius subsignatus* with *Athesapeuta motschulskyi*, the type species. The majority of species were described before 1950. Faust contributed the most with nine species, followed by Marshall (7), Voss (6), Bohemann and Hustache (3 each); Motschulsky (2), and Fabricius, Gerstaecker and Zimmerman (1 each). This genus is predominantly Oriental (18 species) Thirteen are Afrotropical, and five occur in the Palaearctic.

Checklist: Type species: Baridius subsignatus Motschulskyi, 1866 [12] = *Athesapeuta motschulskyi* Voss, 1958 [13]. *(For more details see Table 1)* .

5. Description of Species

5.1. Athesapeuta cyperi Marshall, 1928 [17]. (Figures 2(a), 3(a), 4(a), 5(a), 6(a), 6(b), 8(a), 9(a), 10(a), 11(a), 11(d), 12(a), 13(a)–13(c), 14(a), 14(b), 15(a)–15(d) and 16(a)–16(c)).

Description. Colour shiny black; antennae, tibiae, elytra on lateral and apical margins reddish brown. Head bare, finely alutaceous with sparse punctures, 1.5x as broad as long; eyes 1.28x as wide as long. Rostrum 0.79x as long as head and pronotum combined, strongly curved, smooth, with four rows of small punctures in addition to punctate area above scrobes, sparse vestiture on each side of base, at middle 0.77x as broad as at apex, 0.76x as broad as at base (Figures 2(a) and 3(a)). Antennae inserted at 1.57x of length from base of rostrum; scape slender, long, 9.13x as long as broad, impunctate; funicle with first segment 3.5x as long as second and third combined, third 0.5x as long as broad, segments three to seven slightly transverse, subequal in length and breadth; funicle 1.18x as long as club (Figure 4(a)). Prothorax 1.03x as broad as long, sides subparallel from base to middle, anterior margin truncate, behind apex with tubular constriction, posterior margin shallowly bisinuate, at middle 1.91x as broad as at apex, and 0.98x as broad as at base; dorsum gently convex longitudinally, set with close shallow separated punctures, with a broad impunctate median stripe, punctures on pleurae larger and subreticulate (Figure 5(a)). Scutellum bare, trapezoidal, with two low longitudinal costae, 1.09x as broad as long. Elytra oblong ovate, separately rounded at apex, at middle 1.14x as broad as at apex, 1.13x as broad as at base; striae deep, indefinitely punctate, not diminishing at apex, striae 10 complete; intervals flat, 3x as broad as a striae, with a row of large shallow punctures, each with minute recumbent scale, vestiture small on intervals one to five, larger, scale-like on outer intervals (Figure 8(a)); elytral vestiture whitish, rod-shaped, tapering and pointed at base, blunt at apex, surface with striations reaching apex (Figure 9(a)). Legs coarsely punctate, each puncture containing a grey elongate vestiture; tibia sulcate; profemur with a fringe of long vestiture on ventral surface (Figure 6(a)), 1.12x as long as mesofemur, 1.09x as long as metafemur. Protibia 1.53x and 1.25x as long as meso- and metatibia, respectively, with a sharp tooth on inner edge at about middle in males (Figure 6(b)), females without it. First tarsal segment 1.1x as long as broad, 1.1x as long as second, 0.83x as long as and 0.71x as broad as third, third 1.16x as broad as long, fourth 3x as long as broad. Prosternum with deep transverse sulcus behind apex, base with raised fovea. Procoxa 1.87x as broad as its intercoxal process; mesosternum plate-like, depressed at base, raised at apex, intercoxal process 0.96x as broad as mesocoxa; metasternum depressed in middle with longitudinal impressed line, intercoxal process 1.33x as broad as metacoxa. Venter black, strongly punctate, each with broad vestiture; anterior margin of first ventrite broadly and shallowly ogival, posterior margin straight, 2.92x as broad as long, 1.74x as long as second, second 5x as broad as long, 0.65x as long as three and four combined, ventrites third and fourth subequal in length, five 2.18x as broad as long,

Table 1

Sl No	Species	Distribution
(1)	*affinis* Faust, 1898 [3]	India
(2)	*amoena* Voss, 1958 [13]	China
(3)	*armata* Hustache, 1932 [14]	Madagascar
(4)	*atronuda* Marshall, 1941 [15]	Uganda
(5)	*aurantiaca* Faust, 1894 [2]	Myanmar
(6)	*bengalica* Faust, 1894 [2]	India
(7)	*chinensis* Faust, 1894 [2]	China
(8)	*conradti* Hustache, 1932 [16]	Cameroun
(9)	*cyperi* Marshall, 1928 [17]	Philippines
(10)	*dodonis* (Marshall) = *Baris dodonis* Marshall, 1936 [18]; Pajni and Kohli, 1990 [19]	Uganda
(11)	*famula* (Fabricius) = *Curculio famula* Fabricius, 1798 [20]; Hustache, 1938 [21] = *Rhynchaenus famula* (Fabricius, 1798 [20]); Fabricius, 1801 [22]; Hustache, 1938 [21] = *Baridius famula* (Fabricius, 1798 [20]); Boheman in Schoenherr, 1836 [23]; Hustache, 1938 [21] = *centrodentatus* Desbrochers des Loges, 1891 [24]; Hustache, 1938 [21]	India
(12)	*flavicornis* (Boheman in Schoenherr) = *Baridius flavicornis* Boheman in Schoenherr, 1836 [23]; Hustache, 1938 [21]	USA
(13)	*gyrosicollis* Marshall, 1948 [25]	Southern Shan States
(14)	*immaculata* Faust, 1898 [3]	India
(15)	*latifasciata* Voss, 1958 [13]	China
(16)	*lineolatofasciata* (Motschulsky) = *Baridius lineolatofasciata* Motschulsky, 1866 [12]; Faust, 1894 [2]	India
(17)	*madugodana* Voss, 1957 [26]	Sri Lanka
(18)	*meghalayensis* sp. nov.	India
(19)	*motschulskyi* Voss, 1958 [13]	China
(20)	*oryzae* Marshall, 1916 [4]	India
(21)	*pinguis* Faust, 1894 [2]	Myanmar
(22)	*politirostris* Voss, 1962 [27]	Congo
(23)	*richardi* sp. nov.	India
(24)	*sculptilis* Gerstaecker, 1871 [27] = *scutellaris* Faust, 1896: 145	Africa
(25)	*secura* Faust, 1894 [2]	Myanmar
(26)	*semirubra* (Hustache) = *Titanobaris semirubra* Hustache, 1935 [28]; Marshall, 1941 [15]	Angola
(27)	*soror* Faust, 1898 [3]	India
(28)	*spinulatus* sp. nov.	India
(29)	*subcalva* Marshall, 1941 [15]	Uganda
(30)	*subsignatus* (Boheman in Schoenherr) = *Baridius subsignatus* Boheman in Schoenherr, 1836 [23]; Faust, 1894 [2]	Africa
(31)	*subsignata* (Motschulsky) not Boheman = *Baridius subsignata* Motschulsky, 1866 [12]; Faust, 1894 [2]	India
(32)	*sculpticollis* Voss, 1958 [13]	China
(33)	*ulvae* Zimmerman, 1942 [29]	Guam
(34)	*varicolor* Marshall, 1941 [15]	Uganda
(35)	*versicolor* (Boheman) = *Baridius versicolor* Boheman, 1859 [30] = *Baris versicolor* (Boheman, 1859 [30]); Hustache, 1938 [21]; Pajni and Kohli, 1990 [19]	Indonesia
(36)	*vinculata* Faust, 1894 [2]	Myanmar

posterior margin truncate; pygidium distinctly punctate with fringes of vestiture, exposed on ventral side, with an arch-shaped marking at middle in males (marking being the junction of tergites VII and VIII), females without it, 1.03x as broad as long (Figure 10(a)).

Female Genitalia. Spermatheca not sclerotised, distal arm as long and as broad as proximal arm, angle between proximal and distal arms not acute, nodulus small, ramus flat, cornu blunt (Figures 11(a) and 14(a)). Spiculum ventrale with shaft elongate, 1.5x as long as basal plate, basal plate 5x as long as

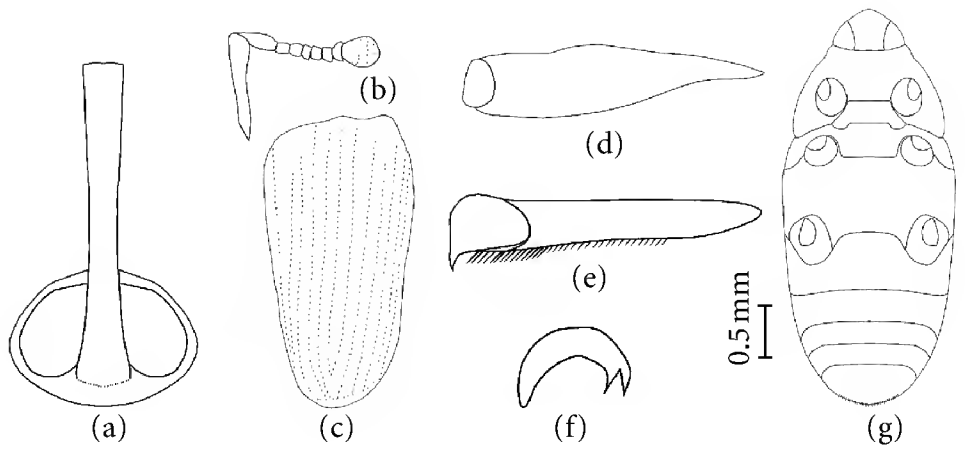

FIGURE 1: *Athesapeuta*. Genus characters: (a), rostrum, dorsal view; (b), antennae; (c), elytron, dorsal view; (d), femur, lateral view; (e), tibiae; (f), tarsal claw; (g), habitus; ventral view.

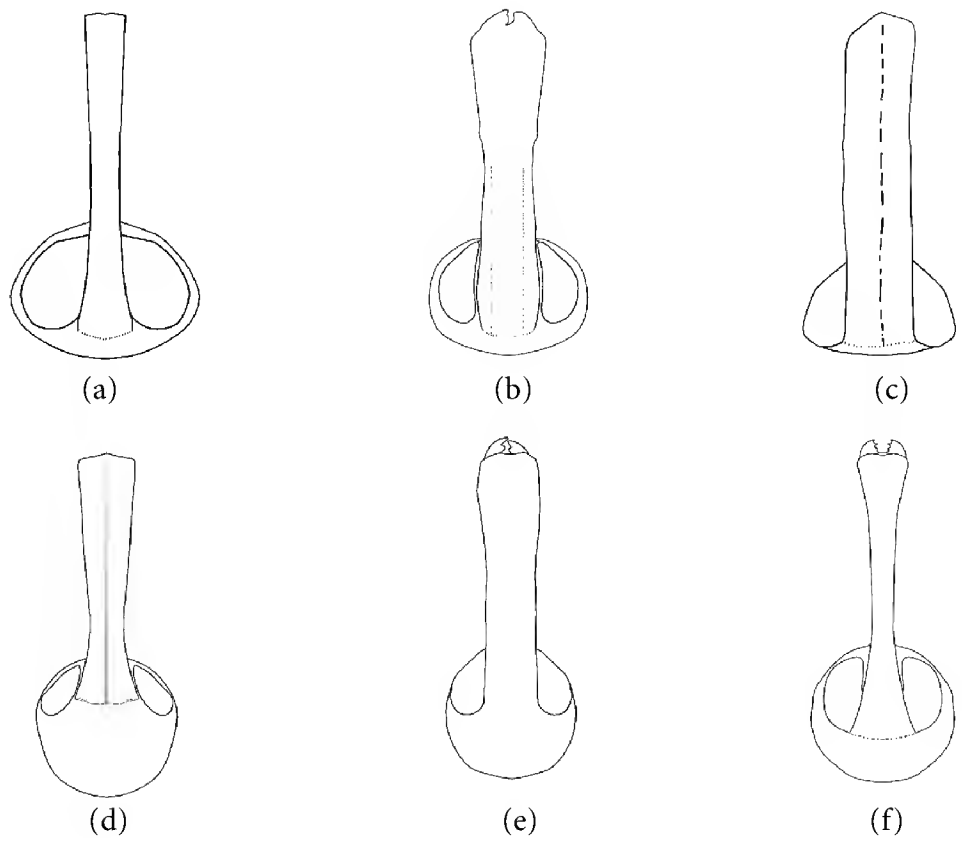

FIGURE 2: Rostrum, dorsal view: (a), *A. cyperi*; (b), *A. immaculata*; (c), *A. meghalayensis* sp. nov.; (d), *A. oryzae*; (e), *A. richardi* sp. nov.; (f), *A. spinulatus* sp. nov.

broad, apical end truncate, without hairs (Figures 11(d) and 14(b)).

Male Genitalia. Apophyses 0.25x as long as median lobe, 0.25x as long as spiculum gastrale; median lobe moderately sclerotised, parallel sided from base to behind middle, apex sinusoidal, at middle 1.42x as broad as at apex and as broad as at base (Figures 13(a)–13(c) and 15(a)–15(d)). Spiculum gastrale uniformly thick, curved at apex, 11.6x as long as broad (Figures 12(a) and 15(d)).

Measurements. Total length: 3.25 ± 0.23 mm; standard length: 3–3.30 mm; breadth: 1.36 ± 0.07 mm.

Material Examined. India: 6♂♂, Maharashtra: Phaltan, feeding on nut sedge, x.1999, Coll. Nimbkar; 4♂♂, 3 ♀♀, West Bengal: Kolkata, 11.i.2011, Coll. Ramasubramanian, larvae boring on *Cyperus rotundus*.

Distribution. India: Maharashtra; West Bengal. Philippines: Los Banos. Hawaii: Honolulu, Ohau.

5.2. Athesapeuta immaculata Faust, 1898 [3]. (Figures 2(b), 3(b), 4(b), 5(b), 8(b), 9(b), 9(c), and 16(d)–16(f)).

Description. Colour shiny black. Head with sparse punctures, 3.2x as broad as long; eyes 1.38x as wide as long. Rostrum 0.98x as long as head and pronotum combined, strongly curved, with two dorsal carinae from base to antennal insertion and then fading out, at middle 0.85x as broad as at apex, 0.92x as broad as at base (Figures 2(b) and 3(b)). Antennae inserted at 1.8x of length from base of rostrum; scape brown, impunctate, 8.33x as long as broad; funicle with first segment 1.16x as long as second and third combined, third as long as broad, segments three to seven slightly transverse and subequal in length and breadth; funicle 1.81x as long as club; club ovate (Figure 4(b)). Prothorax as broad as long, with granular punctures, anterior margin truncate, behind the apex without tubular constriction, posterior margin shallowly bisinuate, at middle 1.78x as broad as at apex, and 0.75x as broad as at base; dorsum gently convex longitudinally, set with shallow regular punctures, confluent in curves, without any median line. Scutellum raised, trapezoidal, sparsely punctate, as long as

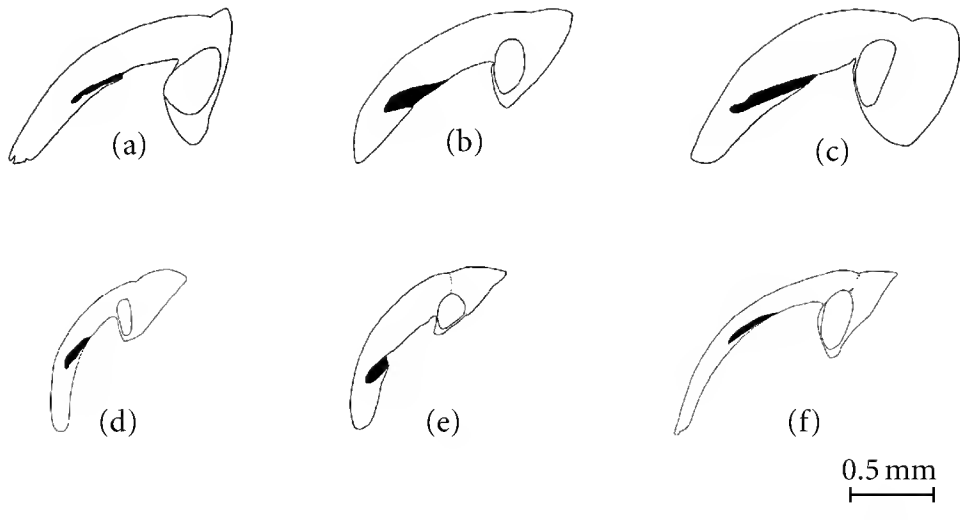

FIGURE 3: Rostrum, lateral view: (a), *A. cyperi*; (b), *A. immaculata*; (c), *A. meghalayensis* sp. nov.; (d), *A. oryzae*; (e), *A. richardi* sp. nov.; (f), *A. spinulatus* sp. nov.

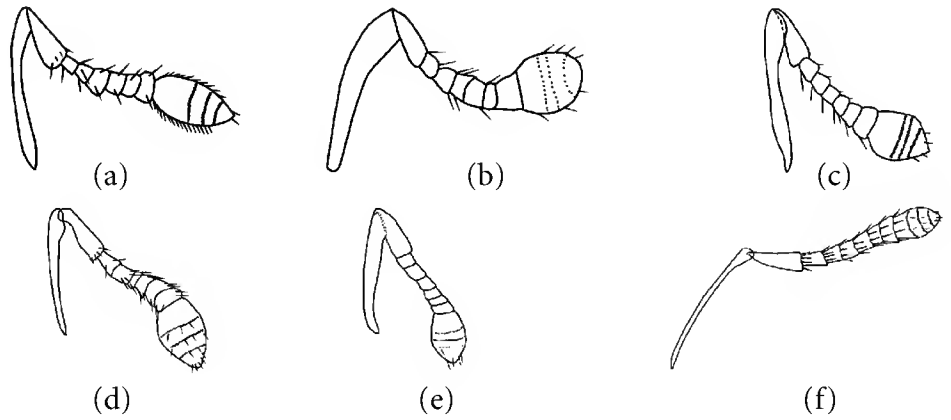

FIGURE 4: Antenna: (a), *A. cyperi*; (b), *A. immaculata*; (c), *A. meghalayensis* sp. nov.; (d), *A. oryzae*; (e), *A. richardi* sp. nov.; (f), *A. spinulatus* sp. nov.

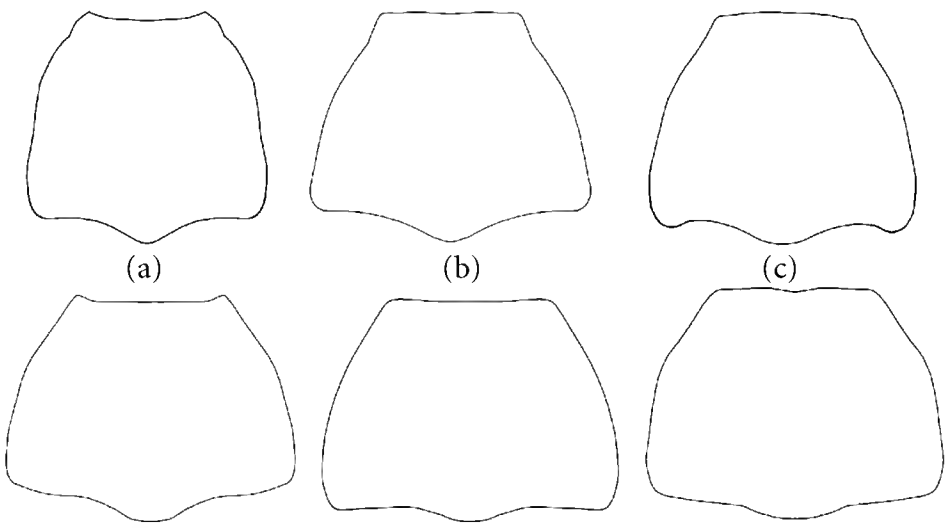

FIGURE 5: Prothorax, dorsal view: (a), *A. cyperi*; (b), *A. immaculata*; (c), *A. meghalayensis* sp. nov.; (d), *A. oryzae*; (e), *A. richardi* sp. nov.; (f), *A. spinulatus* sp. nov.

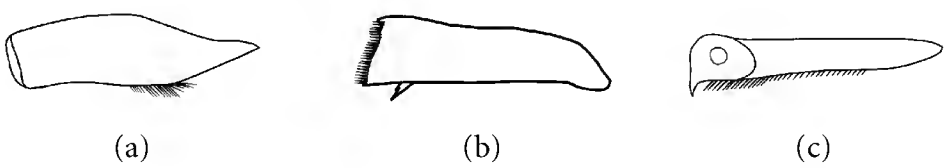

FIGURE 6: (a, b) Profemur and metatibia: *A. cyperi*; (c) Protibia: *A. oryzae*.

FIGURE 7: Protibia and metatibia: *A. richardi* sp. nov.

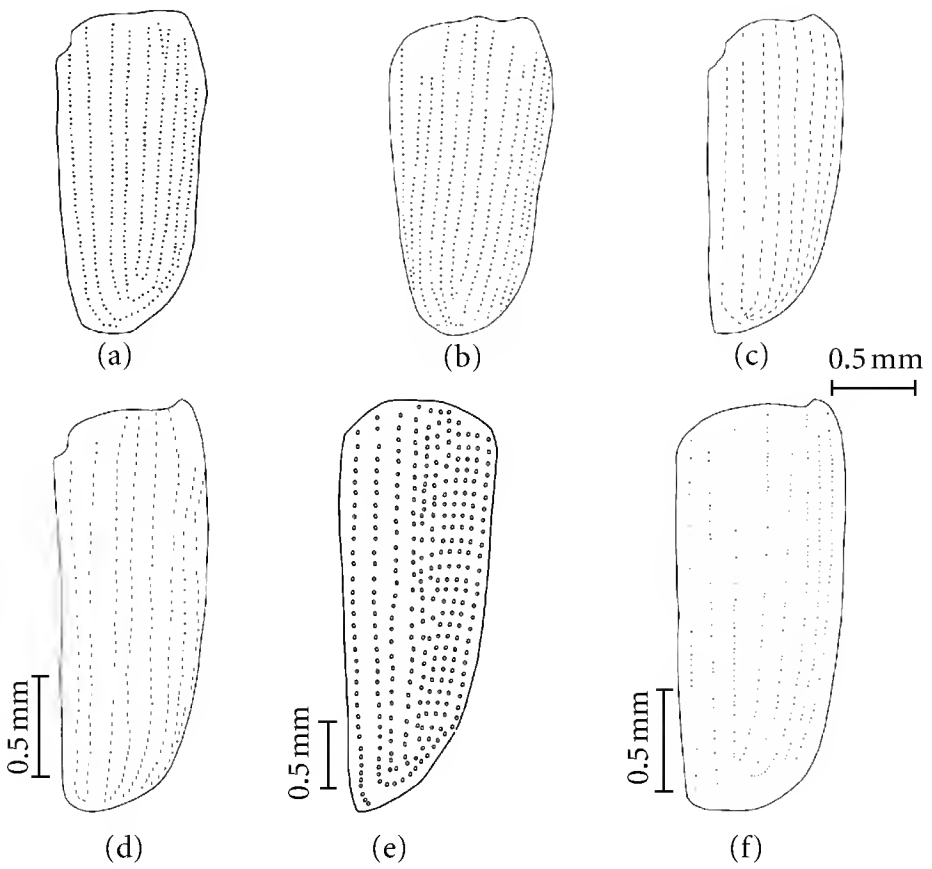

FIGURE 8: Elytron, dorsal view: (a), *A. cyperi*; (b), *A. immaculata*; (c), *A. meghalayensis* sp. nov.; (d), *A. oryzae*; (e), *A. richardi* sp. nov.; (f), *A. spinulatus* sp. nov.

broad (Figure 5(b)). Elytra oblong ovate, separately rounded at apex, without any posterior calli, at middle 1.32x as broad as at apex, 1.22x as broad as at base; striae shallow, with distant catenulate punctures which do not encroach on intervals, striae 10 complete; intervals flat, 1.5x as broad as a striae, with a row of transverse punctures, each containing a minute recumbent scale (Figure 8(b)). Elytral vestiture predominantly of two types; either yellowish white, base tapering and apex concave, surface with granular serrations (Figure 9(b)), or brownish yellow, both ends broader and surface with dense granular serrations (Figure 9(c)). Legs coarsely punctate, each puncture containing grey vestiture; tibia sulcate; profemur 0.90x as long as mesofemur, 0.83x as long as metafemur. Protibia 1.2x and 0.93x as long as meso- and metatibia, respectively. First tarsal segment 1.66x as long as broad, 1.66x as long as second, as long as and 0.54x as broad as third, third 0.90x as broad as long, fourth 4x as long as broad. Prosternum with deep transverse sulcus behind apex, base of prosternum with raised fovea, with definite punctures all over sternum and each punctures with a grey recumbent vestiture. Procoxa 2.5x as broad as intercoxal process; mesosternum plate like, depressed at base and apex, intercoxal process 1.25x as broad as mesocoxa; metasternum depressed in middle with longitudinal impressed line, intercoxal process 1.08x as broad as metacoxa.

Measurements. Total length: 5.4–5.5 mm; standard length: 4.7 mm; breadth: 2.2–2.4 mm.

Material Examined. 2 specimens, location unknown, from Nagasilla grass (abdomen damaged), 12.vii.1985, Coll. unknown.

Distribution. India.

5.3. Athesapeuta meghalayensis sp. nov. (Figures 2(c), 3(c), 4(c), 5(c), 8(c), 9(d), 9(e), 10(b), 12(b), 13(d)–13(g), 15(e)–15(i), and 16(g)–16(i)).

Diagnosis. It is closely related to *A. richardi* sp. nov., but differs in prothorax with broad stripe of yellow vestitures (white in *A. richardi* sp. nov.), with a smooth median line (absent in *A. richardi* sp. nov.); posterior end of tibia does not carry sharp tooth (present in *A. richardi* sp. nov.), metatibia lateroventrally without fringes of grey hairs (present in *A. richardi* sp. nov.).

Description. Colour black. Head with close regular punctures, 4x as broad as long; eyes ventrally placed, 2.12x as wide as long. Rostrum 0.9x as long as head and pronotum combined, strongly curved, gradually widening, irregularly punctate, each punctures with yellow vestiture, more prominent in basal region, irregular punctures become reticulate and rugose beyond antennal insertion, with a median smooth impunctate line, almost parallel sided, without any subbasal dilation (Figures 2(c) and 3(c)), at middle 1.09x as broad as at apex, 0.92x as broad as at base. Antennae inserted at 1.22x of length from base of rostrum; scape robust, 6.25x as long as broad, almost impunctate; funicle with first segment 1.2x as long as second and third combined, segments second to seven carry sharp spines all over surface, third as long as broad, segments three to seven transverse and subequal in length and breadth; funicle 1.46x as long as club; club ovate (Figure 4(c)). Prothorax 1.03x as broad as long, with granular punctures, with a stripe of yellow vestiture on lateral aspect just behind the anterior margin on both sides, which is continuous with scaling of lower surface, tubular constriction at apex, sides gently rounded, posterior margin shallowly bisinuate, at middle 1.8x as broad as at apex, 0.95x as broad as at base, dorsum gently convex

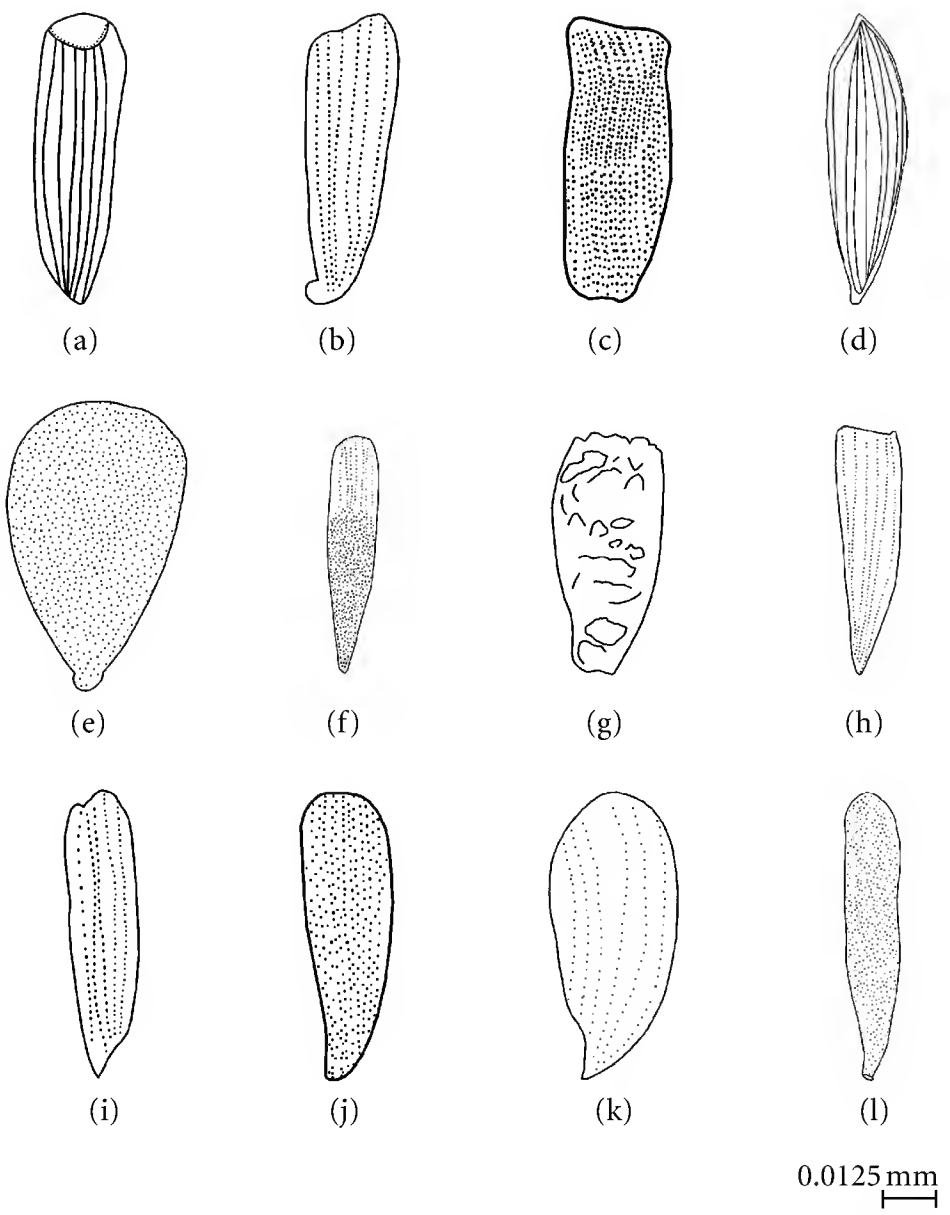

FIGURE 9: Elytral vestiture: (a), *A. cyperi*; (b)-(c), *A. immaculata*; (d)-(e), *A. meghalayensis* sp. nov.; (f)-(g), *A. oryzae*; (h)–(j), *A. richardi* sp. nov.; (k)-(l), *A. spinulatus* sp. nov.

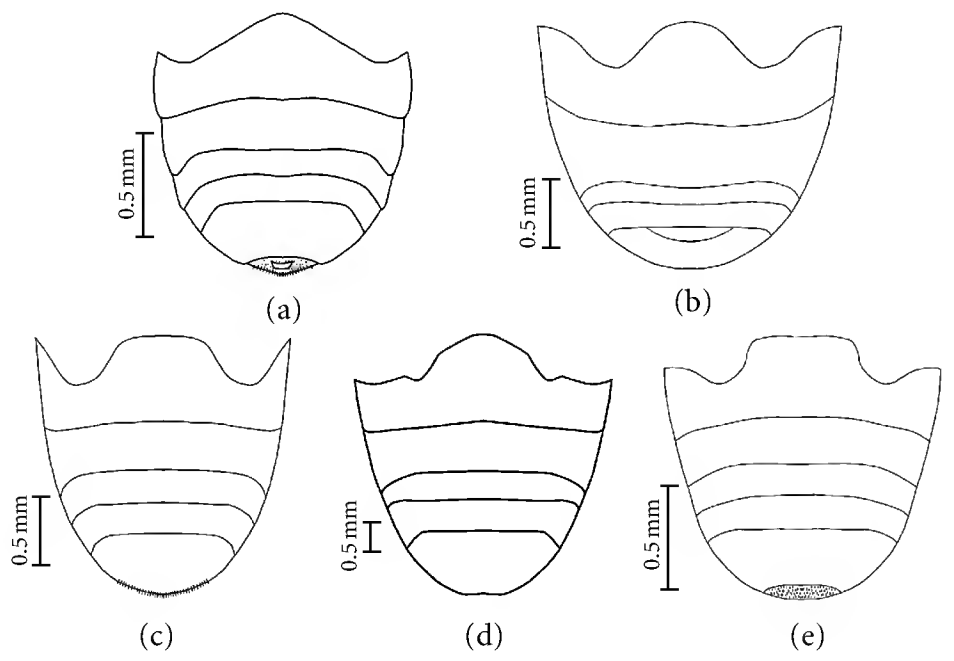

FIGURE 10: Venter: (a), *A. cyperi*; (b), *A. meghalayensis* sp. nov.; (c), *A.oryzae*; (d), *A. richardi* sp. nov.; (e), *A. spinulatus* sp. nov.

longitudinally, set with close granular punctures, confluent in curves, with smooth median line, patches of yellowish white vestiture just above posterior margin of prothorax (Figure 5(c)). Scutellum strongly transverse, square shaped, not punctate, without median impression, as broad as long. Elytra ovate, without deep subapical impressions, without posterior calli, apices rounded, at middle 1.14x as broad as at apex, 1.33x as broad as at base; striae shallow, with distant separate punctures which do not encroach on intervals, striae 10 complete; intervals 4x as broad as a striae, with a row of catenulate transverse punctures, each containing a minute black recumbent scale, lateral margin smooth at apex, interval five with a patch of yellowish vestiture on basal end, middle of elytra with larger patch of vestiture on interval 4 and 5 (Figure 8(c)); elytral vestiture either brownish yellow, with basal end blunt, apex pointed with lines on surfaces reaching apex (Figure 9(d), or greyish white, pear shaped, broad at apex with granular striations on surface (Figure 9(e)). Legs coarsely punctate, each puncture containing a grey vestiture; tibiae sulcate; profemur 1.31x

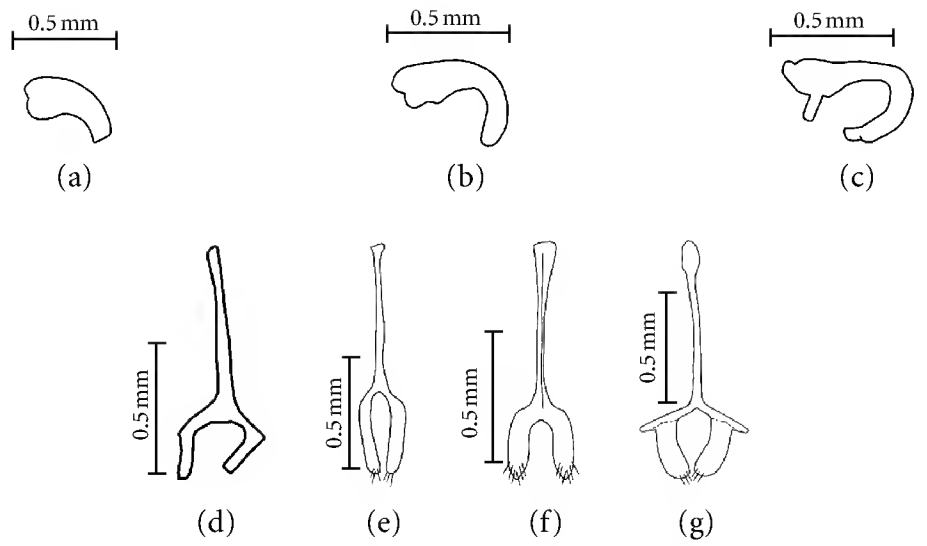

FIGURE 11: Female genitalia, spermatheca, and spiculum ventrale: (a), (d), *A. cyperi*; (b), (e), *A. oryzae*; (c), (f), *A. richardi* sp. nov.; (g), *A. spinulatus* sp. nov.

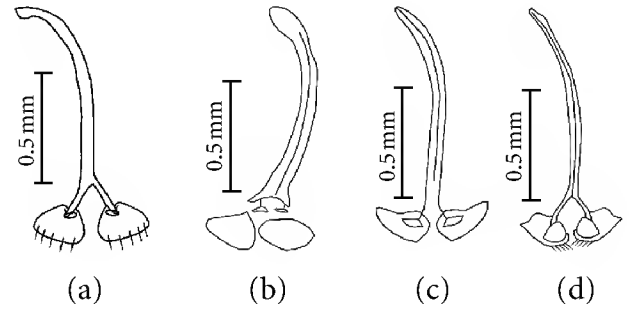

FIGURE 12: Male genitalia, spiculum gastrale: (a), *A. cyperi*; (b), *A. meghalayensis* sp. nov.; (c), *A. richardi* sp. nov.; (d), *A. spinulatus* sp. nov.

as long as mesofemur, 0.92x as long as metafemur. Protibia 0.97x as long as mesotibia, and as long as metatibia. First tarsal segment as long as broad, 1.25x as long as second, 0.83x as long as third, third 1.66x as broad as long, fourth 2.75x as long as broad. Prosternum with deep transverse sulcus behind apex, base of prosternum with raised fovea. Procoxa 1.98x as broad as intercoxal process; mesosternum plate like, depressed at base and apex, intercoxal process 1.03x as broad as mesocoxa; metasternum shallowly depressed at middle with impressed longitudinal line, intercoxal process 2.01x as broad as metacoxa. Venter black, clothed with broad greyish yellow vestiture, strongly punctate, each with broad white scale, anterior margin of first ventrite ogival, posterior margin subtruncate, 2.7x as broad as long, 1.66x as long as second, second 4.22x as broad as long, 1.12x as long as three and four combined, third and fourth subequal in length, ventrite five 3.12x as broad as long, posterior margin rounded; pygidium distinctly punctate, broadly exposed with long hairs from each puncture, 1.53x as broad as long (Figure 10(b)).

Male Genitalia. Apophyses 1.06x as long as median lobe, 0.7x as long as spiculum gastrale, 1.84x as long as tegmen; median lobe moderately sclerotised, parallel sided from base to behind middle, apex truncate, at middle 1.25x as broad as at apex, and 1.42x as broad at base (Figures 13(d)–13(f) and 15(e)–15(g)). Tegmen 1.9x as long as manubrium, 1.58x as long as parameroid lobe; manubrium flat (Figures 13(g) and 15(i)). Spiculum gastrale uniformly thick, curved at apex, with a median line, 12.5x as long as broad (Figures 12(b) and 15(h)).

Measurements of Holotype. Total length: 4.20 mm; standard length: 3.80 mm; breadth: 1.78 mm.

Material Examined. Holotype ♂, India: Meghalaya: Tura, date and coll. unknown, from wild plant (latitude: 25° 30′ N; Longitude: 90° 16′ E). Paratypes (2♂♂): 1♂, India: Meghalaya: Ambashi, from wild plant, 26.v.1988, Coll. D. Kumar; 1♂, Assam: Nagora, from wild plants, 23.v.1988, Coll. Baljinder.

Distribution. India: Meghaya; Assam.

Etymology. The specific epithet refers to the type locality.

5.4. Athesapeuta oryzae Marshall, 1916 [4]. (Figures 2(d), 3(d), 4(d), 5(d), 6(c), 8(d), 9(f), 9(g), 10(c), 11(b), 11(e), 14(c), 14(d)).

Description. Colour shiny black. Head convex, finely shagreened with regular close puncture, 1.75x as broad as long; eyes 1.47x as wide as long. Rostrum 0.92x as long as head and pronotum combined, strongly curved, without any subbasal dilation, with coarse punctures especially at sides, and with an impunctate median carinae, at middle 0.83x as broad as at apex, 0.67x as broad as at base (Figures 2(d) and 3(d)). Antennae black, with whorls of stout yellowish vestiture, inserted at 1.1x of length from base of rostrum; scape slender, 10x as long as broad; funicle with first segment 1.42x as long as second and third combined, third as long as broad, segments three to seven transverse and subequal in length and breadth; funicle 1.85x as long as

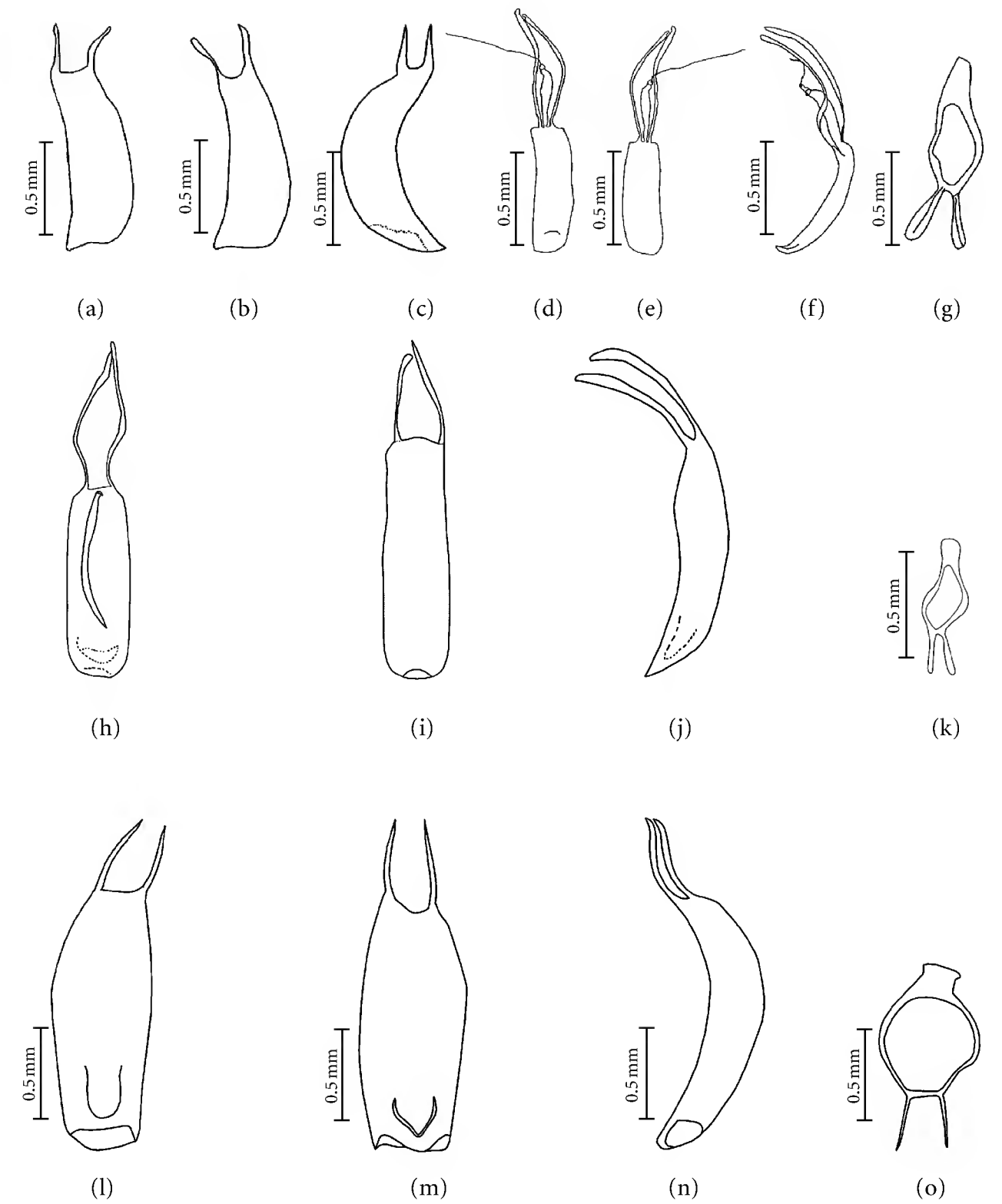

FIGURE 13: Median lobe (dorsal, ventral, and lateral view) and tegmen: (a)–(c), *A. cyperi*; (d)–(g), *A. meghalayensis* sp. nov.; (h)–(k), *A. richardi* sp. nov.; (l)–(o), *A. spinulatus* sp. nov.

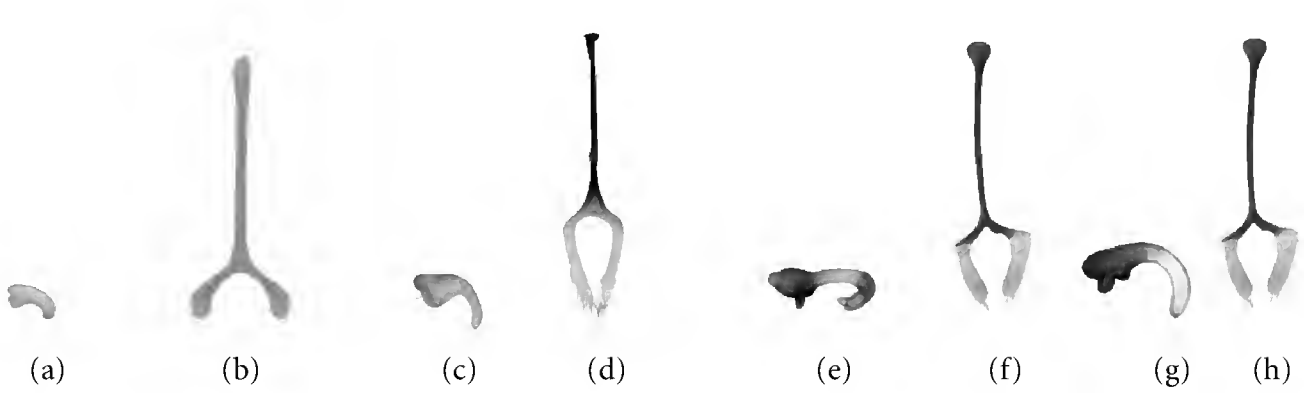

FIGURE 14: Female genitalia, spermatheca, and spiculum ventrale: (a), (b), *A. cyperi*; (c), (d), *A. oryzae*; (e), (f), *A. richardi* sp. nov.; (g), (h), *A. spinulatus* sp. nov.

club; club with whorls of vestiture (Figure 4(d)). Prothorax 1.19x as broad as long, anterior margin truncate, posterior margin bisinuate, at middle 1.9x as broad as at apex, 0.97x as broad as at base, with broad lateral stripe, which is continuous with scaling of lower surface, interrupted about middle by a small bare kidney-shaped spot, with shallow constriction at apex, gently rounded at sides, set with close coarse punctures, with an abbreviated impunctate median line (Figure 5(d)). Scutellum strongly transverse, coarsely punctate, with narrow vestiture, 1.2x as broad as long. Elytra oblong, separately rounded at apex, at middle 1.21x as broad as at apex, 1.21x as broad as at base, with a large basal patch consisting of lines of vestiture on intervals three to eight, those on fifth and sixth longest, those on four, three,

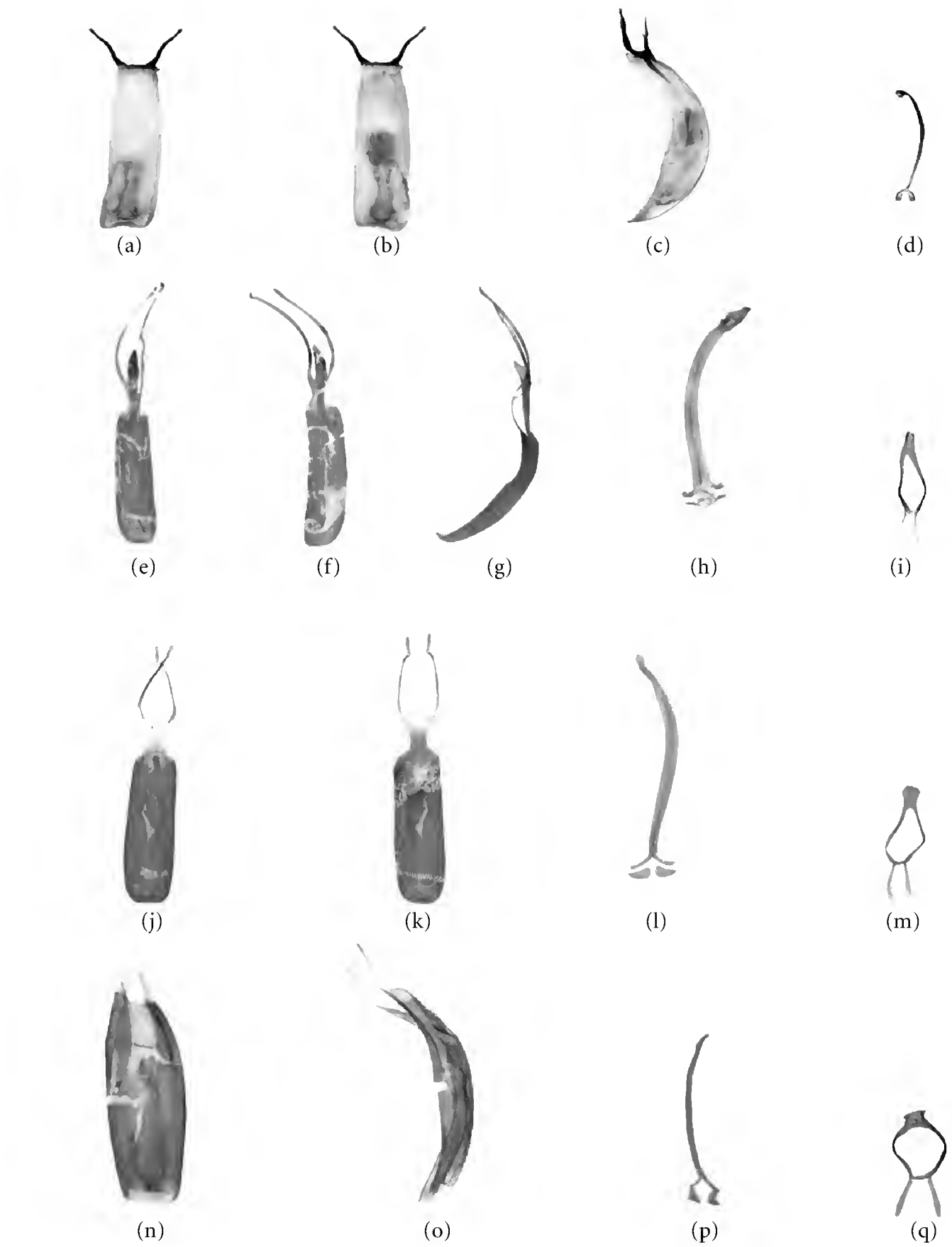

FIGURE 15: Male genitalia, median lobe (ventral, dorsal, and lateral view), spiculum gastrale, and tegmen: (a)–(d), *A. cyperi*; (e)–(i), *A. meghalayensis* sp. nov.; (j)–(m), *A. richardi* sp. nov.; (n)–(q), *A. spinulatus* sp. nov.

seven, and eight diminishing in the order given, whole patch covering about one-third of elytra and leaving shoulder bare, close behind it another large irregular patch, lines of which intervals 2, 3, 4, 8, and 9 are short, with 5, 6, and 7 being longer, that on 5 longest, with a "v"-shaped apical patch extending from interval 3 to 8; striae deep, striae 10 complete; intervals almost plane, 4x as broad as a striae (Figure 8(d)); elytral vestiture elongate, and with sparse punctures on it (Figure 9(f)), or with irregular patches on it (Figure 9(g)). Legs with densely clothed yellowish white vestiture; all tibiae at apical end on sides with fringes of hairs (Figure 6(c)); profemur 1.13x as long as mesofemur, 1.09x as long as metafemur. Protibia 1.25x and 1.12x as long as meso and metatibia respectively. First tarsal segment 0.85x as long as broad, 0.75x as long as second, 0.46x as long as and 0.53x as broad as third, third as long as broad, fourth 3.75x as long as broad. Prosternum with deep transverse sulcus behind apex, whole lower surface densely scaled, base of prosternum with fovea. Procoxa 2.42x as broad as intercoxal process; mesosternum plate like, raised at apex, intercoxal process 0.9x as broad as mesocoxa; metasternum flat without median line, vestiture closer on meta-episternum, intercoxal process 2x as broad as metacoxa. Venter black, strongly punctate, each with broad scale, anterior margin of first ventrite ogival, posterior margin shallowly straight, 3.73x as broad as long, 1.03x as long as second, second 4.5x as broad as long, 0.92x as long as three and four combined, third and fourth subequal in length, ventrite five 5x as broad as long, posterior margin rounded; pygidium indistinctly punctate, exposed on dorsal side, 1.33x as broad as long (Figure 10(c)).

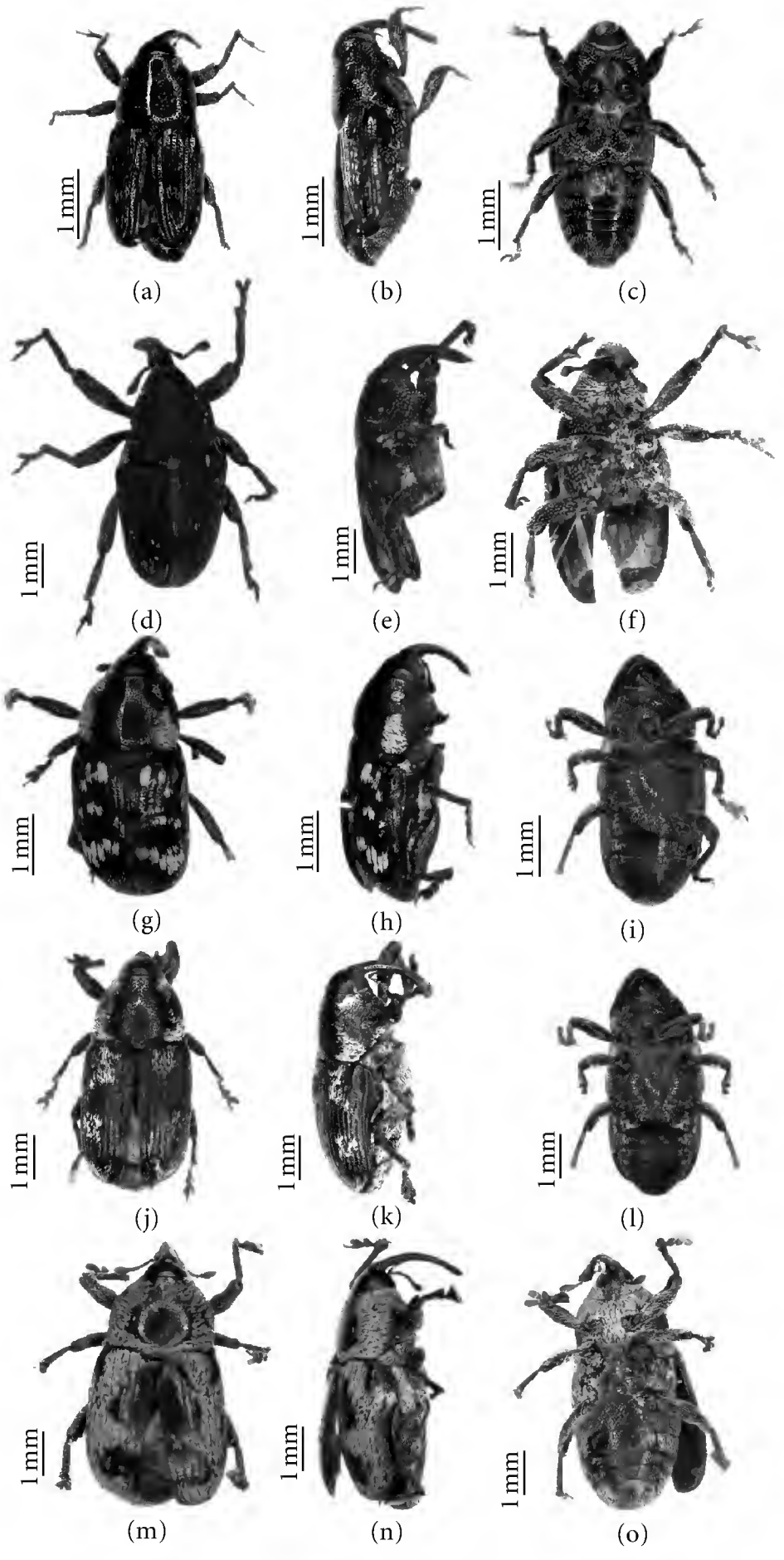

FIGURE 16: Habitus: dorsal, lateral, and ventral view: (a)–(c), *A. cyperi*; (d)–(f), *A. immaculata*; (g)–(i), *A. meghalayensis* sp. nov.; (j)–(l), *A. richardi* sp. nov.; (m)–(o), *A. spinulatus* sp. nov.

Female Genitalia. Spermatheca not sclerotised, distal arm as long as and as broad as proximal arm, angle between proximal and distal arms not acute, nodulus small, ramus flat, cornu slightly bent, not pointed (Figures 11(b) and 14(c)). Spiculum ventrale with shaft elongate, 1.84x as long as basal plate, basal plate 4.75x as long as broad, apical end rounded with hairs (Figures 11(e) and 14(d)).

Measurements. Total length: 5.38 mm; standard length: 4.90 mm; breadth: 2.28 mm.

Material Examined. 1 ♀, Coimbatore, Coll. and host unknown (written as Paratype and determined by G. A. K. Marshall).

Distribution. India: Tamil Nadu; Andhra Pradesh.

5.5. Athesapeuta richardi sp. nov. (Figures 2(e), 3(e), 4(e), 5(e), 7(a), 7(b), 8(e), 9(h)–9(j), 10(d), 11(c), 11(f), 12(c), 13(h)–13(k), 14(e), 14(f), 15(j)–15(m), and 16(j)–16(l)).

Diagnosis. This species is closely allied to *Athesapeuta oryzae* but differs in vestiture which is greyish white as compared to yellowish in *A. oryzae*; rostrum which is without impunctate median line, whereas in *A. oryzae* it is with impunctate median line; tibia with a sharp tooth just above mucro on lateral side and this is absent in *A. oryzae*, and female with cornu of spermatheca having finger-like projection in this species.

Description. Colour shiny blackish yellow. Head with close punctures, 1.76x as broad as long; eyes ventrally placed, 1.53x as wide as long. Rostrum 0.80x as long as head and pronotum combined, strongly curved, gradually widening, punctate, without median line, almost parallel sided, without any subbasal dilation, at middle 0.92x as broad as at apex, 0.63x as broad as at base (Figures 2(e) and 3(e)). Antennae inserted at 1.68x of length from base of rostrum; scape robust, 8.45x as long as broad, almost impunctate; funicle with first segment 0.76x as long as second and third combined, third subequal in length and breadth, segments three to seven transverse, subequal in length and breadth; funicle 2.56x as long as club; club ovate (Figure 4(e)). Prothorax 1.17x as broad as long, with granular punctures, with a broad lateral stripe of white vestiture, which is continuous with scaling of lower surface, interrupted about middle by a small bare kidney-shaped spot, apex with tubular constriction, sides gently rounded, anterior margin truncate, posterior margin shallowly bisinuate; at middle 1.80x as broad as at apex, 0.96x as broad as at base; dorsum gently convex longitudinally, set with close granular punctures, not confluent in curves, without any smooth median line (Figure 5(e)). Scutellum strongly transverse, square shaped, not punctate, without median impression, 1.5x as broad as long. Elytra ovate, without deep subapical impressions and posterior calli absent, apices rounded; at middle 1.62x as broad as at apex, 1.63x as broad as at base; striae shallow, with distant separate punctures which do not encroach on intervals, striae 10 complete; intervals 5.45x as broad as striae, with a row of irregular transverse punctures, each containing a minute vestiture, lateral margin smooth at apex (Figure 8(e)); elytral vestiture either white, with granular serrations reaching apex (Figure 9(h)), or brown with serrated granular ridges on it (Figure 9(i)), or surface without regular serrations but with scattered punctures (Figure 9(j)). Legs coarsely punctate, each puncture containing a grey vestiture; tibiae sulcate, posterior end of all tibiae just before mucro carries sharp tooth (Figure 7(a)); metatibia with fringes of grey hairs on lateroventral side (Figure 7(b)); profemur 1.06x as long as mesofemur, 0.93x as long as metafemur. Protibia 1.19x and 1.07x as long as meso- and metatibia, respectively. First tarsal segment 1.35x as long as broad, 0.98x as long as second, 0.76x as long as third, third 1.24x as broad as long, fourth 4.80x as long as broad. Prosternum with deep transverse sulcus behind apex, base with raised fovea. Procoxa 1.98x as broad as intercoxal process; mesosternum plate like, depressed at base and apex, intercoxal process 1.03x as broad as mesocoxa; metasternum shallowly depressed in middle with longitudinal line, intercoxal process 2.01x as broad as metacoxa. Venter black, strongly punctate, each with broad white vestiture, anterior margin of first ventrite ogival, posterior margin subtruncate, 3.09x as broad as long, 1.64x as long as second, second 4.70x as broad as long, 0.89x as long as three and four combined, third and fourth subequal in length, ventrite five 3.48x as broad as long, posterior margin rounded; pygidium with indistinct punctures, 1.53x as broad as long (Figure 10(d)).

Female Genitalia. Spermatheca more sclerotised at proximal arm, distal arm 0.93x as long as proximal arm, angle between proximal and distal arms acute, nodulus small, tapering towards apex, ramus tubular and long, cornu strongly pointed and with a finger-like projection (Figures 11(c) and 14(e)). Spiculum ventrale with shaft elongate, 2x as long as basal plate, basal plate 3x as long as broad, apical end pointed with hairs (Figures 11(f) and 14(f)).

Male Genitalia. Apophyses 1.06x as long as median lobe, 0.7x as long as spiculum gastrale, 1.84x as long as tegmen; median lobe moderately sclerotised, parallel sided from base to behind middle, apex truncate, at middle 1.25x as broad as at apex, and 1.42x as broad as at base (Figures 13(h)–13(k), 15(j) and 15(k)). Tegmen 1.9x as long as manubrium, 1.58x as long as parameroid lobe, manubrium flat (Figures 13(k) and 15(m)). Spiculum gastrale uniformly thick, curved at apex, with a median line, 12.5x as long as broad (Figures 12(c) and 15(l)).

Measurements of Holotype. Total length: 5.42 mm; standard length: 5.20 mm; breadth: 2.30 mm.

Material Examined. Holotype ♂: India: Meghalaya: Tura, Date and Coll. unknown, from wild plant (latitude: 25° 30′ N; longitude: ° 16′ E). Paratypes: 16 specimens (2 ♂♂, 1 ♀): India: 2 ♂, Meghalaya: Tura, date and coll. unknown, from wild plant; 1 ♀, 12 specimens, Andhra Pradesh: Patancheru, 18.ix.1985, Coll. M.Haq, from agricultural plants.

Distribution. India: Meghalaya; Andhra Pradesh.

Etymology. The name is derived from and in recognition of Dr. Richard Thompson for his contribution towards baridine weevils.

5.6. Athesapeuta spinulatus sp. nov. (Figures 2(f), 3(f), 4(f), 5(f), 8(f), 9(k), 9(l), 10(e), 11(g), 12(d), 13(l)–13(o), 14(g), 14(h), 15(n)–15(q), and 16(m)–16(o))

Diagnosis. This species is closely related to *A. immaculata* but differs in its larger size, with dense vestitures all over body; antennae with all funicular segments with four rows of spines; elytra ovate (oblong ovate in *immaculata*).

Description. Colour black. Head with shallow sparse punctures, 1.8x as broad as long; eyes ventrally placed, 1.48x as wide as long. Rostrum 0.91x as long as head and pronotum combined, curved, gradually widening, broadest at apex, closely punctate at basal side on lateral aspect, each puncture with yellow vestiture, dorsal surface smooth, at middle 0.75x as broad as apex, 0.66x as broad as at base, without any subbasal dilation (Figures 2(f) and 3(f)). Antennae inserted at 1.36x of length from base of rostrum; scape slender, long, 8.75x as long as broad, almost impunctate; funicle with first segment 1.1x as long as second and third combined, third 1.3x as long as broad, segments three to seven transverse

and subequal in length and breadth; funicle 2.53x as long as club, all segments carry sharp four rows of spines on its surface, seventh funicular segment broadest, which is not in continuous with club; club ovate (Figure 4(f)). Prothorax 1.28x as broad as long, with granular punctures at centre, a stripe of yellow vestiture on lateral aspect on both sides, which is continuous with scaling of lower surface, without tubular constriction at apex, broadly rounded at sides, anterior margin truncate, posterior margin shallowly bisinuate, at middle 2.22x as broad as at apex, 1.03x as broad as at base, dorsum gently convex longitudinally without any median line (Figure 5(f)). Scutellum strongly transverse, not punctate, without median impression, 1.5x as broad as long. Elytra ovate, without subapical impressions and without posterior calli, apices rounded, at middle 1.2x as broad as at apex, 1.5x as broad as at base; striae shallow, with distant separate punctures which do not encroach on intervals, striae 10 complete; intervals 6x as broad as a striae, with a row of catenulate transverse punctures, each containing a minute black recumbent vestiture, lateral margin smooth at apex, a patch of yellowish vestiture starts from interval 2 which continues up to 10 (Figure 8(f)); elytral vestiture yellow, either with broad parallel striae running from base to apex (Figure 9(k)), or with granular striations on surface (Figure 9(l)). Legs coarsely punctate, each puncture containing a grey vestiture; tibiae sulcate; profemur as long as mesofemur, 0.8x as long as metafemur. Protibia 1.25x and 1.1x as long as meso- and metatibia, respectively. First tarsal segment 1.66x as long as broad, 1.5x as long as second, and as long as third, third 1.66x as broad as long, fourth 6.66x as long as broad. Prosternum with deep transverse sulcus behind apex, base of prosternum with raised fovea. Procoxa 1.53x as broad as intercoxal process; mesosternum plate like, depressed at base and apex, intercoxal process 1.1x as broad as mesocoxa; metasternum shallowly depressed in middle with impressed longitudinal line, intercoxal process 2.05x as broad as metacoxa. Venter black, clothed with broad greyish yellow vestiture, strongly punctate and each with broad vestiture, anterior margin of first ventrite sinusoidal, posterior margin subtruncate, 2.91x as broad as long, 1.72x as long as second, second 4.75x as broad as long, 0.75x as long as three and four combined, third and fourth subequal in length, ventrite five 2.48x as broad as long, its posterior margin rounded; pygidium distinctly punctate, broadly exposed with long hairs from each puncture, 1.02x as broad as long (Figure 10(e)).

Female Genitalia. Spermatheca more sclerotised at proximal arm, distal arm as long as proximal arm, angle between proximal and distal arms acute, nodulus small, tapering towards apex, ramus small, cornu bent and blunt at apex (Figure 14(g)). Spiculum ventrale with shaft elongate, 2.5x as long as basal plate, basal plate 2.85x as long as broad, apical end truncate with hairs, basal end with a lateral projection (Figures 11(g) and 14(h)).

Male Genitalia. Apophyses 0.44x as long as median lobe, 0.4x as long as spiculum gastrale, as long as tegmen; median lobe moderately sclerotised, parallel sided from base to behind middle, apex truncate, at middle 1.9x as broad as at apex, 1.35x as broad at base (Figures 13(l)–13(n), 15(n), and 15(o)). Tegmen 2.85x as long as manubrium, 2x as long as parameroid lobe, manubrium short and flat (Figures 13(o) and 15(q)). Spiculum gastrale uniformly thick, not curved at apex, with a median line, 16.66x as long as broad (Figures 12(d) and 15(p)).

Measurements of Holotype. Total length: 5.69 mm; standard length: 5.30 mm; breadth: 2.33 mm;

Material Examined. Holotype ♂: India: Haryana: Jind, 9.vii.1986, coll. unknown, from grass (latitude: 29° 48′ N; longitude: 78° 26′ E). Paratypes (2 ♂♂, 2 ♀♀): India: 1 ♀, Haryana: Jind, 9.vii.1986, coll. unknown, from grass; 2 ♂♂, Andhra Pradesh: Patancheru, 18.ix.1985, coll. unknown, from wild aquatic plant; 1 ♀ (no data).

Distribution. India: Haryana; Andhra Pradesh.

Etymology. The specific name is given after the funicular segments which have sharp spines in four rows.

6. Key to the Indian Species of *Athesapeuta*

(1) (a) Rostrum without carinae—2.

(b) Rostrum with carinae—3.

(2) (a) Rostrum with four rows of small punctures; prothorax with a broad impunctate median stripe; profemur with fringes of long vestiture on ventral surface (Figure 6(a)); protibia with sharp tooth on inner edge at about middle in males (Figure 6(b)), females without it—*cyperi*.

(b) Rostrum without four rows of small punctures; prothorax without a broad stripe; profemur without fringes of long vestiture on ventral surface; protibia without tooth in males or females—4.

(3) (a) Tibiae at apical end on sides with fringes of hairs (Figure 6(c)); elytra oblong, with large basal patch consisting of lines of vestiture on intervals three to eight, those on fifth and sixth longest, those on four, three, seven, and eight diminishing in the order given, whole patch covering about one third of the elytra and leaving the shoulder bare, close behind it another large irregular patch, lines of which intervals 2, 3, 4, 8, and 9 are short, and 5, 6 and 7 being longer, that on 5 longest, with a "v"-shaped apical patch extending from interval 3 to 8—*oryzae*.

(b) Tibiae without fringes of hairs; elytra not oblong but ovate or oblongovate—5.

(4) (a) Funicular segments without spines; posterior end of all tibiae carries sharp tooth (Figure 7(a)), metatibia with fringes of grey hairs on lateroventrally (Figure 7(b)); apex of prothorax with tubular constriction, granular punctures all over, sides gently

rounded; apophyses 1.06x as long as median lobe; spiculum gastrale 12.5x as long as broad—*richardi*, sp. nov.

(b) Funicular segments with spines (Figure 4(f)); posterior end of all tibia does not carry sharp tooth; apex of prothorax without tubular constriction, granular punctures only at centre, sides broadly rounded; apophyses 0.44x as long as median lobe; spiculum gastrale 16.66x as long as broad—*spinulatus*, sp. nov.

(5) (a) Prothorax with broad lateral stripe, with smooth median line; elytra ovate (Figure 8(c)); elytal vestiture either brownish yellow, with basal end blunt, apex pointed with lines on surfaces reaching apex (Figure 9(d)), or greyish white, pear shaped, broad at the apex with granular striations on the surface (Figure 9(e))—*meghalayensis*, sp. nov.

(b) Prothorax without broad lateral stripe, without median line; elytra oblongovate (Figure 8(b)); elytral vestiture either yellowish white, base tapering, apex concave, surface with granular serrations (Figure 9(b)), or brownish yellow, both ends broader, surface with dense granular serrations (Figure 9(c))—*immaculata*.

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Research Article

Arboreal Burials in *Nicrophorus* spp. (Coleoptera: Silphidae)

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Nicrophorus beetles are well known for interring small vertebrates below ground for the purpose of rearing their young. However, the *arboreal* use of carrion has not been previously investigated. Nest boxes were suspended in the canopy of two forest habitats in Nova Scotia, Canada, to determine if this microhabitat fostered the same behaviour. Although four species of *Nicrophorus* as well as *Oiceoptoma noveboracense* (Forster) were recorded in association with carrion, *arboreal* reproduction was recorded exclusively and for the first time in *N. tomentosus* Weber and *N. defodiens* Mannerheim. Both *N. sayi* Laporte and *N. pustulatus* Herschel were associated with the arboreal carrion but did not reproduce on it during these experiments.

1. Introduction

Carrion found on the forest floor is a sporadic and ephemeral resource causing rigorous competition among scavengers. This competition is especially intense between the many species of scavenging insects [1, 2]. Other than flies, the most likely insects to arrive at a carcass within the first 24 hours are the burying beetles (Coleoptera: Silphidae: *Nicrophorus*; [3]). Their common name refers to how they inter small vertebrate carcasses in order to use them as a brood-rearing resource.

These beetles invest extensive energy into carrion burial and the care of the larvae [4, 5]. Typically, both parents regurgitate flesh from the brood ball for the begging, early instar larvae, while preventing competing flies from ovipositing in the carrion [6]. This brooding behaviour is unparalleled by any other social interaction seen in the Coleoptera [7, 8] and is essential for larval development [9, 10].

Carrion burial, although essential and well-studied for most *Nicrophorus* life cycles, is variable among the species. Pukowski [5] and L. J. Milne and M. H. Milne [4] were among the first to document the extensive burial process which took pairs of beetles 5–8 hours to completely entomb a small vertebrate carcass under a few centimetres of soil. Since these early observations, researchers have shown that differences in the size of both the beetle and the carrion can affect how efficiently carcasses are buried. Smaller species have more difficulty securing larger resources from competitors and keeping them free from fly infestation [11]. As a result, multiple beetles can be observed cooperatively burying larger carcass and providing joint parental care for the brood [12, 13].

The smaller nicrophorines do not completely bury carrion according to the process described in L. J. Milne and M. H. Milne [4]. *Nicrophorus tomentosus* and *N. vespilloides* dig shallow pits and make use of leaf litter and debris as cover [5, 14]. *Nicrophorus defodiens* may not bury carrion at all but simply conceals the resource under leaf litter [15]; Wilson and Fudge [7] report this species also digging a shallow pit prior to covering the carcass with leaves.

Carrion beetles have been traditionally caught using ground-based methods, such as carrion-baited pitfall traps and ground-level carrion [7, 16, 17]. Neither the use of eggs as a brood-rearing resource (recently demonstrated for *N. pustulatus* in Ontario [18] and Illinois [19]) nor the collaborative use of salmon carcasses by *N. investigator* Zetterstedt [20] could have been predicted using the traditional ground-level carrion techniques.

Additionally, studies over a vertical gradient in the forest reveal patterns of species' distribution which would have gone unnoticed using the traditional, ground-based techniques [21, 22]. Shubeck [23] was the first to compare the response of burying beetles between ground-level carrion

and carrion suspended 1.5 m above the ground. Vertical preferences at heights greater than a couple of metres have only been recently investigated [21, 24–26]. The authors suggested that *N. pustulatus* might preferably search for carrion in the canopy to avoid the competition of the larger *N. orbicollis* on the ground. Using traps at several heights above the ground, Ulyshen and Hanula [25] showed that the vertical distribution of beetles, even within a family, can be diverse. Schroeder et al. [22] recorded carrion beetles over 3–25 m, the greatest vertical gradient investigated so far. The evidence from these studies lend insight into which species can be caught in the canopy, but studies investigating why some beetles occur in the canopy have yet to be conducted.

Nicrophorus spp. have been found within the cavity nests of two raptors, the Northern Saw-Whet Owl (*Aegolius acadicus*, [Gmelin]) [27] and the American Kestrel (*Falco sparverius* L., RFL unpublished data). Whether their presence in raptor nests simply reflects an attraction to uneaten prey or deceased nestlings is unknown. Although the extensive work by Krištofík et al. [28] and Majka et al. [29] found several dozen beetle species in almost 100 Boreal (Tengmalm's) Owl (*A. funereus*) and Northern Saw-whet Owl nests, no nicrophorines were among them.

Despite the diversity of burying behaviour among *Nicrophorus* being well documented, field studies of carrion beetle burials have been limited to ground-based carrion. Foraging in the canopy may be preferred by some species [24, 25], but reproduction on arboreal carrion remains undocumented. With evidence of a height preference in some species and a documented presence in raptor nests, it is possible that of the multiple strategies used by burying beetles, arboreal reproduction is included. The current study will focus on the use of this understudied microhabitat for reproduction in *Nicrophorus* species found on mainland Nova Scotia, Canada.

2. Materials and Methods

2.1. Study Sites. This study was located on Crown Land along the Beaver River at The Keppoch, 14 km southwest of Antigonish, NS. The mixed woods site (centered at N 45° 32′ 4.5″, W 62° 8′ 13.6″) was made up of 50% tolerant hardwoods (yellow birch (*Betula alleghaniensis* Britt), sugar maple (*Acer saccharum* Marshall)), 30% eastern hemlock (*Tsuga canadensis* (L.) Carr.), 10% other hardwood, 10% other conifer (GIS data obtained from NS Department of Natural Resources); the trees at the site averaged 16 m in height. The hemlock site (centered at N 45° 31′ 57.9″, W 62° 8′ 19.9″), approximately 250 m upstream from the mixed wood site, was dominantly eastern hemlock with 10% tolerant hardwood, 10% spruces (*Picea* spp.), and 10% other conifer; the average tree height was 15 m. There were approximately 150 m between the closest traps of the two sites.

2.2. Nest Box Design and Placement. The nest boxes were made from inverted 2 L milk jugs which were cut into equal top and bottom sections; the sections were then nested together with about two centimetres of overlap. The halves were hinged together using two wire loops, one of which was permanent, the other was removable to allow access to the inside of the nest box (Figure 1).

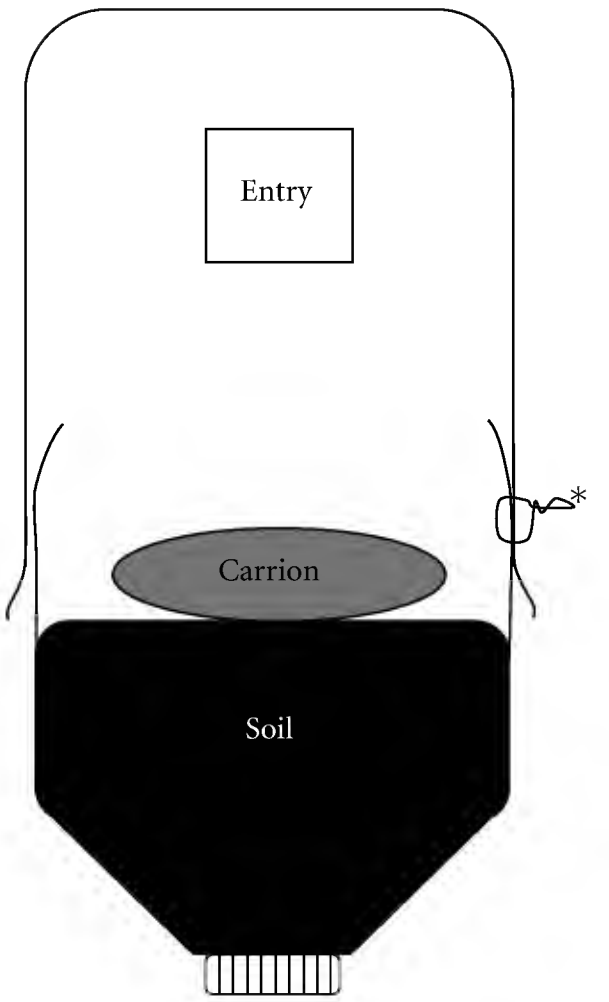

Figure 1: A nest box with soil and carrion in place. Only one of two wire hinges (∗) holding the two halves in place is shown; the other was affixed to the diagonally opposite corner, not visible in this diagram. Not to scale.

The bottom half of the nest box (i.e., the top of the jug) was filled with potting soil (Premium Nature Mix, Hortibec) up to 2 cm below the cut. Although soil is not frequently expected in the canopy, the occurrence of natural, mineral-reduced, canopy soil is typical of old rot holes or woodpecker cavities. This soil comes from the use of the cavity for purposes such as the nesting of small owls, whose young regurgitate and defecate in the cavity. The wastes build up for over a month and with time that organic matter composts into a mineral-free soil. We sought to mimic this largely mineral-free organic matter by using the potting soil which is also mineral-free.

A small passerine or other similarly sized bird with an average mass of 24.1 ± 10.1 g (mean ± standard deviation, see Section 2.3) was placed on the soil surface before wiring the nest boxes shut (Figure 1). Beetles accessed the carrion through a 3×3 cm entry cut on one side of the nest box. Small holes were punctured into the jug caps to allow for drainage.

The nest boxes were individually strung from large tree branches near the bottom of the canopy layer. At the mixed woods site, the boxes were hung at an average height of 9.5 ± 0.6 m above the forest floor, and, at the hemlock site, the boxes were at 9.7 ± 0.5 m; the nest box heights at the two sites were not significantly different from each other ($P > 0.05$; Student's t-test). Care was taken to ensure the nest boxes were at least a metre from both the trunk and the branch from which it was hung; this was done to reduce scavenging by vertebrates, predominantly raccoons (*Procyon lotor* [L]).

The nest boxes at the mixed woods site were spaced 21.4 ± 6.9 m from each other. Five of six trees containing nest boxes were yellow birch; the remaining box was hung in a white spruce (*Picea glauca* (Moench) Voss). Five of six nest boxes at the hemlock site were strung in eastern hemlock; the remaining was in a yellow birch. The average distance between neighbouring nest boxes at this site was 22.0 ± 9.2 m. The spacing of nest boxes at the two sites were not significantly different from each other ($P > 0.05$; Student's t-test).

2.3. Bait Animals. Most birds used for this study were collected from community members as window kills or from a rehabilitation centre. As such, the majority were species typical of bird feeders in the area. The species and weight of each bird used were recorded (see Table 2).

2.4. Nest Box Inspection and Analysis. The nest boxes were erected on 23 May 2009. Observations from the nest boxes were then made twice weekly for eight consecutive weeks between May 26 and August 28, 2009.

The status of the bird was recorded as being with or without larvae or pupae, buried or unburied. The soil was only replaced if infested with maggots; birds were replaced based on the same criterion, or if they had been consumed. Once a burial was established, it was necessary to carefully dig for adults, larvae, or pupae during checks. Burying beetles were identified to species [14] and quantified during each check. The carrion, along with its inhabitants, was reburied.

Nest boxes containing final instar larvae or pupae were brought into the lab (at approximately 21°C) if no parent beetles were found. Nest box exits were then covered with 6 mm hardware cloth to allow for air flow but prevent escape of newly eclosed adults. The broods were checked periodically, and eclosed adults were identified. All specimens were preserved in 70% isopropanol or pinned and deposited into the collection of RFL. A nesting was considered successful if at least one adult eclosed.

2.5. Statistical Analysis. The comparisons made between the use of the two habitats for burial were tested for significance using a Pearson's Chi-squared test with $k = 1$ degrees of freedom and a P value of 0.05.

3. Results

Four *Nicrophorus* species (*N. sayi*, *N. pustulatus*, *N. tomentosus*, and *N. defodiens*), as well as *Oiceoptoma noveboracense*, were observed in the nest boxes (Table 1). No adult beetle was observed in a nest box which contained pupae. No species had a significant forest habitat preference (Chi-squared analyses, $P > 0.05$ in all cases).

Of the 160 beetle observations within nest boxes, *N. tomentosus* was the most frequently found, with almost 70% of all the observations being of this species. *Nicrophorus tomentosus* was found in nest boxes with either unburied or buried carcasses, with or without larvae. Just over half were observed in nest boxes of the mixed woods site (Table 1).

Nicrophorus defodiens was represented by 10% of the observations and was associated with the same stages of carrion burial as was *N. tomentosus*. Ten of the sixteen *N. defodiens* (62.5%) were found at the hemlock site.

The 24 specimens of *N. sayi* were associated with unburied carrion in 87% of the observations and were never found in nests containing larvae or pupae of burying beetles. This species was distributed between the two forest types almost evenly, in a pattern very similar to that of *N. tomentosus*.

The least recorded burying beetle, *N. pustulatus*, was recorded in nest boxes six times, representing less than 4% of encounters. The observations were distributed between both forest types. Most were in nests that did not have brood balls, except on August 25, 2009, when one *N. pustulatus* was found in the presence of four adult *N. tomentosus* and a partially buried carcass. There were unidentified nicrophorine larvae on the carcass.

The only Silphid found which was not from the genus *Nicrophorus* was *O. noveboracense*; the six specimens were equally divided between habitats and were only observed with unburied carcasses (Table 1).

Nicrophorus defodiens and *N. tomentosus* each had single successful broods in the hemlock site, while the latter was also successful in the mixed woods site. The bait bird was discovered buried for the successful *N. defodiens* nest on June 30, 2009. The bait birds were discovered buried on August 7, 2009 and August 11, 2009, for the successful *N. tomentosus* nests in the mixed woods and hemlock woods site, respectively. The carcasses in each of the three successful nest boxes had all been completely buried. The only burying beetles found in successful nest boxes prior to the eclosion of the next generation were of the same species which eventually eclosed.

The other local burying beetles, *N. orbicollis* Say and *N. vespilloides* Herbst, were not found in any of the canopy nest boxes.

4. Discussion

Oiceoptoma noveboracense is expected to be attracted to carrion, though it was not found in association with buried carcasses. *Oiceoptoma noveboracense* is not a burying beetle and does not bury carcasses. The purpose of carcass burial by nicrophorines is to hide the resource from competitors [30] such as *O. noveboracense*; the absence of this beetle from the nest boxes at postburial stages suggests the nicrophorines' strategy is successful.

It is likely that two local nicrophorines, *N. vespilloides* and *N. orbicollis*, were not recorded in the nest boxes for different reasons. *Nicrophorus vespilloides* is a specialist of open habitats such as bogs [14, 31] and is therefore not expected in the forest. *Nicrophorus orbicollis* is uncommon in the study area and, when found, avoids the canopy [23]. Additionally, it was expected that no adult beetles of any species would be observed in nests with next-generation pupae since pupae no longer need care.

Although not likely as rich in carrion as the ground, the canopy still has the potential to provide significant amounts

Table 1: Observations of adult beetles in nest boxes at the different carcass stages. The adults may or may not have been parents of offspring, if any, from a nest; however, these adults were not next-generation beetles. Since adult beetles were not marked, the values represent maxima. H: hemlock woods; MW: mixed woods.

	Unburied		Buried, no larvae		Buried, with larvae		Buried, with pupae		
	H	MW	H	MW	H	MW	H	MW	Totals
N. tomentosus	28	45	15	11	5	4	0	0	108
N. defodiens	0	6	4	0	6	0	0	0	16
N. sayi	8	13	3	0	0	0	0	0	24
N. pustulatus	3	2	0	0	1	0	0	0	6
O. noveboracense	3	3	0	0	0	0	0	0	6
Totals	42	69	22	11	12	4	0	0	160

Table 2: Bait birds.

Species		Mean mass (g)	*n*
Duckling	*Anas* sp.	27.6	1
Spotted sandpiper	*Actitis macularius*	17.5	1
Downy woodpecker	*Picoides pubescens*	25.7	1
Yellow-bellied flycatcher	*Empidonax flaviventris*	12.5	1
Flycatcher	*Empidonax* sp.	13.4	1
Black-capped chickadee	*Poecile atricapillus*	13.6	7
Blue-headed vireo	*Vireo solitarius*	22.0	1
Red-eyed vireo	*Vireo olivaceus*	18.9	1
Cedar waxwing	*Bombycilla cedrorum*	35.0	2
Swainson's thrush	*Catharus ustulatus*	35.8	2
Hermit thrush	*Catharus guttatus*	34.5	1
Ovenbird	*Seiurus aurocapilla*	20.9	4
Yellow-rumped warbler	*Setophaga coronata*	14.0	1
Savannah sparrow	*Passerculus sandwichensis*	25.8	1
White-throated sparrow	*Zonotrichia albicollis*	24.6	2
Dark-eyed junco	*Junco hyemalis*	22.6	8
Purple Finch	*Carpodacus purpureus*	29.6	18
White-winged crossbill	*Loxia leucoptera*	26.8	1
Common redpoll	*Acanthis flammea*	21.4	1
Pine siskin	*Spinus pinus*	13.0	4
American goldfinch	*Spinus tristis*	13.5	6
Evening Grosbeak	*Coccothraustes vespertinus*	58.2	2
Finch sp.	Carduelinae	24.9	1
Unknown chick		29.0	4

of carrion. There are many species of cavity nesting, altricial birds including small raptors (e.g., American Kestrel and Northern Saw-whet Owl), seven species of woodpecker (Piciformes), and more. Two species of cavity-nesting squirrels are also in the study area, Red Squirrel (*Tamiasciurus hudsonicus* (Erxleben)) and Northern Flying Squirrel (*Glaucomys sabrinus* (Shaw)). In all of the birds, the rearing of the nest-bound young is the responsibility of both parents. If a predator kills either parent, the nestlings are destined to perish as well. If the female squirrel is depredated, the milk-dependant young will die. Should either of these scenarios happen, burying beetles are one of several canopy-foraging groups of carrion exploiters to potentially use the newly available resource.

Terrestrial-based work has revealed that burying beetles may take advantage of abandoned cavities and burrows rather than dig a new crypt for a carcass [31, 32]. Therefore, using an existing tree cavity may simply be an extension of a behaviour first utilized on the ground.

Nicrophorus tomentosus was the most abundant species found in the canopy nest boxes and was a confirmed breeder there. Although this species has one of the widest foraging niche breadths of all *Nicrophorus* species [33], their frequent presence in elevated traps [23] alluded to life history aspects beyond what was known. Complete burial of carcasses which is typical of most nicrophorines is not characteristic of *N. tomentosus* [14]. Normally, they only dig a shallow pit and cover the carcass with leaves. In the current study, no leaves were provided which may have obligated the beetles to completely bury the carcass.

Smaller species of burying beetle, such as *N. defodiens*, are often out-competed for breeding resources by larger species

[34]. Wilson et al. [3] showed a decrease in the proportion of successful broods on the ground for *N. defodiens* during mid July, correlating with an increased ambient temperature facilitating searches by the larger *N. orbicollis*. As the number of intrageneric competitors increase with midsummer [14, 35], the decreased ability of *N. defodiens* to produce successful broods on the ground may have stimulated a shift in breeding habitat [3, 16] and provided pressures favouring canopy search behaviour and breeding.

Only one brood was produced by *N. defodiens* in this study. Similar to the unconventional burial noted for *N. tomentosus*, the carcass used for the *N. defodiens* brood was also completely buried. Although this has been documented as a rare occurrence, normally, this species simply covers its carcasses with leaf litter [7].

The two other beetles recorded in canopy nest boxes, *N. sayi* and *N. Pustulatus,* may reproduce in the canopy, although they were not confirmed as breeders in this study. Both species were associated with brood balls in the nest boxes at least once, but this association was less common than observations with unburied carrion. Sexually mature beetles are differentially attracted to small vertebrate carcasses [36], like those used in this study, which supports the potential for breeding by *N. sayi* and *N. pustulatus*. The timing of this study was initiated towards the end of the breeding season for *N. sayi*, which may account for the small number of observations and lack of confirmed breeding for this species.

Carcass use has never been documented for *N. pustulatus* in the wild, despite hundreds of bait animals being placed out [7, 31] though Smith et al. [37] and others have documented *N. pustulatus* breeding on mice in the lab. This species is infrequently caught in conventionally baited pitfall traps [38], suggesting it is either truly uncommon or has a different life history which does not involve carrion at ground level. Serendipitous discoveries of *N. pustulatus* rearing its young on ground-nesting snake eggs [18, 19] has solved part of the conundrum surrounding this beetle's unusual life history. Other apparently contradictory data show *N. pustulatus* routinely in the canopy [25, 26, Table 1], suggesting the canopy as a foraging habitat, or an as yet undocumented alternate breeding habitat.

The novel observation of one *N. pustulatus* and four *N. tomentosus* found together in association with larvae of a partially buried carcass suggests brood parasitism, even though the identity of the species responsible for the burial could not be determined. Although Trumbo [13] was unable to record brood parasitism in the field, he did document the ability of *N. pustulatus* to parasitically lower the brood size of *N. orbicollis* in lab [39]. However, parasitism is a common behaviour among most genus members and occurs both inter- and intraspecifically, [38, 40–43].

Previous canopystudies used either unbaited flight intercept traps [25] or rat-baited window traps [26] and dominantly caught *N. pustulatus* in the elevated traps. No previous study has provided *N. pustulatus* with both carrion and a nest for brood rearing in its apparently preferred canopy microhabitat. This documented canopy preference [25, 26] and occurrences of *N. pustulatus* in the nest boxes from this study, combined with previous findings of this species in the nest cavities of a Northern Saw-Whet Owl [27] and an American Kestrel (Lauff unpublished data), provide compelling evidence that *N. pustulatus* may reproduce in the canopy.

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Editorial

Advances in Neotropical Myrmecology

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... I believe Myrmecology is even more significant for tropical ecology than Ornithology, because the impact of ants in tropical habitats is tremendous. In addition ants served for many disciplines as model systems, for example, Sociobiology, Communication Biology, Chemical Ecology, and in recent years ants and other social insects have become model organisms for the study of Epigenetics ... (Bert Hölldobler in a message sent to the organizers of the XX Simpósio de Mirmecologia (2011) which was held at Petrópolis, Brazil)

Knowledge about Neotropical ants began to accumulate ever since European colonization, when the Portuguese owner of a sugarcane factory in the Reconcavo region of Bahia, Brazil, Gabriel Soares de Souza in 1587 [1] and the Spaniard José Celestino Mutis in 1780s [2, 3] made the first observations about ants in their American habitats. These observations were soon followed by more texts published by naturalist travelers in the XIX Century reporting ant behavior, among whom Bates [4] is one between the best known. Myrmecology as a discipline of entomology took its roots when these travelers and a myriad of correspondents distributed throughout the American continent sent biological material to European collections where taxonomists such as Auguste Forel, Carlo Emery, or Felix Santschi could study and describe considerable amounts of new ant material [5]. In the meantime, Forel, Santschi (as Forel's secretary), and Edouard Bugnion (Forel's brother-in-law) made a memorable travel (1896) through the Sierra Nevada de Santa Marta region in the northeast of Colombia, where they subsequently accumulated ant observations and experiences which they will use throughout their lives. Interestingly, all the three produced independent memories of the Colombian expedition ([6, 7], Santschi's notes in [8]). Other important contributors to earlier Neotropical myrmecology in the XIX and XX centuries were the German mycologist Alfred Möller in southern Brazil and Franciscan priests Thomas Borgmeier and Walter Kempf; the Brazilians Herman Luederwaldt, Karol Lenko, Mario Autuori, Elpídio Amante, and Cincinnato Rory Gonçalves; the Argentineans Carlos Bruch, Angel Gallardo, and Nicolas Kusnezov; and the North Americans William M. Mann, Neal Albert Weber, and William Morton Wheeler [9–14]. Research output from the Neotropical Region still remained modest until the Second World War. This began to change with the rapid development of national infrastructures that started to occur in the 1960s and the burgeoning of new universities in the 1970s and 1980s. Most of the older scientific contributions were historically devoted to taxonomy and leaf-cutter ant damage and control, but the topics which have called the attention of myrmecologists in recent years have been much more diverse and concerned essentially with ant communities, ant-plant relations, mutualisms, biomonitoring, biogeography, morphology and anatomy, genetics and cytogenetics, and taxonomy.

Selection of exculsive identifiers for publications from the Neotropics makes it difficult to measure output from publication databases, but taking leaf cutter ants as a surrogate, and using key words *Acromyrmex*, *Atta,* or Attini, as search identifiers in the ant database *Formis 2011* [15],

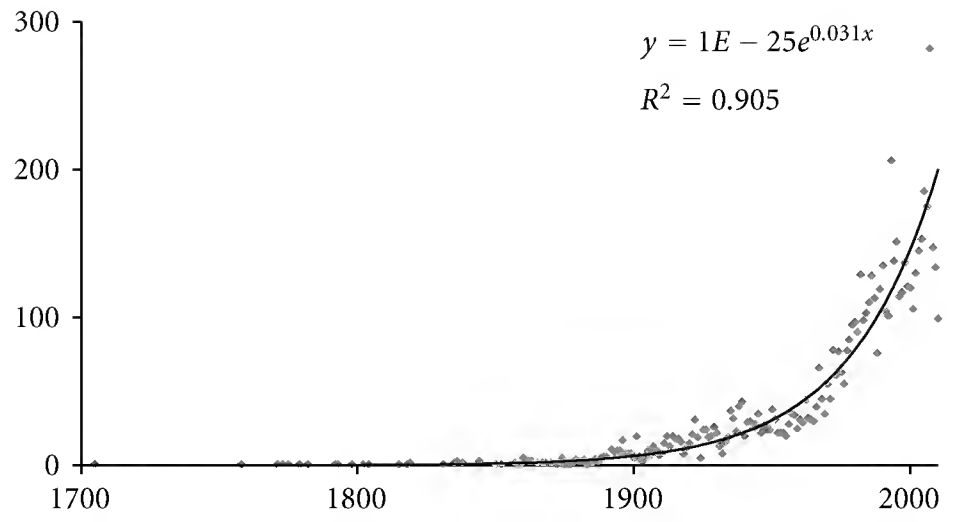

FIGURE 1: The exponential spread of papers containing the words *Acromyrmex*, *Atta*, or Attini in the title, abstract, or key words ($n = 6,561$). The regression is calculated for the period from 1850–2010. Although the tribe is not exclusively Neotropical, we estimate an annual output of 300 leaf-cutter ants papers by 2020. *Formis 2011* [15].

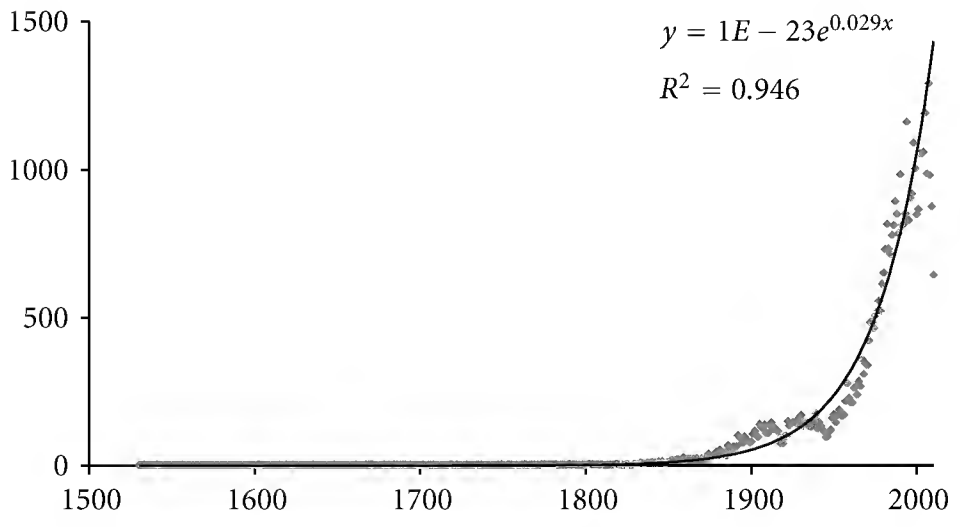

FIGURE 2: The exponential spread of World ant literature ($n = 46,182$). The regression is based on annual production and is calculated for the period between 1800 and 2010. An extrapolation estimates to 1,700 for the number of projected papers on ant biology for 2020. *Formis 2011* [15].

indicates an almost exponential increase in output from Latin America during the latter part of the last century through to the present time (Figure 1). This pattern closely follows World trends (Figure 2) and, assuming that for every paper published on leaf cutter ants, another four to six papers are currently produced on other Neotropical ant species, the Latin American ant literature could soon account for one third of annual World ant literature. So great is this increase, that extrapolation suggests an annual output of 1700 papers (World lower estimation) per year by the time of 2020. This enthusiasm for ants is matched by the existence of a Latin American Sections of the International Union for the Study of Social Insects, and also by a very attractive biennual Simpósio de Mirmecologia, which is traditionally attended by hundreds of scientists and students from Brazil and, in the recent years, by an increasing number of scientists from elsewhere in the Americas, Europe, and Australia.

This volume brings together some of the recent research on ants in the Neotropical Region and includes contributions by authors from Argentina, Australia, Belgium, Brazil, Colombia, Costa Rica, Ecuador, France, French Guiana, Mexico, Venezuela, and USA. Six of the contributions are concerned with taxonomy and systematics of the ant fauna and reflect the spread of interest in ant taxonomy and the new integrative taxonomy approach [16]. One is concerned with ant morphology and continues the long tradition in Brazil of identifying special features within local ants. A further three papers address aspects of ant biology, including division of labour, chemical recruitment, and behavioural differentiation between castes, with emphasis on reproduction.

Three of the papers are concerned with the ecology of individual species, while a further five take a community ecology approach to the fauna. In addition, since the Neotropics contain some of the World's biodiversity hotspots, five other papers describe various aspects of the interactions between ants and some of the unique plant species which occur in this region. There is one paper on leaf cutter ants which, interestingly, indicates that leaf cutting activity can influence the local spread of fire in Amazon ecosystems.

The influence of the ALL protocol [17] and the use of Winkler sampling in studies of community ecology are evident in some of the papers, although one contribution discusses the potential for a new sampling method, subterranean trapping, to augment existing techniques. Finally, the use of ants as bioindicators, originally pioneered in Australia [18], has escalated throughout countries in the Neotropical Region, and one paper reviews such studies in Brazil, presenting ideas for improvements to the procedure.

Perusal of the papers in this volume, and those elsewhere, indicates that a sizeable proportion of Neotropical ant species have yet to be described, and researchers in these groups tend to assign ants to morphospecies; they may even use morphospecies codes for described species if they do not have ready access to the main museums where reference collections are held. This unsatisfactory situation is confounded by the fact that each research group tends to adopt its own morphospecies coding system, or even a separate coding system for each individual study! Thus we have endless papers featuring *Pheidole* sp. 1, *Pheidole* sp. 2, and *Pheidole* sp. 3, but we have no idea whether they are the same species or not.

This imposes serious limitations for the making of comparisons between studies—a lost opportunity indeed. It is a relatively simple matter to determine an ant to genus level and assign codes to perceptibly different morphospecies, but obtaining determinations requires access to keys, museums, or specialists, and producing a uniform morphospecies coding system requires a system of voucher specimens, deposited in secure and accessible locations, which is a requirment to be inserted in "material and methods" by many of the entomological journals. All of this takes time and money.

What can we do to overcome this impediment? We suggest that each of the major countries have at least one, and preferably more, central reference collections, comprising formally determined material plus vouchers of coded morphospecies, all compiled using a standardised numbering and data-based system. Attempts should be made to "clone" these collections, or at least regional subsets of the material,

and placing the subcollections in strategic regions of the country where they are accessible to local research groups. As an adjunct to this, keys to the material should be produced, illustrated with line drawings or photographs, and rapidly made available on the internet. The existence of undescribed "morphospecies" need be no bar to the production of keys. Heterick's [19] recent book *A guide to the Ants of South-Western Australia* features keys and line diagrams of the 500 or so species which exist there, of which almost half are only known by morphospecies codes. We admit that coordination of collections from the various research groups would be an enormous task, especially if cross-national coordination was involved. At the very least, integrated collections for each country could be assembled, with each having a prefix letter before the code number (e.g., *Pheidole* sp. B1, *Pheidole* sp. B2, and *Pheidole sp.* B3, etc. for Brazil, e.g.). Subsequent integration of the national collections, at least for individual genera, could then be undertaken as specialist projects or could be coordinated through organized groups, possibly under the direction of the International Union for the Study of Social Insects (IUSSI).

To assemble these "national" collections and keys would require dedicated staff, a committed and guaranteed amount of resources, with security of tenure. However, considering the importance of ants in our natural and cultural landscapes, and their increasing importance in the disciplines linked to Neotropical entomology as a whole, this might well be a good investment. Major research organisations, governments, and national or international philanthropic funding bodies should seriously consider this option. An embryo of this effort is the site http://www.antweb.org/ maintained by the Californian Academy of Sciences, which has the explicit mission of documenting, through high-quality imagery, the whole ant diversity of the Planet, beginning with the types. Besides offering the option of "digital curation" for countries or areas, initiatives like this can encourage taxonomists to have consensus on the delimitation of morphospecies of large or problematic genera, together with the well-known genera with broad morphological variation. It is critical that the few researchers with access to types can offer high-quality photos of them, in order to assist those who have to rely less on loans and risky mail.

The numerous internet sites which currently exist about ants, specialized symposia, ant field courses, and many other activities clearly show that the tropical myrmecology lives a golden era with a very promising future.

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Research Article

Temporal Dynamics and Electronic Nose Detection of Stink Bug-Induced Volatile Emissions from Cotton Bolls

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Management decisions for stink bugs (Pentatomidae) in *Bt* cotton are complicated by time-consuming sampling methods, and there is a need for more efficient detection tools. Volatile compounds are released from cotton bolls in response to feeding by stink bugs, and electronic nose (E-nose) technology may be useful for detecting boll damage. In this study, we investigated the temporal dynamics of volatile emissions in response to feeding by stink bugs and tested the ability of E-nose to discriminate between odors from healthy and injured bolls. Feeding by stink bugs led to an approximate 2.4-fold increase in volatile organic compound (VOC) emissions. Principal components analysis of E-nose sensor data showed distinct (100%) separation between stink bug-injured and healthy bolls after two days of feeding. However, when E-nose was used to randomly identify samples, results were less accurate (80–90%). These results suggest that E-nose is a promising technology for rapid detection of stink bug injury to cotton.

1. Introduction

Phytophagous stink bugs (Heteroptera : Pentatomidae) are major pests of food and fiber crops worldwide [1]. In the USA, stink bugs are increasingly destructive in cotton, *Gossypium hirsutum* (L.). Prior to the introduction of transgenic cotton varieties expressing insecticidal proteins from *Bacillus thuringiensis* (*Bt*), growers were afforded coincidental control of stink bugs through the use of broad-spectrum insecticides for major pests such as bollworm, *Helicoverpa zea* (Boddie), and tobacco budworm, *Heliothis virescens* (F.). With the widespread adoption of *Bt* varieties, eradication of the boll weevil, *Anthonomus grandis grandis* (L.), and the subsequent reduction in the use of broadspectrum insecticides, stink bugs have emerged as important pests threatening cotton production, especially in the southeastern USA [2]. Since 1995, insecticide use targeting these pests has risen from zero to millions of applications, and crop losses recently exceeded 50 million dollars [3]. Stink bugs feed directly on cotton bolls resulting in boll abscission and lint staining and reduced fiber quality and yield [2, 4–6]. Scouting techniques for stink bugs require destructive sampling of cotton bolls with visual inspection for internal lint staining and callus warts on internal carpal walls [7]. Due to difficulties in assessment and time-consuming scouting practices, there is a need for a more reliable, rapid, and nondestructive method for determining boll injury from these pests.

It is well known that herbivory results in the induced synthesis of volatile organic compounds (VOCs) from plant tissues including a variety of terpenoids, phenylpropanoids, and fatty-acid derivatives [8, 9]. In cotton, terpenoids are stored in lysigenous glands, and herbivore feeding causes the release of stored terpenes, as well as the induction of novel compounds through a combination of physical damage and elicitors from salivary components of the attacking herbivore [10–13]. Stink bug feeding has been shown to induce VOC emissions from cotton [14], corn [15], and soybean [16]. Recently, it was shown that stink bug feeding on cotton bolls led to a 2-3-fold increase in VOC emissions compared with healthy bolls and that induced emissions were similar in response to feeding by different hemipteran species, including southern green stink bug, *Nezara viridula* (L.), and brown stink bug, *Euschistus servus* (Say) [17].

In the last decade, electronic nose (E-nose) technology has been developed to detect VOC emissions at the level of parts-per-million to parts-per-billion [18]. These devices characterize the overall profile of an odor as a digital "smellprint" generated by the change in resistance of several nonspecific gas sensors when exposed to components of the VOC mixture [18]. E-noses have been used for a range of applications, including detection of diseases [19], microbes [20], hazardous chemicals [21], and changes in food quality [22]. E-nose technology has also been successfully applied to monitoring pest damage in several systems. Based on brief (7 to 12 sec) samples from the headspace of tomato plants, E-nose was capable of discriminating pathogen infection, herbivore damage, and mechanical damage based on changes in VOC emissions among treatments [23]. In a laboratory study, it was demonstrated that an E-nose was 90% accurate at differentiating excised healthy boll from those injured by stink bugs [24].

Because stink bugs are known to induce VOC emissions from cotton bolls [17], E-nose technology could potentially serve as a rapid, nondestructive tool for monitoring stink bug injury to cotton bolls; however, this technology has yet to be tested for this purpose under field conditions. The overall goal of this study was to determine the feasibility of using an E-nose to detect and differentiate between VOCs from healthy (undamaged) bolls and those exposed to stink bug feeding (damaged) under field conditions. The specific objectives were to (1) determine the temporal dynamics in VOC emissions from cotton bolls in response to feeding by stink bugs, (2) determine the ability of an E-nose to differentiate between undamaged bolls and those damaged by stink bugs, and (3) determine if an E-nose can discriminate between VOCs induced in response to feeding by *E. servus* and *N. viridula*.

2. Materials and Methods

2.1. Plants and Insects. Experiments were conducted during August 2010 at the Edisto Research and Education Center (EREC) in Blackville, SC, USA. Field trials were carried out in a 1.5-hectare field planted with *G. hirsutum* var. Delta and Pine Land 161 B2RF.

Adults and nymphs of *E. servus* and *N. viridula* were initially collected from field populations in soybean and maintained separately in an insect rearing chamber at EREC. Insects were fed on a source of fresh green beans and provided with water on moistened cotton pads until initiation of experiments. Male and female adult stink bugs (5–7 d in stage) were used in all experiments.

2.2. E-Nose Instrumentation. The E-nose used in all experiments was the Cyranose 320 (Smiths Detection, Inc., Pasadena, CA, USA). The Cyranose 320 is a conductimetric, chemiresistive E-nose containing 32 sensors constructed of an aluminum substrate coated with a thin film of variable carbon composite polymers. Preliminary tests indicated that four sensors were sensitive to water vapor, and these sensors were deactivated for all experiments to improve discrimination capability. A typical sample cycle for the E-nose consisted of a sensor baseline purge, sample collection, and a sample purge. In this study, a high pump speed ($180\,\text{mL}\cdot\text{min}^{-1}$) was used for baseline and sample purges, and a medium pump speed ($120\,\text{mL}\cdot\text{min}^{-1}$) was used for all sample collections. When exposed to chemical vapors during sample collection, the film on each sensor swells causing an increase in resistance. The change in resistance for each sensor ($\Delta R/R$) is represented as the difference between electrical resistances (ΔR) recorded during sample collection and baseline purge during the sample cycle and the resistance (R) recorded at the end of the baseline purge. The resistance from all 32 sensors is measured, and the overall composition of individual sensor responses represents a "smellprint" for an odor. The resistance data from all sensors is analyzed by onboard pattern recognition algorithms including K-nearest neighbor, K-means clustering, and canonical discriminant analysis. The canonical algorithm was used for all analyses in this study.

2.3. VOC Collection and Analysis. A randomized complete block design was used to evaluate the temporal dynamics in VOC emissions and E-nose detection (described below) of undamaged and damaged bolls. In each block, three white blooms at the same node on individual plants were enclosed in cages to protect developing bolls from insect damage prior to experiments. Cages were constructed from polystyrene foam cups with the base of the cup removed and nylon stocking stretched over the cup. Cages were placed over blooms, and the nylon stocking was secured around the peduncle using a 24-gauge steel wire. The nylon stocking was also secured around the top of each cage using another piece of 24-gauge steel wire. For analysis of stink bug-induced VOC emissions, a single adult *N. viridula* or *E. servus* was placed in cages on bolls 10–12 d postanthesis and allowed to feed *ad libitum*. Stink bugs were removed in 24 hr intervals to collect VOC emissions from damaged and undamaged bolls. Volatiles were sampled using a dynamic headspace sampling method. A polyacetate oven bag (Reynolds, Inc., Richmond, VA) modified to a volume of 300 mL was placed over a boll and loosely fastened with a small cable tie at the base of the boll to permit airflow through the bag. A volatile trap was connected to the top corner of each bag using a small cable tie. Volatile collection traps were constructed from glass Pasteur pipettes (10 cm long, 0.5 cm [o.d.]) and contained 35 mg of Porapak Q adsorbent polymer (Alltech Assoc., Deerfield, IL, USA) held in place by two small plugs of glass wool. A battery-operated air-sampling pump (SKC, Inc. Eighty Four, PA, Model 224-44XR) fitted with an independently controlled, adjustable, 4-way splitter (SKC, Inc. Model 224-26-04) was used to draw ambient air through the bag across the boll and directly onto the trap at a rate of $300\,\text{mL}\cdot\text{min}^{-1}$. Volatiles were collected for 3 hr during each sampling interval. Ambient air blanks were collected simultaneously with boll volatile collections to correct for any volatile contaminants contained in the air pulled through the collection bag. Emissions from damaged and undamaged bolls were collected in 24 hr intervals over a 4 d period.

Volatiles were extracted from adsorbent traps by washing with 150 μL of analytical grade hexane and collecting the extract directly into a 2 mL autosampler vial containing a 150 μL insert. Two μL of each sample were analyzed by gas chromatography (GC) on a Hewlett-Packard 6890 gas chromatograph equipped with a RTX-5 column (30 m $\times$ 0.25 mm [i.d.], 0.25 μm film thickness) (Restek, Bellefonte, PA). Injections were made in the splitless mode for 0.5 min with an inlet temperature of 250°C. The oven was held at 50°C for 10 minutes and then increased to 150°C at 5°C·min^{-1}, followed by an increase to 250°C at a rate of 15°C·min^{-1}, and a final increase to 300°C at a rate of 10°C·min^{-1}. Helium was used as a carrier gas at a flow rate of 1 mL·min^{-1}. Samples were subsequently analyzed by mass spectrometry (MS) using a Varian VG-70S (Waters Corp., Milford, MA) operated in electron impact mode. Compound identities were confirmed by comparison with mass spectra and retention indices of the library of essential oil components identified by GCMS [25], with spectra obtained from known standards (Sigma-Aldrich, Inc., Milwaukee, WI), and from high-resolution mass spectra from solvent extracts of boll material. Quantification of compounds was based on external standard curves of α-pinene for monoterpenes and β-caryophyllene for sesquiterpenes. The C11 homoterpene, DMNT, and the C16 homoterpene, TMTT, were quantified based on the standard curve of α-pinene and β-caryophyllene, respectively.

2.4. E-Nose Training on Damaged and Undamaged Bolls. Before an E-nose can be used for discrimination between treatments, it must be trained to recognize the odor profile among replicates of treatment groups. The temporal dynamics in E-nose detection of stink bug feeding damage were examined by training the E-nose on VOC emissions from bolls damaged by *N. viridula*, as well as undamaged bolls, in 24 hr intervals over a 4 d feeding period. During each 24 hr training period, cages and stink bugs were removed, and bags were placed over bolls (described previously) prior to E-nose sampling. Headspace VOCs were allowed to accumulate inside the bag for 30 minutes prior to E-nose training. To train the E-nose, the snout of the E-nose was inserted into the top corner of a bag, and VOCs were sampled using a 10 sec baseline purge, a 15 sec sample collection, and a 30 sec postsample purge. VOCs were sampled from 8 damaged and 8 undamaged bolls during each training period, and the same bolls were sampled on consecutive training periods. Following E-nose training, bugs and cages were placed back over bolls. This process was repeated for each 24 hr training set. Smellprint data from each temporal training set were analyzed separately by canonical discriminant analysis followed by cross-validation to assess the accuracy of each training set.

2.5. Identification Accuracy of E-Nose Temporal Training Sets. To evaluate the accuracy of E-nose training data, each temporal training set was used to randomly identify a separate set of 10 bolls damaged by *N. viridula*, and 10 undamaged bolls immediately following collection of training data. Bolls used for E-nose identification were exposed to stink bugs for an equivalent amount of time as those used for training. The same bolls were used for consecutive training set evaluations. The identification quality of the E-nose was set to "highest," which included a range of potential responses including correct identifications (undamaged or damaged), as well as incorrect identifications (incorrect treatment, confused, or unknown). The number of correct and incorrect identifications was recorded after each sample exposure, and the identification accuracy was determined based on the percentage of correct identifications.

2.6. E-Nose Discrimination of Species-Specific Feeding Damage. To examine the ability of E-nose to discriminate between feeding damage by different species of stink bugs, a single adult of *E. servus* or *N. viridula* (5–7 d in stage) was placed on individual 10–12 d old bolls inside cages (described previously). Stink bugs were allowed to feed *ad libitum* for 3 d, after which cages and stink bugs were removed and a polyacetate oven bag was placed over the bolls. E-nose was used to randomly sample VOCs from undamaged bolls and those damaged by *E. servus* and *N. viridula* using the sampling protocol described previously.

2.7. Statistical Analysis. A mixed model, repeated measures analysis of variance (PROC MIXED) [26] was used to test for differences in total and individual volatile emissions using plant treatment (control, *N. viridula*-damaged, and *E. servus*-damaged) and time (0, 24, 48, 72, and 96 hr) as main effects in the model statement, with plant assigned as the repeated subject. Volatile emissions data were $\log(x + 1)$ transformed prior to analysis to satisfy assumptions of normality. Differences in the emissions of individual compounds among treatments were analyzed by Tukey post hoc comparisons. On-board data analysis software was used for canonical discriminant analysis and cross-validation analysis of E-nose training sets. The proportions of correct identifications of damaged and undamaged bolls following each temporal training session were statistically analyzed using *chi*-square analysis (PROC FREQ) [26].

3. Results

3.1. Volatile Emissions from Cotton Bolls in Response to Stink Bug Feeding. Herbivory by *N. viridula* and *E. servus* had a significant effect on the emission of VOCs over time (Table 1; Figure 1). A significant interaction between treatment and time (sampling period) indicated that the effect of stink bug feeding on VOC emissions varied significantly among sampling periods (Table 1). Prior to placement of stink bugs in cages (time 0), total VOC emissions were not significantly different among treatment (Figure 1). Significantly greater emissions were detected from bolls exposed to *N. viridula* and *E. servus* compared with controls 48 hr after feeding (Figure 1). Total emissions showed the strongest increase between the 48 and 72 hr sampling period, and emissions remained significantly elevated 96 hr after initial exposure (Figure 1). Feeding by stink bugs resulted in approximately a 1.3-, 1.9-, 2.2-, and 2.4-fold increase in total VOC emissions

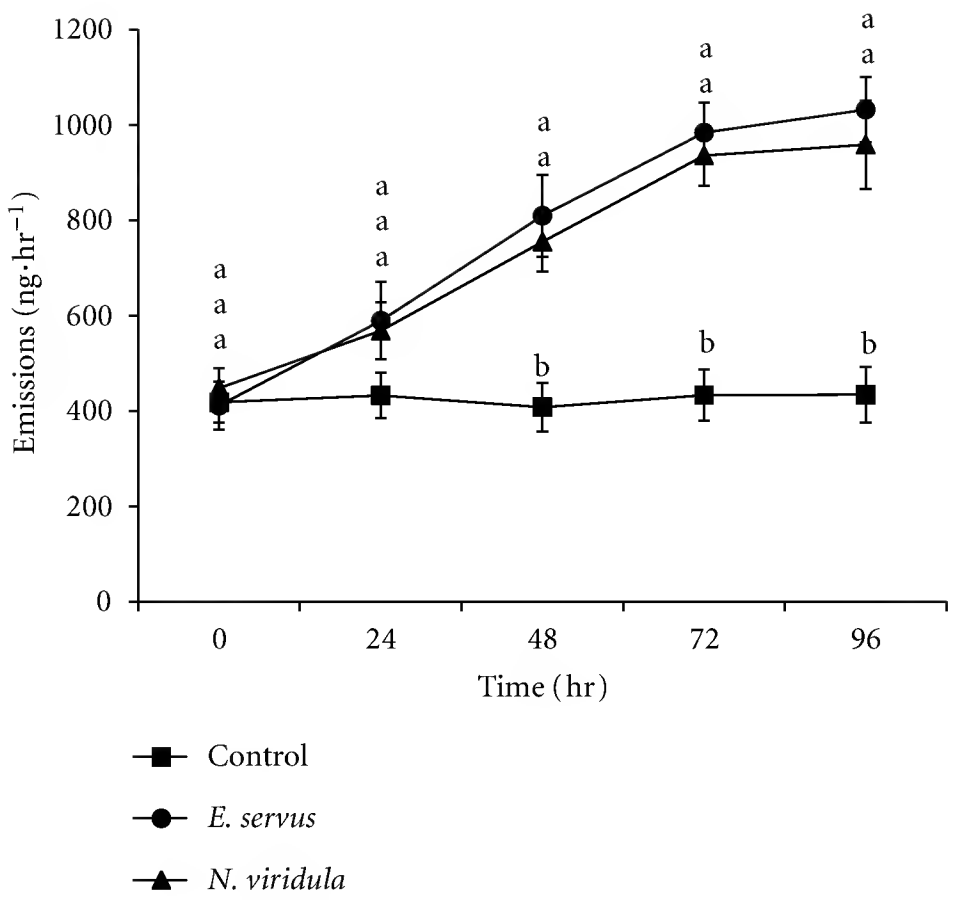

FIGURE 1: Temporal dynamics of total VOC emissions from cotton bolls exposed to *Euschistus servus and Nezara viridula* and unexposed (control) bolls under field conditions. Data points represent mean VOC emissions (±1 SE; $n = 5$). Different letters indicate a significant difference in total VOC emissions between treatments at each sampling interval (repeated measures ANOVA $P < 0.05$).

TABLE 1: Repeated measures analysis of variance on total volatile emissions released from cotton bolls exposed to *Euschistus servus* and *Nezara viridula* and unexposed bolls collected over a 4 d feeding period.

Effect	Num DF	Den DF	*F* value	*P* value
Treatment	2	12	41.54	<0.0001
Time	4	48	16.1	<0.0001
Treatment ∗ Time	8	48	3.98	0.0011

24, 48, 72, and 96 hr, respectively, from initial exposure to bolls (Figure 1). No significant difference was detected in total VOC emissions between *E. servus*- and *N. viridula*-damaged bolls at any sampling period (Figure 1).

Herbivory by *E. servus* and *N. viridula* resulted in a strong increase in the emission of several acyclic terpenes compared with controls, including the C10 monoterpene β-ocimene, the C15 sesquiterpene β-farnesene, and the C11 and C16 homoterpenes 4,8-dimethyl-1,3,7-nonatriene (DMNT) and 4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT), respectively (Figure 2). Emissions of β-ocimene, β-farnesene, and DMNT increased significantly in bolls after 48 hr of exposure to stink bugs and remained elevated 72 and 96 hr after introduction of bugs (Figure 2). A significant increase in emissions of TMTT was not detected until 72 hr after exposure to stink bugs (Figure 2). Furthermore, while small quantities of β-ocimene, β-farnesene, and DMNT were detected in controls, TMTT was not detected in the headspace emissions of controls during any sampling interval (Figure 2).

3.2. E-Nose Training on Damaged and Undamaged Bolls. According to principal components analysis and cross-validation of training data, smellprints of damaged bolls were separated from smellprints of undamaged bolls with 81.25% accuracy after 24 hr of exposure under field conditions (Figure 3(a); Table 2). Smellprints of bolls exposed to *N. viridula* for 48, 72, and 96 hr were separated with 100% accuracy from smellprints of undamaged bolls (Figures 3(b), 3(c), and 3(d); Table 2). Interclass Mahalanobis distances (*M*-distance) provided an indication of the degree of separation between classes following each training session (Table 2). Longer durations of exposure led to greater separation of smellprints from damaged and undamaged bolls according to the interclass *M*-distance values generated from the cross-validation analysis (Table 2).

3.3. Identification Accuracy of E-Nose Temporal Training Sets. Regardless of the length of feeding exposure, identification accuracy was always lower compared with training set accuracy (Tables 2 and 3). When using the smellprints from training sets collected after 24 and 48 hr of feeding, the E-nose correctly identified bolls (damaged or undamaged) 60 and 65% of the time, respectively (24 hr: *chi*-square $P = 0.37$; 48 hr: *chi*-square $P = 0.18$; Table 3). Identification accuracy was markedly improved when using the smellprints from training sets collected after 72 and 96 hr of exposure to stink bugs. The E-nose correctly identified bolls (damaged or undamaged) with 95% accuracy after 72 hr of feeding exposure (*chi*-square $P < 0.0001$; Table 3) and 90% accuracy after 96 hr of exposure (*chi*-square $P = 0.0003$; Table 3).

3.4. E-Nose Detection of Stink Bug Species-Specific Feeding Injury. Overall, nine of the 28 active sensors showed a strong change in resistance in response to headspace emissions from cotton bolls (Figure 4). Smellprints collected from the headspace of unexposed bolls (Figure 4(a)) were distinct compared with smellprints from bolls exposed to *E. servus* and *N. viridula* (Figures 4(b) and 4(c)). Differences among smellprints were analyzed by canonical discriminant analysis followed by cross-validation (Figure 5; Table 4). Treatment groups were separated mainly along the first canonical component, with smellprints from unexposed bolls located along positive values of the first component and exposed bolls along negative values (Figure 5). A greater separation was observed between control and exposed bolls than between bolls exposed to *E. servus* or *N. viridula* (Figure 5). Cross-validation of the dataset indicated that all treatment groups were separated with 70% accuracy, but smellprints from bolls exposed to either stink bug species were separated with much less accuracy (65%) than control and damaged bolls (87.5%) (Table 4).

4. Discussion

In this study, it was demonstrated that E-nose technology can readily detect and distinguish VOCs emitted from cotton bolls damaged by stink bugs under field conditions. Herbivory by *N. viridula* and *E. servus* resulted in a significant

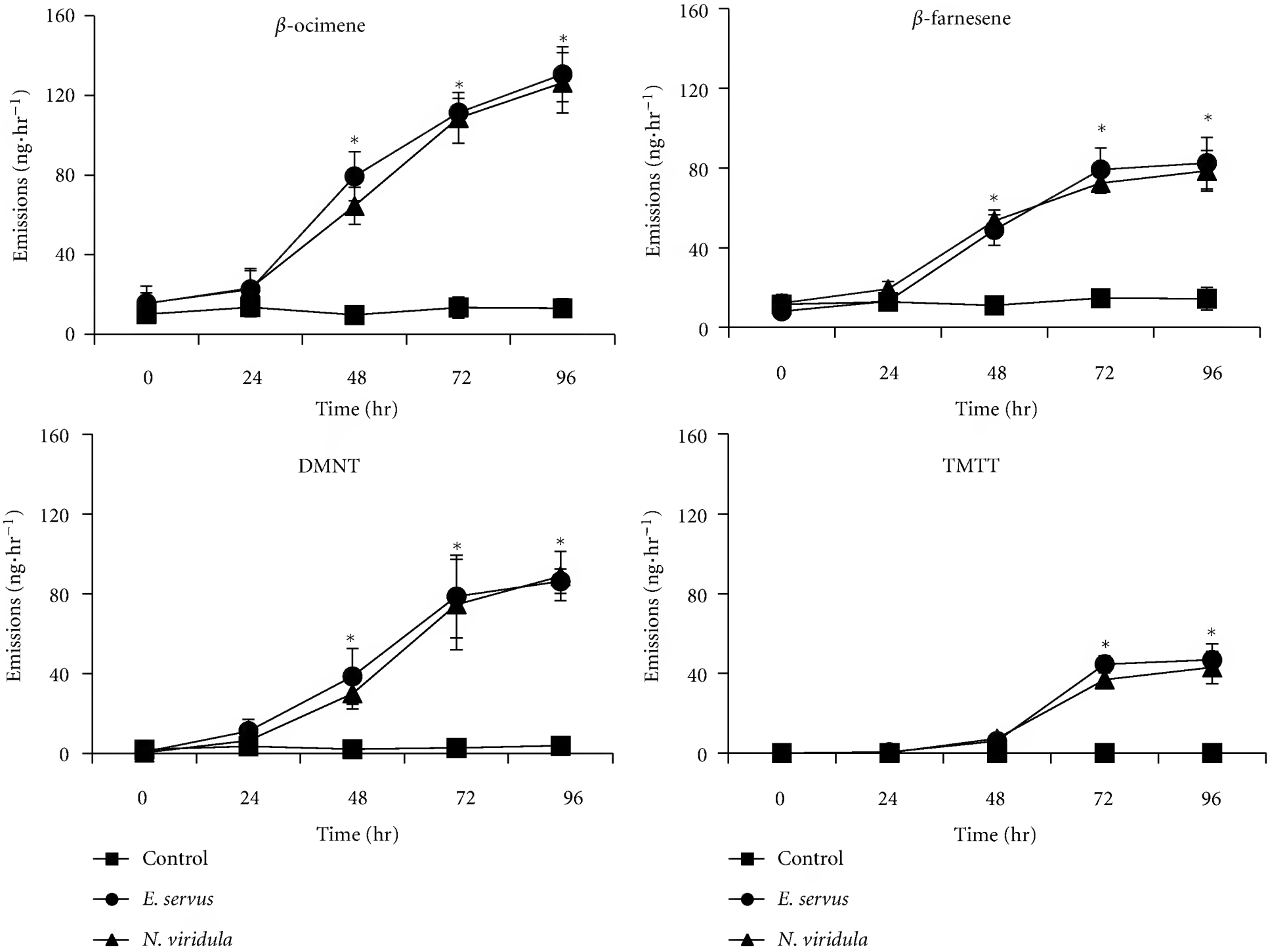

FIGURE 2: Temporal dynamics of induced VOC emissions from cotton bolls exposed to *Euschistus servus* and *Nezara viridula* and unexposed (control) bolls under field conditions. Data points represent mean VOC emissions (±1 SE; $n = 5$). Asterisks indicate significant difference ($P < 0.05$) between control and infested bolls.

TABLE 2: Cross-validation, training accuracy, and interclass Mahalanobis distance of training sets based on E-nose sensor response to headspace VOC emissions from 8 unexposed (control) bolls and 8 bolls exposed to *Nezara viridula*.

Time			Identified as		Training accuracy	Interclass M-distance
			N. viridula	Control		
Trained as	24 hours	*N. viridula*	7	1	81.25%	2.339
		Control	2	6		
			N. viridula	Control		
	48 hours	*N. viridula*	8	0	100%	5.117
		Control	0	8		
			N. viridula	Control		
	72 hours	*N. viridula*	8	0	100%	5.125
		Control	0	8		
			N. viridula	Control		
	96 hours	*N. viridula*	8	0	100%	5.837
		Control	0	8		

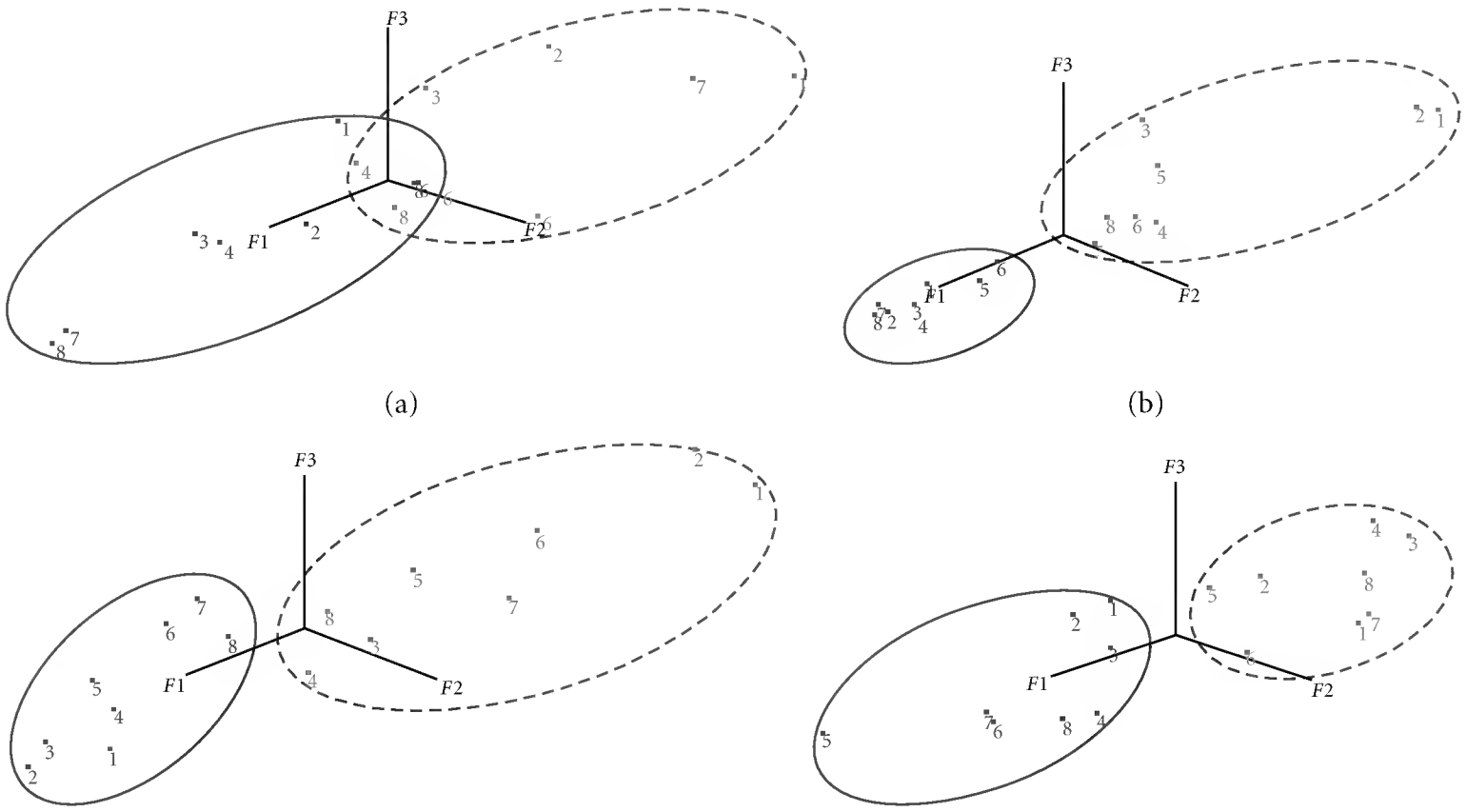

FIGURE 3: Principal components plot of E-nose sensor responses to headspace VOC emissions from unexposed cotton bolls (solid ellipsoids) and bolls exposed to *Nezara viridula* (dashed ellipsoids) after 24 (a), 48 (b), 72 (c), and 96 (d) hr of feeding.

TABLE 3: Accuracy of E-nose training sets used to identify 10 unexposed bolls (control) and 10 bolls exposed to *Nezara viridula* collected during a 96 hr feeding trial.

Time	Treatment	Correct[a]	Incorrect[b]	Identification accuracy
24 hr	*N. viridula*	5	5	50%
	Control	7	3	70%
48 hr	*N. viridula*	4	6	40%
	Control	9	1	90%
72 hr	*N. viridula*	9	1	90%
	Control	10	0	100%
96 hr	*N. viridula*	8	2	80%
	Control	10	0	100%

[a]Classification includes total number of bolls correctly identified by E-nose (damaged or control).
[b]Classification includes total number of bolls incorrectly identified by E-nose (false, confused, unknown).

TABLE 4: Cross-validation of the training set used to train the E-nose to recognize headspace VOC emissions from undamaged (control) bolls and bolls damaged by *Euschistus servus* and *Nezara viridula* following 3 days of feeding damage.

		Identified as		
		Control	*E. servus*	*N. viridula*
Trained as	Control	8	2	0
	E. servus	0	8	2
	N. viridula	1	4	5

increase in VOC emissions 24–48 hr after initial exposure, and temporal dynamics in VOC emissions were similar between the two species. Furthermore, E-nose was capable of accurately (90% success) identifying bolls damaged by stink bugs from undamaged bolls, and the degree of separation between treatments increased with increasing exposure of stink bugs. Finally, E-nose was much less accurate (65% success) at differentiating VOC emissions induced by different species of stink bugs.

In response to stink bug feeding injury, cotton bolls released VOC emissions in significantly greater quantities compared with controls between 24–48 hr of exposure. Herbivory by stink bugs has been shown to induce VOC emission from leaves of several plant species [14–16], as well as from cotton bolls [17]. This study provides a more detailed analysis of the timing of induced VOC emissions in response to stink bug feeding damage. Similar quantitative changes were observed in overall emissions as well as the emissions of specific volatiles in response to feeding by both *N. viridula* and *E. servus*, suggesting that induced VOCs were not species-specific for stink bugs, at least for the level of sensitivity in this experiment. The specificity of induced VOC emissions is determined in part by the feeding mode of herbivores [27, 28] but also by the presence of elicitors in the oral secretion of insects [29]. Oral secretions from stink bugs applied to leaves in the absence of physical injury caused a 2-fold increase in sesquiterpene emissions in corn seedlings [3], indicating that stink bugs contain bioactive compounds in their regurgitant that induces VOCs. The similarities of induced VOC emissions between bolls damaged by *N. viridula* and *E. servus* suggest that, in addition to similar types of physical damage from piercing-sucking mouthparts, these species may also contain bioactively similar elicitors.

E-nose was not capable of accurately discriminating between VOC profiles induced in response to feeding damage from closely related stink bug species. In a previous study, it was shown that E-nose could differentiate between the

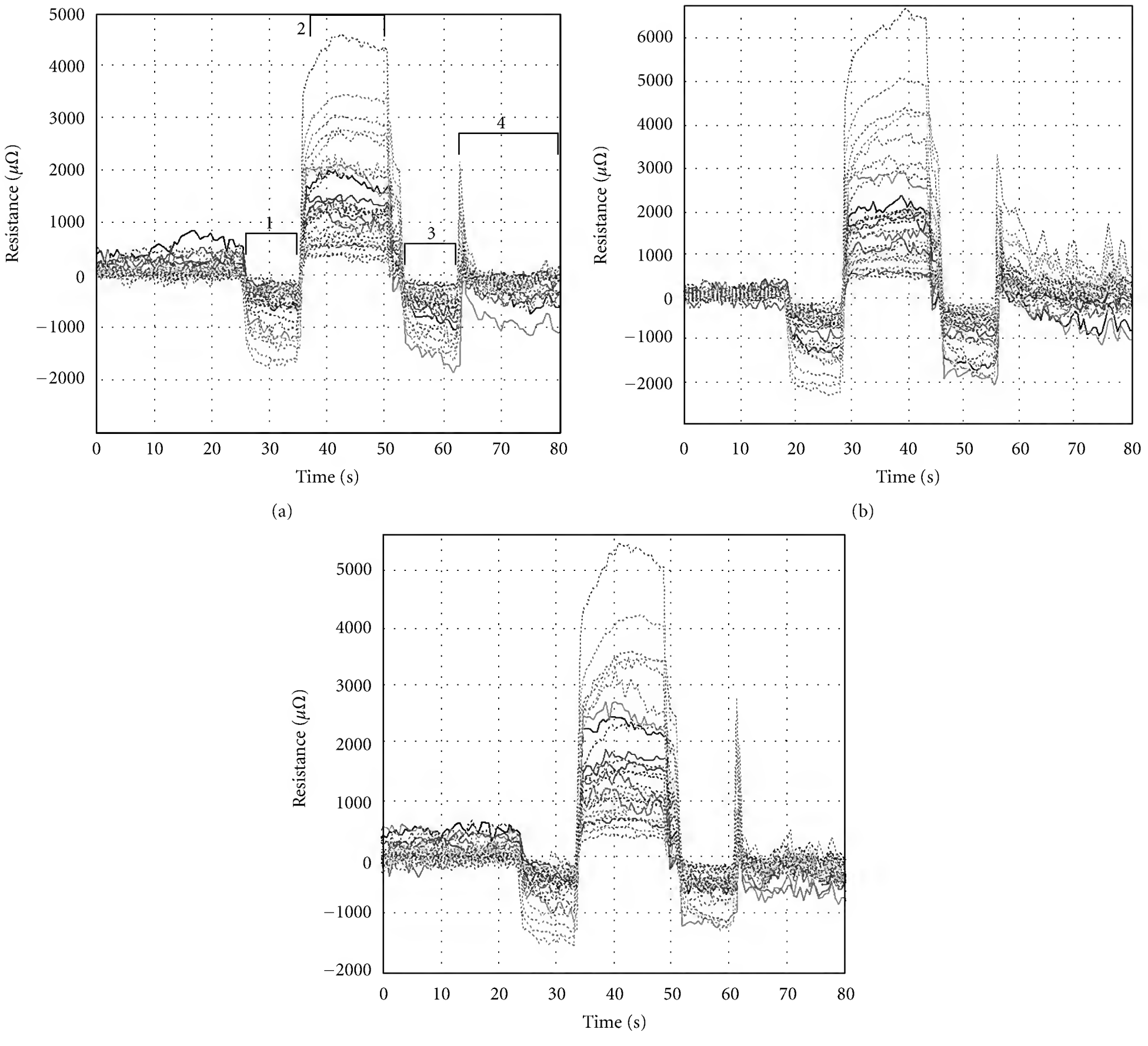

FIGURE 4: Representative E-nose sensor response patterns (smellprints) based on a 15 sec sample of headspace VOC emissions from unexposed (control) cotton bolls (a), or bolls exposed to *Euschistus servus* (b), and *Nezara viridula* (c) following 3 d of feeding damage. 1: baseline purge; 2: sample draw; 3: air purge; 4: sample purge.

defensive secretions released by *N. viridula* and green stink bug, *Acrosternum hilare* (Say) [24]. While E-nose technology was sufficiently sensitive to discriminate species-specific stink bug odors, the results presented here suggest that E-nose is not capable of accurately differentiating sources of damage based on plant VOCs induced by different stink bug species. This is most likely due to the lack of differences in VOC emissions from bolls damaged by *N. viridula* and *E. servus* detected in this study. These results are similar to those reported in a previous study, which indicated that VOC emissions were similar in response to feeding by three hemipteran species [17]. It has been suggested that sufficient specificity in VOC emissions may enable E-nose to detect particular host plant-pest interactions [23]. In a study investigating different types of damage to rice plants, it was demonstrated that E-nose could discriminate between damage by striped stem borer, *Chilo suppressalis* (Walker), and the rice brown plant hopper, *Nilaparvata lugens* (Stäl) [30]; however, these herbivores are from different feeding guilds (leaf chewer versus piercing sucking) and likely cause significant differences in VOC emissions due to differences in elicitors and physical damage inflicted during feeding [28, 31]. The results presented here indicate that feeding by different species of stink bugs does not result in sufficient

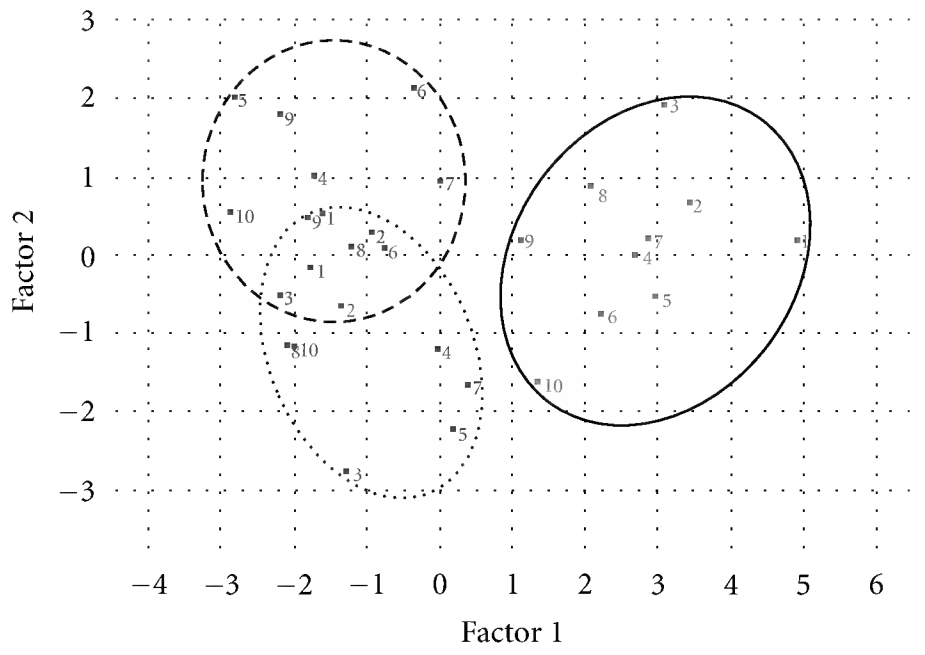

Figure 5: Canonical projection plot of E-nose sensor responses after training the E-nose to recognize VOC emissions from 10 unexposed (control) bolls (solid ellipsoid) and 10 bolls exposed to *Euschistus servus* (fine-dashed ellipsoid) and *Nezara viridula* (broad-dashed ellipsoid) following a 3 d exposure.

specificity in VOC emissions to allow E-nose to discriminate between species-specific sources of damage when the attacking herbivores have similar feeding modes.

While E-nose could not accurately differentiate between plant VOCs released in response to feeding by different species of stink bug, it was highly accurate when discriminating between damaged and undamaged bolls. Prolonged feeding exposure led to greater separation between treatments based on E-nose training sets. Training set data indicated that E-nose was capable of separating damaged and undamaged bolls with 100% accuracy. Identification of known (damaged or undamaged) samples based on E-nose training sets revealed that identification accuracy was consistently 10–15% less than training set accuracy over the course of the experiment. This is likely due to the modest separation of treatment groups as indicated by the low interclass M-distances. In previous research, it was demonstrated that E-nose was capable of identifying damaged bolls with 90% accuracy using bolls excised from plants and measured under laboratory conditions [24]. In this study, all tests were performed on intact plants under field conditions to more accurately demonstrate the potential of this technology as a nondestructive, in-field assessment tool for stink bug damage. In several cases, E-nose technology has been successfully applied to discriminate between healthy and pest-damaged plants [23, 24, 30], and, to our knowledge, this is the first study to demonstrate that E-nose technology is capable of accurately distinguishing between stink bug-damaged and undamaged bolls under field conditions based on a rapid sample of headspace VOC emissions.

While the results of this study are promising, it remains to be determined how the temporal dynamics in VOC emissions (and subsequently E-nose detection) observed from these feeding assays relate to natural variation in stink bug feeding dynamics. Under field conditions, cotton bolls are likely not injured by a single individual over 96 hr, but, rather, visited by one or multiple foraging stink bugs over time. As a result, VOC emissions under broader spatiotemporal variation in feeding dynamics may inhibit the ability to accurately distinguish damaged and healthy bolls using a predetermined training set. Whether induced VOC emissions reach significantly different levels from bolls exposed to much broader (and variable) spatiotemporal feeding dynamics from stink bugs and, subsequently, whether E-nose training sets are capable of differentiating those emissions remain to be determined. Preliminary data suggest that this may not be a major complication, as training sets based on 96 hr of feeding damage were successful in identifying stink bug damage in naturally infested fields (unpublished data); however, this is still under investigation.

The ability to differentiate pest injury based on brief samples of VOC emissions makes E-nose an attractive technology for monitoring stink bug feeding damage in cotton because it could potentially serve as a non-destructive monitoring tool, increase the accuracy of monitoring, and reduce the time and effort associated with current techniques. Further separation of treatment groups by E-nose could be achieved by optimizing sensor technology for the detection of stink bug induced VOCs. For example, sensor chemistries have been designed to respond specifically to VOCs induced by bark beetle attack [32]. In addition to specific sensor chemistries, research also suggests that longer durations of absorption and desorption cycles from E-nose may increase the ability to differentiate among treatments [23]. In this study, E-nose discrimination accuracy was achieved using standard sampling protocols that were not optimized specifically for detecting stink bug damage. Further sampling modifications and/or incorporation of VOC-specific sensor technology would likely improve the discrimination accuracy of E-nose. Nevertheless, the results of this study support the conclusion that E-nose is a promising technology for development of a rapid, nondestructive monitoring tool for stink bug feeding damage in cotton.

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Review Article

The Sexual Behaviour of Chagas' Disease Vectors: Chemical Signals Mediating Communication between Male and Female Triatomine Bugs

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Chemical communication mechanisms that mediate sexual behaviour in triatomine bugs are reviewed with regard to source, identity, and function of sex pheromones. Males attempt to copulate but may be rejected, depending on female age and nutritional status. Triatomine males locate partners through sex pheromones emitted by the metasternal glands (MGs) of females. These activate males, inducing them to leave their refuges and initiate flight. Wandering males display anemotactic orientation modulated by chemical signals emitted from female MGs. Analyses of the MG secretions of several species resulted in the identification of numerous ketones, acetals, and alcohols. Occlusion experiments showed that Brindley's gland products were not required for mating. Metasternal gland volatiles are emitted by virgin male and female bugs, with detection over females occurring more consistently, especially during the early scotophase, suggesting female calling behaviour. Mating triatomine females have been reported to attract males that tend to copulate successively with them. Mating males prolong mating and postcopulatory mate guarding in the presence of other males. This is indicative of a polyandrous mating system in several triatomine species. Its potential advantages remain unknown, and comparative studies are required to increase our understanding of triatomine reproductive strategies.

1. Introduction

Triatomine bugs have great epidemiological relevance because they transmit *Trypanosoma cruzi* Chagas 1909, the etiological agent of Chagas' disease [1–3]. This disease afflicts ~15 million people, with a further 75–90 million at risk from potential transmission in Latin America [4]. The vectorial capacity of these insects relies largely on their ability to invade habitations and develop large colonies, coupled with their being efficient intermediate hosts of trypanosomes. The risk of transmission is greatly enhanced in the case of poorly maintained thatch and mud houses where cracks provide abundant shelter in close proximity to humans and their domestic animals. Vector control is currently the only feasible means of reducing Chagas' disease transmission because of the lack of vaccines and effective therapeutic drugs [4].

Triatomine bugs have been shown to use a diverse range of chemical cues and signals to detect their hosts and communicate with conspecifics, respectively [5]. Host-related cues include stimuli such as body heat, CO_2, and volatile compounds from the skin and breath [6]. Insects in this group also utilize pheromones for marking refuges, locating mates, and for alerting conspecifics to danger [5]. The following sections review our current knowledge of triatomine chemical communication, particularly with regard to reproductive behaviours.

2. Triatomine Mating Behaviour

The mating behaviour of several triatomine species has been described in detail [7–12]. The general mating pattern is similar across species and consists of several behavioural steps performed by males. Initially, a male approaches and either jumps onto or slowly mounts a female. The male then places itself in a dorsolateral position in relation to the female. Once in this position, the male grasps the female, both dorsally and ventrally using all three pairs of legs, releases its paramers, and immobilizes the genitalia of the female to allow the introduction of the aedeagus. The duration of copulation varies between species, ranging from *ca.* 6 min for *Triatoma infestans* Klug, 1834, to *ca.* 50 min for *Rhodnius prolixus* Stål, 1859 [12]. Even after the genitalia of both insects are separated, the males occasionally remain on top of females for several minutes.

Males may perform several copulatory attempts with the same female in order to engage in mating. These copulatory attempts are not always successful because female receptivity depends on age and nutritional status, among other factors [8–10]. Females can display different rejection behaviours in response to copulation attempts [8–10]. These include flattening (the female lowers her body against the substrate to prevent a male from moving into the copulatory position), abdominal movements (females shake their bodies up and down), evasion (females walk away), and stridulation (females rub the tip of their rostrum against the prosternal groove) [12]. The term male-deterring stridulation has been used to describe this behaviour by nonreceptive females. This behaviour has been reported as source of a substrate-borne signal which plays a role in intraspecific communication in *T. infestans* and *R. prolixus* [13, 14].

It has been demonstrated that odours from the metasternal glands promote mating in *T. infestans* and *R. prolixus* [15, 16]. In addition to the volatile compounds secreted by the metasternal glands, it has been suggested that epicuticular lipids may play a role as female recognition signals (i.e., contact pheromones) in *T. Infestans*, but this needs to be confirmed [17].

3. Signals Mediating Copulation Behaviours

Following moulting to the adult stage, triatomine bugs search for host cues to obtain blood meals for nutrition and egg development by females, but they also need to find mates as they become sexually mature. The various species in this subfamily inhabit diverse sylvatic habitats where they associate with vertebrates. Both immatures and adults usually feed at night on a wide array of warm- and cold-blooded animals such as birds, mammals, reptiles, and amphibians. Generally, ecotopes such as birds' nests offer limited and unpredictable access to blood, for example, due to seasonal chick rearing. In addition, coinhabiting the den of a mammal or a bird's nest with a host represents an increased predation risk. Such circumstances might have induced the evolution of the strong tendency of triatomines to hide in cracks and crevices, at the cost of simultaneously affecting the chances of finding mates.

The location, frequency, and timing of mating in triatomines remain unknown, but it is likely that mating occurs around dusk when general locomotion patterns are enhanced [18]. Similarly, bugs probably leave their refuges to search for mates around this time, and it is likely that chemical signals could mediate mate location (see the following). In fact, without long distance signals to mediate encounters between males and females, reproduction would be limited to small sylvatic colonies where adult pairs coexist.

Recently it has been shown that males of two triatomine species are stimulated to leave their shelters by female-produced volatile signals [19, 20]. Interestingly, males may attempt copulation with other males, and according to these authors, this tendency is greatly increased in the presence of chemical signals emitted by the female. These results indicate that odours from the female act as activators, attractants, and aphrodisiacs.

Many authors have attempted to determine whether triatomine bugs use chemical signals to communicate between sexes, often with contradictory results which in many cases are difficult to interpret or place in context due to methodological flaws. For example, Antich [21] reported the attraction of adult *R. prolixus* to volatile pheromones. Later, Neves and Paulini [22] presented similar results describing sexual attraction mediated by chemical signals in adult *T. infestans* and *Panstrongylus megistus* Burmeister, 1835. Additionally, Ondarza and collaborators [23, 24] presented results indicating that a volatile signal mediated the sexual behaviour of *T. mazzotti* Usinger, 1941, but conflicting results were subsequently presented by Rojas and collaborators [25]. Closer inspection of some of these pioneering studies suggests that the experimental designs might not have been sufficient to be able to conclude that chemical signals were mediating encounters between males and females.

Many years later, Brindley's glands became the target of several studies aimed at elucidating communication between adult triatomine bugs. Fontan and collaborators [26], as well as Guerenstein and Guerin [27], presented results showing that a subset of the compounds in Brindley's glands could be recovered from the headspace over groups of copulating *T. infestans* and *R. prolixus*, respectively. The first report provided evidence of behavioural or electrophysiological effects of single components from the secretion of Brindley's glands on adult triatomines. However, it remains unclear whether these effects are related to reproductive behaviours, given that the elicitation of an electrophysiological response or a change in the level of activity does not necessarily indicate a sexual role for the compounds tested. Even attraction of adults to single compounds is not a certain demonstration of a sexual role, unless only one sex is attracted and immatures do not display similar behavioural responses. Two additional points weaken the argument that Brindley's gland products are related to sexual behaviour. First, Brindley's glands are the proposed source of alarm pheromones in all species in the subfamily [28–30], including *R. prolixus*, *T. infestans*, *T. phyllosoma*, *P. megistus*, *T. maculata*, *T. brasiliensis*, and *T. vitticeps*. Therefore, the inadvertent release of Brindley's gland compounds by handling bugs before or during bioassays may have confounded the results of those

assays, and in fact, some of the studies suggesting a sexual role for Brindley's glands secretions did not report how they avoided the emission of the alarm pheromone while handling bugs. Second, reports proposing such a role for Brindley's gland odours have used groups of adults for odour collection; however it is uncertain whether similar results can be obtained when working with pairs of insects, the basic unit of reproductive behaviour. Addressing these questions may help to clarify unresolved issues in the study of triatomine sexual communication.

4. Chemical Signals Mediating Possible Attraction between Adult Triatomine Bugs

It had been suggested that males of several triatomine species might be captured more frequently in light traps than females [31], and this proved to be the case with light trapping of *T. infestans* in Northern Argentina [32]. A recent report also suggested a link between male flight initiation and sexual communication for *R. prolixus* [33], indicating that males may undergo dispersal flights as a mechanism for locating females via volatile chemical signals. Zacharias and collaborators [33] have shown that *R. prolixus* females emit volatile compounds that induce males to initiate flight, and that these compounds are likely associated with the metasternal glands. In addition, these authors showed that excised metasternal glands elicited similar responses from males, confirming that these glands are the source of a volatile signal from the female. A similar increase in take-off frequency was also observed for male *T. brasiliensis* Neiva, 1911, when presented airstreams containing female odour [19]. Females of both species showed no response to male odours. Preliminary results with *T. vitticeps* Stål, 1859, have shown that males of this species only engage in flight if volatile signals emitted by females are present (H.H.R. Pires, *personal communication*). It is also worth noting that flight initiation by males has a clear directional component, with males typically flying upwind [33]. The introduction of female metasternal gland odours into an airstream only induces an increase in male take-off frequency. However, the female-produced chemicals that cause this increase remain unknown.

5. Attraction between Walking Adult Triatomine Bugs

In addition to flying, triatomine adults may detect and walk towards sexual partners from a distance. Whereas the reaction of males to the presence of odours from females has already been described, two recent publications investigated the anemotactic responses of wandering males. Vitta and collaborators [34] used an olfactometer to show that male *T. brasiliensis* move upwind in airstreams laden with the odour of females, exhibiting odour-modulated anemotaxis in response to a sexual pheromone. In addition, they reported that the bioactive compounds were associated with metasternal gland products from females. Interestingly, males also exhibited similar responses in the presence of airstreams containing odours from males. However, blocking the metasternal gland orifices of the males used as the stimulus source did not impair the response of tested males, suggesting that the test males were responding to odours from a different source, and possibly being used in a different context. In contrast, *T. brasiliensis* females showed no response to odours from either sex.

A different experimental approach allowed Pontes [20] to show that *R. prolixus* males are attracted and orient towards airstreams laden with the odour of females. This study used a locomotion compensator [35] to evaluate the responses of males and females presented with odours from adult bugs of either sex. The study showed that *R. prolixus* males were attracted by volatile compounds from the metasternal glands of females.

6. The Role of Triatomine Exocrine Gland Secretions

Most Reduviidae have several exocrine glands in the thorax and abdomen, such as the metasternal glands, Brindley's glands, dermal glands, ventral glands, and abdominal glands [38, 39]. Ventral and abdominal glands are apparently absent in the subfamily Triatominae, and only adult insects possess both metasternal and Brindley's glands [40]. The paired metasternal glands are widespread among the Heteroptera. They are ventrally located at the anterior margin of each metacoxal cavity [41]. Each gland consists of an unbranched secretory tubule and a small pear-shaped reservoir opening laterally to the sternal apophyseal pit [42, 43]. The sac-like paired Brindley's glands are dorsally located, extending into the lateral portion of the second abdominal segment and opening onto the metathoracic epimeron [39, 41, 44, 45]. These glands, which secrete isobutyric acid as their most abundant product, are likely to be associated with alarm and defense functions [29, 30, 45, 46]. As stated previously, it has also been suggested that compounds produced by Brindley's glands are involved in sexual signalling in triatomines [26, 27, 45]. However, no behavioural evidence has been reported that associates compounds from Brindley's glands with behavioural responses specifically related to sex. Further investigation is required to clarify the role of Brindley's glands secretions in the reproductive behaviour of triatomines.

The secretion from the metasternal glands contains several compounds, some of which are highly volatile aliphatic ketones and alcohols (Table 1). The first compound identified from these glands, 3-methyl-2-hexanone, from *Dipetalogaster maxima* (Uhler, 1894), may function as an alarm pheromone or defensive secretion [36] and can be detected when bugs of this species are disturbed [27]. Volatile components emitted from these glands have now been identified in other triatomine species such as *T. infestans, R. prolixus*, and *T. brasiliensis*. Table 2 summarizes metasternal gland compounds detected in the headspace odours of triatomine bugs in different behavioural contexts. As mentioned, it has been proposed that metasternal gland components may mediate sexual communication between

Table 1: Compounds identified from metasternal glands of different triatomine species (references cited in brackets).

	D. maxima [36]	*T. infestans* [30]	*R. prolixus* [16]	*T. brasiliensis* [34]	*T. brasiliensis* *T. infestans* [37]
2-butanone		+	+	+	
2-pentanone			+		
(*S*)-2-butanol			+	+	
(*R*)-2-pentanol				+	
2-methyl-3-buten-2-ol			+		
3-methyl-2-butanol			+		
3-pentanone		+		+	
3-methyl-2-hexanone	+				
3-pentanol		+	+	+	
(*S*)-2-pentanol			+		
(*E*)-2-methyl-3-penten-2-ol			+		
(*S*)-4-methyl-2-pentanol			+		
(*S*)-3-hexanol			+	+	
(*R*)-3-hexanol				+	
3-methyl-2-hexanol				+	
(*R*)-4-methyl-1-hexanol				+	
3-hexanol	+				
2-methyl-1-propanol				+	
2-methyl-1-butanol	+	+			
(*S*)-2-methyl-1-butanol				+	
4-methyl-3-penten-2-ol			+		
1-heptanol				+	
6-methyl-1-heptanol				+	
(*R*)-4-methyl-1-heptanol				+	
(*R*)-1-phenylethanol				+	
(4*S*,5*S*)-2,2,4-triethyl-5-methyl-1,3-dioxolane					+
(4*S*,5*S*)-2,4-diethyl-2,5-dimethyl-1,3-dioxolane					+trace
(2*R*/*S*,4*S*,5*S*)- and (2*R*/*S*,4*R*,5*R*)-4-ethyl-5-methyl-2-(1-methylethyl)-1,3-dioxolane					+
(2*R*/*S*,4*S*, 5*S*)-4-ethyl-5-methyl-2-(1-methylpropyl)-1,3-dioxolane					+trace
(2*R*/*S*,4*S*, 5*S*)-4-ethyl-5-methyl-2-(2-methylpropyl)-1,3-dioxolane					+trace

Table 2: Compounds associated with metasternal gland secretions of different triatomine species emitted in different behavioural contexts (references cited in brackets).

	D. maxima (disturbed adults: [27])	*T. infestans* (disturbed adults: [30])	*T. infestans* (mating: [30])	*R. prolixus* (female calling: [16])	*R. prolixus* (mating: [16])
2-butanone		+		+	
2-pentanone				+	+
3-pentanone		+	+		
3-methyl-2-hexanone	+				
(*S*)-2-butanol				+	
2-methyl-3-buten-2-ol					+
3-methyl-2-butanol				+	
(S)-2-pentanol				+	
2-methyl -1-butanol		+		+	+

adults of these species [30, 34, 47]. In *T. infestans,* the metasternal glands produce a number of volatiles, including the main component 3-pentanone, which was detected in the odours from copulating pairs [30]. This suggests that *T. infestans* may use this and possibly other compounds for signalling during mating. The quantities of 3-pentanone in the metasternal glands of *T. infestans* varied between 10 and 100 μg per adult [30], whereas other compounds of the secretion such as 2-methyl-1-butanol and 3-pentanol were present at 1 and 10 μg per insect, and 3-hexanol between 0.1 and 1 μg per insect. Surprisingly, no apparent differences were found in the composition of the scent produced by these glands between males and females [30]. More recently, further compounds have been identified from two triatomine species, including the acetal (4*S*,5*S*)-2,2,4-triethyl-5-methyl-1,3-dioxolane and related compounds [37, 48]. Occlusion of female metasternal gland orifices resulted in a significant decrease in copulation frequency and prevented the male aggregation behaviour described for this species, suggesting that metasternal gland odours mediate the sexual behaviour of *T. infestans* [15]. Because the attractiveness of mating pairs decreased after occluding female metasternal glands, it was suggested that females emit volatile compounds that promote both copulation and male aggregation behaviour. Similar results were obtained with *R. prolixus*, in which occlusion of male or female metasternal gland orifices induced a decrease in copulation success [16]. Recently, Pontes and Lorenzo [47] have shown that the occlusion of *R. prolixus* female metasternal glands also prevented male aggregation around mating pairs, whereas the occlusion of male metasternal glands did not affect male aggregation. In addition, occlusion experiments also showed that Brindley's gland products are not required for normal mating and male aggregation [15]. In sum, these experiments suggest that volatile compounds from metasternal glands may be among the key signals used in mating interactions.

The metasternal glands of *R. prolixus* produce a variety of volatile compounds, including at least 12 ketones and alcohols. Of these, the most abundant are 2-methyl-3-buten-2-ol, 2-pentanol, (*E*)-2-methyl-3-penten-2-ol, 4-methyl-3-penten-2-ol, and the enantiomers of 4-methyl-3-penten-2-ol [16]. Further analysis suggested that the minor compounds 2-butanol, 2-pentanol, 4-methyl-2-pentanol, and 3-hexanol were produced as the (*S*)-enantiomers, whereas 4-methyl-3-penten-2-ol (mesityl alcohol) was a mixture of the (*R*)- and (*S*)-enantiomers. As with *T. infestans*, the metasternal gland components of male and female *R. prolixus* appeared to be the same. For *R. prolixus,* these volatile compounds were also detected in the odours of virgin male and female bugs [16], with detection being more frequent from headspace odours from females. Furthermore, females released these substances more frequently during the early hours of the scotophase. In addition, their detection, albeit in limited quantities (e.g., 10–100 pg for 2-pentanol), over copulating pairs of *R. prolixus* suggests that these compounds may be involved in sexual communication [16]. Interestingly, 2-methyl-1-butanol is the most consistently detected compound found over mating pairs, even though it is not the most abundant product of metasternal gland secretions.

The metasternal gland secretions of *T. brasiliensis* were found to contain at least 16 ketones, acetals, and alcohols [34, 37, 48], with 3-pentanone being one of the most abundant compounds, as seen for *T. infestans* [30]. In addition to 3-pentanone, other abundant components included 3-pentanol and (*R*)-4-methyl-1-heptanol. The configurations of the chiral compounds varied; 2-methyl-1-butanol was present as the (*S*)-enantiomer, whereas 4-methyl-1-hexanol, 4-methyl-1-heptanol, and 1-phenylethanol were present as (*R*)-enantiomers, and 3-hexanol and 3-methyl-2-hexanol were present in all isomeric forms [34]. GC-EAD recordings performed with *T. brasiliensis* showed that male antennae responded to a number of volatile compounds in the metasternal glands of females, including 3-pentanone, (*R*)-4-methyl-1-heptanol, (4*S*,5*S*)-2,2,4-triethyl-5-methyl-1,3-dioxolane, (*S*)-2-methyl-1-butanol, and (*R*)-1-phenylethanol [34]. Electrophysiological experiments still need to be carried out with other important vector species such as *T. infestans, T. dimidiata*, and *R. prolixus* in order to determine which of the components of the secretions have associated receptors on the bugs' antennae.

Paired glandular areas have been described on the intersegmental membrane between abdominal segments 8 and 9 of male *T. infestans* and *Triatoma rubrofasciata* [49, 50], along with three areas of glandular structures associated with the basal articulatory apparatus in *T. rubrofasciata* [50]. The identities and roles of the products of these glands have not been determined.

7. Male Aggregation Signals and Their Emission during Copulation

The existence of a chemical signal that is released during mating and promotes the aggregation of males around mating pairs has been consistently reported for *R. prolixus* and *T. infestans* [47, 51–53]. In these species, females were not observed to aggregate around a mating pair, suggesting that female behaviour is not influenced by the presence of copulating pairs [47, 53]. The mating and aggregation behaviour of males is not consistent across all triatomines. *Panstrongylus megistus* females are receptive to copulation only once during their imaginal life, perhaps because the genitalia of males and females remain coupled for an extended period, thus preventing mating with other males [54]. In addition, the apparent absence of signals promoting the aggregation of other males around a mating pair contrasts with the behaviour of *T. infestans* and *R. prolixus* [54]. For the latter two species, pairs remain coupled for shorter intervals, allowing subsequent copulations. For example, aggregated males copulate successively with the same female for *T. infestans* [53], *T. brasiliensis* [55], and *R. prolixus* [47]. Thus, the aggregation of males could be related to a polyandrous mating system [56].

Copulating *T. brasiliensis* males are known to modify their behaviour in response to the presence of other males [55], with the duration of mating being prolonged in pairs copulating in the presence of other males. In addition, long postcopulatory associations were observed with *T. brasiliensis*

and *R. prolixus* males mating in similar circumstances [47, 55]. These postcopulatory associations are likely a form of mate guarding geared towards impeding subsequent copulation by other males [57]. The observation of multiple mating in several triatomine species suggests the likelihood of sperm competition mechanisms, and the possible costs and benefits of multiple mating in triatomines deserve further analysis.

8. Perspectives

Recent advances in identifying and determining the roles of chemical signals in triatomine bugs will aid the development of semiochemical tools for controlling these important disease vectors, but much further work needs to be done to determine which specific compounds mediate sexual behaviours, and their precise roles. If synthetic chemicals are shown to attract males, they could find immediate use as baits for traps to monitor and control populations, thereby limiting the transmission of Chagas' disease. Further detailed analyses of gland contents, including the complete identification of minor components, are essential. The compounds identified will then need to be methodically tested in both laboratory and field bioassays to elucidate their roles as mediators of behaviour. These experiments will need to be followed up with practical field bioassays to determine whether these compounds are sufficiently biologically active that they can be used to trap triatomine bugs effectively. The problem is compounded by the fact that these compounds only affect the behaviours of adult bugs and, in many cases, may only affect one sex. Thus, it remains to be seen whether mass trapping males only, or possible pheromone-based mating disruption, could result in effective control of these bugs. Other aspects, such as cost-effectiveness, can only be evaluated once the key compounds are known. However, because control of triatomine bugs hinges on sensitive detection of low-density populations and foci of vectors, even effective methods of monitoring triatomine bugs would represent a major advance in minimizing disease transmission.

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Review Article

Effects of Abiotic Factors on the Geographic Distribution of Body Size Variation and Chromosomal Polymorphisms in Two Neotropical Grasshopper Species (*Dichroplus*: Melanoplinae: Acrididae)

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We review the effects of abiotic factors on body size in two grasshopper species with large geographical distributions: *Dichroplus pratensis* and *D. vittatus*, inhabiting Argentina in diverse natural habitats. Geographical spans for both species provide an opportunity to study the effects of changes in abiotic factors on body size. The analyses of body size distribution in both species revealed a converse Bergmannian pattern: body size is positively correlated with latitude, altitude, and seasonality that influences time available for development and growth. Allen's rule is also inverted. Morphological variability increases towards the ends of the Bergmannian clines and, in *D. pratensis*, is related with a central-marginal distribution of chromosomal variants that influence recombination. The converse Bergmannian patterns influence sexual size dimorphism in both species but in different fashions. Body size variation at a microspatial scale in *D. pratensis* is extremely sensitive to microclimatic clines. We finally compare our results with those for other Orthopteran species.

1. Introduction: Ecogeographic Rules, Body Size, and Abiotic Factors

Body size, one of the most important characteristics of animals, is strongly influenced by abiotic factors [1, 2]. One of the main causes of the importance of body size in determining many characteristics of the life history of organisms is that it scales with metabolic rate which, in turn, influences the rate at which an individual grows, acquires resources, and reproduces [3]. In Orthoptera, a interspecific analysis using 32 species of 7 families (both Ensifera and Caelifera) showed that the scaling exponent between metabolic rate and body size was 1.06 [3] supporting the model of Kozlowski et al. [4, 5] that cell size and number influence the metabolic scaling of organisms.

The influence of body size in a large number of life history characteristics of organisms including grasshoppers has been repeatedly stressed [2, 6–8]. Examples of this in grasshoppers are the relationship between body size and fecundity [7] or survival [9]. Thus, analyzing large-scale geographic variation of body size in different organisms is of importance in order to understand both the abiotic and biotic factors that may modify it through several mechanisms and the ecological and evolutionary consequences of this variation [2, 10–12].

The concept that abiotic factors are relevant to the evolution and adaptation of living beings has been present since the early days of modern biology [13–15]. Because abiotic factors vary geographically, their effects on organisms should change in consequence. A number of the so-called

"ecogeographic rules" (Bergmann's rule, Allen's rule, and others) try to describe geographic patterns of body size observed across the geographic (essentially latitudinal and altitudinal) distributional range of species. Bergmann's rule [16] was originally formulated for endothermic animals (mammals and birds) at an interspecific scale. This rule states that body size tends to increase with latitude (or altitude) because of the ecological advantage of being larger, thus having lower surface/volume ratio, when temperature is lower. Heat loss is minimised (or heat conservation is increased) due to a larger body size. Allen's rule [17] is usually seen as a complement to Bergmann's rule because it describes geographic patterns of decreases in relative size of protruding parts of animals (ears, tails, wings, and limbs) as latitude increases (and mean temperature decreases), as a means for avoiding heat loss. Another important ecogeographic principle relating to adaptation to different environments both within and between species is Gloger's rule [18], which states that birds in climates with high relative humidity tend to be darker than conspecifics in climates with low relative humidity. However, this rule has not been thoroughly explored, especially in insects. A further ecogeographic rule, Jordan's rule or the Law of Vertebrae [19], states that the number of vertebrae in fish increases with latitude and thus decreases with temperature. In this sense it intersects with Bergmann's rule, but also with many other possible environmentally and genetically related mechanisms [20], and of course is not applicable to insects.

It is important to note that all these ecogeographic rules proposed, at their inception, a thermoregulatory explanation for the observed clinal trends (either latitudinal or altitudinal [21]). However, there has been growing concern that these patterns are probably the result of many factors (abiotic and biotic) acting jointly, which makes the situation far more complex than originally thought [20, 22–24], a suggestion that was already present in an early paper by Scholander [25].

With time, Bergmann's and Allen's rules became modified in their original sense. Both Mayr and Rensch [21, 26–29] transformed the original concept of Bergmann's (and by extension, Allen's) rule to an intraspecific pattern: races or populations (not necessarily species) varied in size according to temperature gradients. Also, a number of studies were undertaken to try to apply these rules to ectotherms, that of Ray being one of the first [30]. However, his results [30] were inconclusive mainly because he freely compared different kinds of ectotherms (including insects and amphibians) and mechanisms underlying ecogeographic patterns in different organisms that probably obey different mechanisms. In fact, in the case of endotherms for which the ecogeographic rules were originally formulated, a number of discrepancies occur and it is not always possible to explain the geographic body size trends by simple thermoregulatory models. This situation becomes more complex when trying to verify the rules in ectotherms that, in most cases, regulate body temperature behaviourally, as is the case of most studied Orthoptera [31–35].

For endotherms, a number of non-Bergmannian physiological or ecological explanations have been developed to explain clinal patterns of body size variation, especially when this variation does not conform to Bergmann's rule [36–38]. This situation is complicated in ectotherms and particularly in insects, which tend to show geographic body size patterns that represent a continuum between fully empirical Bergmannian trends to its converse [39] and where classical thermoregulatory explanations are difficult to apply [10, 11].

We have studied two closely related species of Neotropical Melanopline grasshoppers, *Dichroplus pratensis* and *D. vittatus*, both of wide geographic distribution in Argentina in order to describe and understand their patterns of body size variation across climatic gradients.

2. Study Species

Dichroplus pratensis Bruner, 1900, and *D. vittatus* Brunner, 1900, belong to a large genus of Melanopline grasshoppers widely distributed in South America. Both species have the largest geographic distributions within the group, essentially in Argentina. Although *Dichroplus* has been recently the subject of revision and a number of species were placed in new genera, the *maculipennis* group, containing 9 species, seems to be well based and monophyletic on the grounds of structure of the phallic complex and external morphology [40].

Dichroplus pratensis distribution spans more than 23 degrees latitude, from the Puna highlands of Jujuy province to Santa Cruz province in Southern Patagonia. It is found from 0 to at least 2,500 masl, and longitudinally it is found from the Atlantic Ocean shore to the Andes [10, 12]. *D. pratensis* is more frequent in elevated, dry grasslands. It is found in an astonishing variety of habitats including the Patagonian steppe, the Puna highlands, and the humid Pampas. It is obviously a species of wide ecological tolerance, which is probably related to its nonselective polyphagous herbivorous habits. However, there is some evidence that it prefers forbs, which would explain its presence in nongrassland territory [41].

Dichroplus vittatus also has a wide distribution in Argentina partially overlapping that of *D. pratensis* although only seldom both species are found in strict sympatry, probably due to competitive exclusion. *D. vittatus* is more common in semiarid and arid habitats, and populations have been found at over 3,000 masl in Catamarca and La Rioja provinces [40]. Large populations are found in central Chubut on natural grasses and on "*Jarilla*" (*Larrea divaricata*) in the Monte phytogeographic region of Argentina [40]. Both species are univoltine, and the length of their adult reproductive periods depends largely on latitude and altitude [10, 11].

Twenty five population samples of adult *D. pratensis* Bruner (343 males, 352 females) were collected at localities from Argentina spanning 22° of latitude and 0 to 2,474 m elevation during February and March, 2001. Population samples of adult *D. vittatus* Bruner were obtained at nineteen Argentine localities (190 males and 174 females) spanning almost 20° of latitude and 36 m to 2,758 m above sea level during February and March, 2001. We used SPSS

for Windows (Statistical Package for the Social Sciences) software to perform all statistical tests, mainly OLS regression and parametric and nonparametric correlation between body size estimators and geographic and climatic variables. Reduced major axis (RMA) regression was used in tests of allometry and sexual size dimorphism (SSD). Principal components analysis (PCA) was usually performed to reduce dimensionality of predictors because most environmental variables tend to show a high degree of colinearity. Prior to statistical analysis, all measurements were log-transformed and then tested for normality using the Kolmogorov-Smirnov test to determine the appropriateness of subsequent parametric or nonparametric analysis.

2.1. Clinal Variation of Body Size in Dichroplus pratensis and D. vittatus: The Converse to Bergmann's Rule. Bergmannian patterns in insects remain controversial. Some species or species groups tend to show clinal variation of increasing body size or body mass towards higher latitudes or altitudes and lower temperatures. However, in a large number of cases converse trends (or even the absence of a trend) have been observed at intra- and interspecific levels [39, 42–45].

We studied geographic body-size variation in 25 populations of *Dichroplus pratensis* (along more than 22 degrees of latitude (*S*) and between 0 and almost 2,500 m altitude) and 19 populations of *Dichroplus vittatus* spanning 20 degrees of latitude and 2,700 m altitude. Geographic size variation is wide in both species. Mean male body size (populational means) varied between 18.9 and 26.4 mm in *D. pratensis* and 16.43 and 21.62 mm in *D. vittatus*. For females, size ranges were 22.2–28.2 and 20.26–28.13 mm, respectively.

Using mean body length of each sex and factors obtained from PCA analyses of six morphometric linear characters (body length, length of left hind femur, length of left hind tibia, length of tegmina, middorsal length of pronotum and height of pronotum), it was shown that *D. pratensis* and *D. vittatus* follow the converse to Bergmann's rule, becoming smaller at higher latitudes and altitudes (Table 1). In *D. pratensis* variability of body size increased with latitude and altitude in both sexes (Table 3). Body size trends were significantly correlated with mean ambient temperatures (annual mean, minimum, and maximum), precipitation (annual mean, minimum, and maximum), and two estimators of seasonality, the difference between the maximum and minimum temperatures and the difference between maximum and minimum precipitation; all nonparametric correlations were positive (Table 2). Body size was also positively and significantly correlated with actual evapotranspiration (AET), a measure of primary productivity, and with potential evapotranspiration (PET), a measure of ambient energy (Table 2). Some allometric relationships also showed geographic variation (see the section on Allen's rule) [10, 46]. We proposed that the observed decrease in size with latitude and the increase in morphological variability are joint consequences of the shortening of the growing season, the increasing seasonality and climatic unpredictability and lower primary productivity towards the south (as represented by AET) and that the species exhibits protandry, which contributes, in the south, to smaller and more variably sized males and smaller but more constant body sizes in females. A further factor increasing variability at marginal localities has a genetic component (see below).

A parallel study was performed in the closely allied *D. vittatus*. This species also follows the converse to Bergmann's rule latitudinally but not altitudinally where no significant trends were observed (Table 1). For males, variability of body size increased with latitude but not altitudinally (Table 3). Both trends (size and variability) were significantly and positively correlated with mean annual temperature and minimum annual temperature and two estimators of seasonality: the coefficients of variation of mean annual temperature (negative correlation) and mean annual precipitation (positive correlation) (Table 1). As in *D. pratensis*, some allometric relationships also showed geographic variation. It was suggested that the observed decrease in size with latitude together with the increase in morphological variability is a consequence of a number of factors, which parallel those that predict body size in the sister species: the shortening of the growing season southwards, the increasing seasonality and climatic unpredictability, and the fact that the species exhibits protandry which contributes to smaller and more variable size in males and smaller but more constant body sizes in females [10].

Thus, both species seem to obey the same environmental pressures (either because of phenotypic plasticity or natural selection in different habitats), and the correlations with abiotic factors result from the large geographic distribution of both species which extend progressively into areas of increasing seasonality, lower resource availability, and shorter time for growth, development, and reproduction. These results are in close agreement with those obtained for crickets [43–45] but were reported by us for the first time in species of Acrididae.

2.2. Allen's Rule in D. pratensis and D. vittatus. The relative length of protruding parts of endothermic animals tends to decrease with increasing latitude and altitude as Allen proposed almost a century and a half ago [17, 47]. This pattern, as Bergmann's rule, has been usually regarded as a means of decreasing area/volume ratios in order to minimize heat loss and as a thermoregulatory evolutionary adaptation [48]. It has recently been suggested that a further physiological explanation could explain the effect of temperature on limb length in endotherms. In an experiment using mice it was shown that peripheral tissue temperature closely reflects housing temperature *in vivo*. Also, tissue temperature was significantly correlated with the proliferation of chondrocytes in *in vitro* cultures of metatarsals without vasculature [49]. This provides a novel, nonthermoregulatory explanation to limb length variation by a direct effect of ambient temperature.

However, with very few exceptions, application of Allen's rule to insects has not been explored [30, 48]. We studied the geographic variation of three morphometric characters in relation to body size in *Dichroplus vittatus* and *D. pratensis* to test Allen's rule in these ectotherms. Since both

Table 1: Correlation coefficients and their statistical significance between an estimator of body size (BL: body length) and several geographic and environmental predictors for males (M) and females (F) of two grasshopper species.

Variable*	Correlation coefficient (P)			
	Dichroplus pratensis		*Dichroplus vittatus*	
	$\log_{10}$MBL	$\log_{10}$FBL	$\log_{10}$MBL	$\log_{10}$FBL
LAT	−0.70 (<0.001)	−0.76 (<0.001)	−0.64 (0.003)	−0.65 (0.003)
ALT	−0.39 ns	−0.51 (<0.001)	ns	ns
TMEAN	0.71 (<0.001)	0.65 (<0.001)	0.52 (0.021)	0.60 (0.006)
TMAX	0.70 (<0.001)	0.63 (<0.001)	ns	ns
TMIN	0.59 (0.002)	0.53 (0.007)	0.58 (0.010)	0.70 (0.001)
CVT	ns	ns	−0.67 (0.002)	−0.75 (<0.001)
TMm	0.73 (<0.001)	0.44 (0.029)	ns	ns
PANNU	0.56 (0.004)	0.40 (0.044)	ns	ns
PMAX	0.43 (0.031)	0.42 (0.039)	ns	ns
PMIN	0.48 (0.014)	ns	−0.53 (0.020)	−0.50 (0.028)
CVP	ns	ns	0.56 (0.012)	0.52 (0.024)
PMm	0.49 (0.013)	0.42 (0.039)	0.47 (0.043)	ns
PET	0.59 (0.002)	0.48 (0.016)	ns	0.46 (0.047)
AET	0.48 (0.014)	0.50 (0.011)	ns	ns
WB	ns	ns	ns	ns

* LAT: latitude in decimal degrees; ALT: altitude in metres above sea level; TMEAN: mean annual temperature (in Celcius degrees); TMAX: mean annual maximum temperature; TMIN: mean annual minimum temperature; CVT: coefficient of variation of TMEAN; TMm: mean difference between maximum and minimum monthly temperatures; PANNU: total annual precipitation (in mm/year); PMAX: mean maximum monthly precipitation; PMIN: mean minimum monthly precipitation; CVP: coefficient of variation of PANNU; PMm: mean difference between maximum and minimum monthly precipitation; PET: potential evapotranspiration (the Priestley-Taylor equation); AET: actual evapotranspiration (the Thornthwaite formula); WB: water balance; ns = non-significant.

Table 2: Spearman correlation coefficients and their statistical significance (in parentheses) between the *arsin*-transformed proportions of three morphometric traits (F3L: femur 3 length; T3L: tibia 3 length; TeL: tegmina length) and body length (BL) with respect to latitude (LAT), altitude (ALT), and three selected abiotic factors (TMEAN: mean annual temperature; PMm: mean difference between maximum and minimum mean monthly precipitation; WB: water balance) in males and females of 25 and 19 populations of *D. pratensis* and *D vittatus*, respectively.

Species (sex)	*arsin* Proportion	LAT	ALT	TMEAN	PMm	WB
D. pratensis M	F3L/BL	0.55 (0.004)	−0.47 (0.018)	−0.03 (ns)	0.19 (ns)	0.73 (<0.001)
	T3L/BL	0.55 (0.004)	−0.68 (<0.001)	−0.10 (ns)	0.04 (ns)	0.57 (0.003)
	TeL/BL	0.53 (0.006)	−0.56 (0.004)	0.07 (ns)	0.18 (ns)	0.77 (<0.001)
D. pratensis F	F3L/BL	0.63 (0.001)	−0.46 (0.022)	−0.61 (0.001)	−0.005 (ns)	0.39 (0.005)
	T3l/BL	0.66 (<0.001)	−0.58 (0.002)	−0.61 (0.001)	−0.22 (ns)	0.31 (ns)
	TeL/BL	0.71 (<0.001)	−0.73 (<0.001)	−0.14 (ns)	−0.13 (ns)	0.64 (0.001)
D. vittatus M	F3/BL	0.32 (ns)	−0.10 (ns)	0.10 (ns)	−0.63 (0.004)	0.07 (ns)
	T3/BL	−0.50 (ns)	−0.05 (ns)	0.32 (ns)	0.70 (0.001)	−0.32 (ns)
	Te/BL	−0.34 (ns)	0.10 (ns)	0.43 (ns)	0.66 (0.002)	−0.11 (ns)
D. vittatus F	F3/BL	−0.32 (ns)	0.06 (ns)	0.07 (ns)	0.51 (0.026)	−0.16 (ns)
	T3/BL	−0.49 (ns)	0.08 (ns)	0.26 (ns)	0.34 (ns)	−0.33 (ns)
	Te/BL	−0.60 (0.007)	0.002 (ns)	0.54 (0.016)	0.61 (0.006)	−0.37 (ns)

ns = non-significant.

species follow the converse to Bergmann's rule owing to latitudinal and/or altitudinal variation in time available for growth and reproduction, geographic variation in body size proportions of protruding parts may obey differential allometric growth in different geographic areas owing to time constraints on development and growth imposed by abiotic factors that in turn regulate adult season and time available for reproduction (see above). Alternatively, it could reflect true Allenian variation related to thermoregulation. Body proportions (hind femur, hind tibia, and tegmina with respect to total body length measured from the tip of the head up to the distal portion of the hind femur length

Table 3: Mean body length in mm (BL) and coefficients of variation (CV) in selected marginal and central populations of two species of grasshoppers. LAT: latitude in decimal degrees; ALT: altitude in metres above sea level.

Species	Population	LAT	ALT	Male BL/CV	Female BL/CV
D. pratensis	Volcán	23.92	2574	22.36/5.56	24.34/4.69
	Estación Mazán	28.73	646	23.83/4.56	26.48/5.71
	Don Tomás	36.68	175	24.24/4.00	25.41/3.51
	Olavarría	36.92	162	22.57/3.95	24.34/4.30
	Diadema Argentina	45.78	326	19.29/5.86	22.18/6.21
	Villa Rada Tilly	45.95	0	18.21/6.33	23.17/6.37
D. vittatus	Huacalera	23.43	2758	17.30/4.05	21.66/6.09
	Santiago del Estero	26.02	174	21.62/4.38	24.83/3.71
	Valle Fértil	30.63	828	18.55/4.64	24.68/2.35
	Villa del Rosario	31.57	248	19.19/3.65	26.08/3.26
	Toay	36.57	174	16.77/7.69	20.76/2.89
	Playa Unión	43.07	36	16.43/5.78	20.26/8.29

when parallel to the longitudinal axis of the body) were studied by correlation/regression analyses with geographic and climatic variables (temperature, precipitation, evapotranspiration, and water balance) (Table 2). In *D. pratensis*, body proportions increased with latitude and decreased with altitude (Table 2). These results probably obey the effects of water balance and seasonality on final body size, and on the allometric growth of the three studied characters not being related to thermoregulation. In *D. vittatus*, a generally nonsignificant trend towards the decrease of the mean proportions of all three characters with increasing latitude was observed (Table 2). Nevertheless, also in this species, it is probable that the environmental gradient responds to seasonality factors (although not to water balance) that affect the length of growing season and, in consequence, body size and its allometric relationships. We conclude that the regularities in the geographic distribution of body proportions of *D. pratensis* and *D. vittatus* do not follow Allen's rule in the sense of thermoregulation and result from variables that determine growing season length and the allometric growth of different body parts, closely correlated with the converse Bergmannian body size trends [46].

2.3. The Central Marginal Distribution of Chromosomal Polymorphisms of D. pratensis and Its Relationship with Body Size and Abiotic Factors. *Dichroplus pratensis* has a standard all-telocentric chromosome complement of $2n = 18 + X0♂/18 + XX♀$ but is polymorphic and polytypic for Robertsonian (Rb) fusions that involve the six larger autosomes (L1–L6). Each population may be polymorphic (or eventually may have become fixed) for one to three Rb fusions (except in monobrachial chromosomal hybrid zones in which four fusions may coexist), which vary in quality and frequency in different populations. Fusions in this species produce profound changes in inter- and intrachromosomal genetic recombination by reducing the number of linkage groups that assort independently and by creating, through a reduction of chiasma frequency, large pericentromeric recombination-free chromosomal regions that may house adaptive supergenes [50].

Distribution of Rb polymorphisms is not random in *D. pratensis*: different fusion systems characterize different chromosomal races that inhabit radically different environments. Moreover, the highest number of fusions and their highest frequencies are associated with ecologically optimal (central) environments. In these not highly seasonal habitats, primary productivity is high and resources are abundant in quantity and variety. Populations tend to be very large and extremely dense in some years. Rb frequencies decrease clinally and steeply towards the margins of the geographic distribution until in the most extreme environments (i.e., the Patagonian steppe towards south and the Puna highlands towards north) fusions completely disappear, populations being strictly monomorphic for the standard karyotype. Those extreme habitats are harsh, unpredictable, and highly seasonal. Populations are rare, very small, and of very low density, and the distribution of the species is extremely patchy and not continuous as in central habitats [50, 51].

The central-marginal model relates the complex Rb polymorphisms with the distribution of abiotic and biotic factors along latitudinal and altitudinal gradients and variation in body size which, as stated before, follows the converse to Bergmann's rule. We have proposed that, in central regions, high frequencies of Rb polymorphism would maintain coadapted supergenes adaptive to these stable and favorable environments; thus, restriction of genetic recombination would be essential to impede supergenes breakdown through crossing-over. In marginal habitats, however, which are changing and unpredictable, where resources are low and populations probably endure continuous cycles of extinction and recolonisation, high recombination is essential for the liberation of genetic variability that would be the substrate of natural selection for allowing adaptation to these harsh environments.

The former was in part corroborated by studies of morphological diversity along the range of the species. It has been shown that, although body size decreases clinally

Table 4: Reduced major axis (RMA) regressions of male body length on female body length under the null hypothesis of $\beta = 1.0$ in *Dichroplus pratensis* and *D. vittatus*. r: pearson's correlation coefficient; T: student's t-statistic; df: degrees of freedom; P : probability; β: slope of RMA regression; S_ξ: standard deviation; T: Clarke's T-statistic; CI: confidence interval; a: RMA regression intercept.

		Correlation coefficient				RMA slope					RMA intercept	
SPECIES	TRAIT	r	t	df	P	β (S_ξ)	T	df	P	95% CI	a (S_ξ)	95% CI
D. pratensis	BL	0.79	5.12	23	<0.001	1.328 (0.171)	2.60	20.33	0.009	0.975, 1.681	−0.493 (0.239)	−0.988, 0.002
D. vittatus	BL	0.83	6.15	17	<0.001	0.767 (0.104)	2.40	15.38	0.015	0.549, 0.986	0.221 (0.143)	−0.080, 0.522

towards the margins, morphological variability increases significantly (especially in adult males) despite the fact that, in marginal populations, the time available for development and growth is much lower than in optimal central environments (Figure 1). This fact has been interpreted as a result of increased recombination and release of genetic variability (Figures 1(c)–1(f)). The same phenomenon has been observed in the sister species, *D. vittatus* whose geographic distribution mostly overlaps that of *D. pratensis* (see below).

2.4. Rensch's Rule Is Affected by Bergmann's Rule or Its Converse. In 1950, Berhard Rensch [52] described, in phylogenetically related species (including mammals, birds, and carabid beetles), an interspecific pattern, now called Rensch's rule, by which sexual size dimorphism (SSD) tends to increase as general body size increases. Later, Rensch expanded his definition as follows: "In species of birds in which the male is larger than the female, the relative sexual difference (in size) increases with body size. If by way of exception, the females are larger than the males, as among many species of birds of prey, the opposite correlation applies, that is, the greater sexual difference is found in the smaller species". The latter has become to be considered the standard definition of Rensch's rule [53, 54] but its interpretation is ambiguous. Although in male-biased SSD the rule is usually clearly demonstrated, in the opposite situation (female-biased SSD) the situation is far from clear [55]. This is most relevant because in a vast majority of insects and particularly in grasshoppers females are usually larger than males. In grasshoppers, there are many cases of extreme female-biased SSD in families such as Proscopiidae, Ommexechidae, and Romaleidae but information regarding Rensch's rule is extremely scarce [56]. Furthermore, since patterns of SSD are probably heavily influenced by Bergmannian or converse-Bergmannian body size patterns, which in turn depend on clinal variation of abiotic factors [57], a clarification of Rensch's rule in grasshoppers is needed.

The case of both *Dichroplus* species here reviewed is clear in this respect: both species have overlapping geographic distributions in Argentina, and both are sister species belonging to the same *Dichroplus* species group (the "*maculipennis*" group) and follow the converse to Bergmann's rule. Although Melanoplinae Acrididae do not show extreme SSD, males and females are readily distinguished by size in the field and all species show female-biased SSD. However, both species exhibit completely opposite patterns of SSD regarding Rensch's rule.

SSD can be the result of sexual or natural selection. Due to male-male competition for access to females, SSD could favor an increase in male body size. On the other hand, larger size in females could be favored by natural selection since fecundity is directly correlated with body size [56, 58].

SSD occurs in both species across their geographical distribution ranges, also involving differences in allometry and shorter developmental times in males. In *D. vittatus* the degree of SSD increased significantly with general body size (classical definition of Rensch's rule), whereas in *D. pratensis* SSD decreased as body size increased (as predicted by the extended definition of Rensch's rule) (Table 4). A plausible explanation of SSD is that sexual selection favors a differential increase in female body size related to a preference by males for more fecund females. Given the close phylogenetic relationship between both species, the differences in SSD between them may be the result of differential natural and sexual selective pressures. In *D. vittatus* both sexes may be reacting differently to environmental conditions regarding body size, while in *D. pratensis* protandry could be the main factor behind SSD, although both react to ambient conditions following the converse to Bergmann's rule [58].

Considering that both species exhibit converse latitudinal Bergmannian patterns related to environmental conditions and that SSD depends on general body size according to Rensch's rule (independently of definition), the steeper latitudinal body size cline shown by males of *D. vittatus* with respect to females would be a consequence of male differential responsiveness to seasonality and season length, determining the decrease of SSD towards South.

2.5. Microspatial Body Size Variation in D. pratensis. In a recent study [59], variation in six morphological measurements in *D. pratensis* sampled at a microspatial scale within the Sierra de la Ventana chromosomal hybrid zone was analyzed. The Sierra de la Ventana region (Buenos Aires province, Argentina) is a heterogeneous environment spread over the southern portion of the transitional zone between wet and dry pampas of Argentina. As a consequence of the interaction among climatic and geological factors (i.e., 16 different vegetation units, transitional annual rainfall regime, and diversity of soils, environments, and topographical design) this region displays diverse microclimates (see references in [59]). Despite its habitat heterogeneity, the Sierra de la Ventana area belongs to the central, ecologically favorable, range for *D. pratensis* characterized by higher food availability and less strenuous environmental conditions [58]. In this hybrid zone, two chromosomal races, polymorphic for different Rb fusions, encounter and hybridize [60, 61].

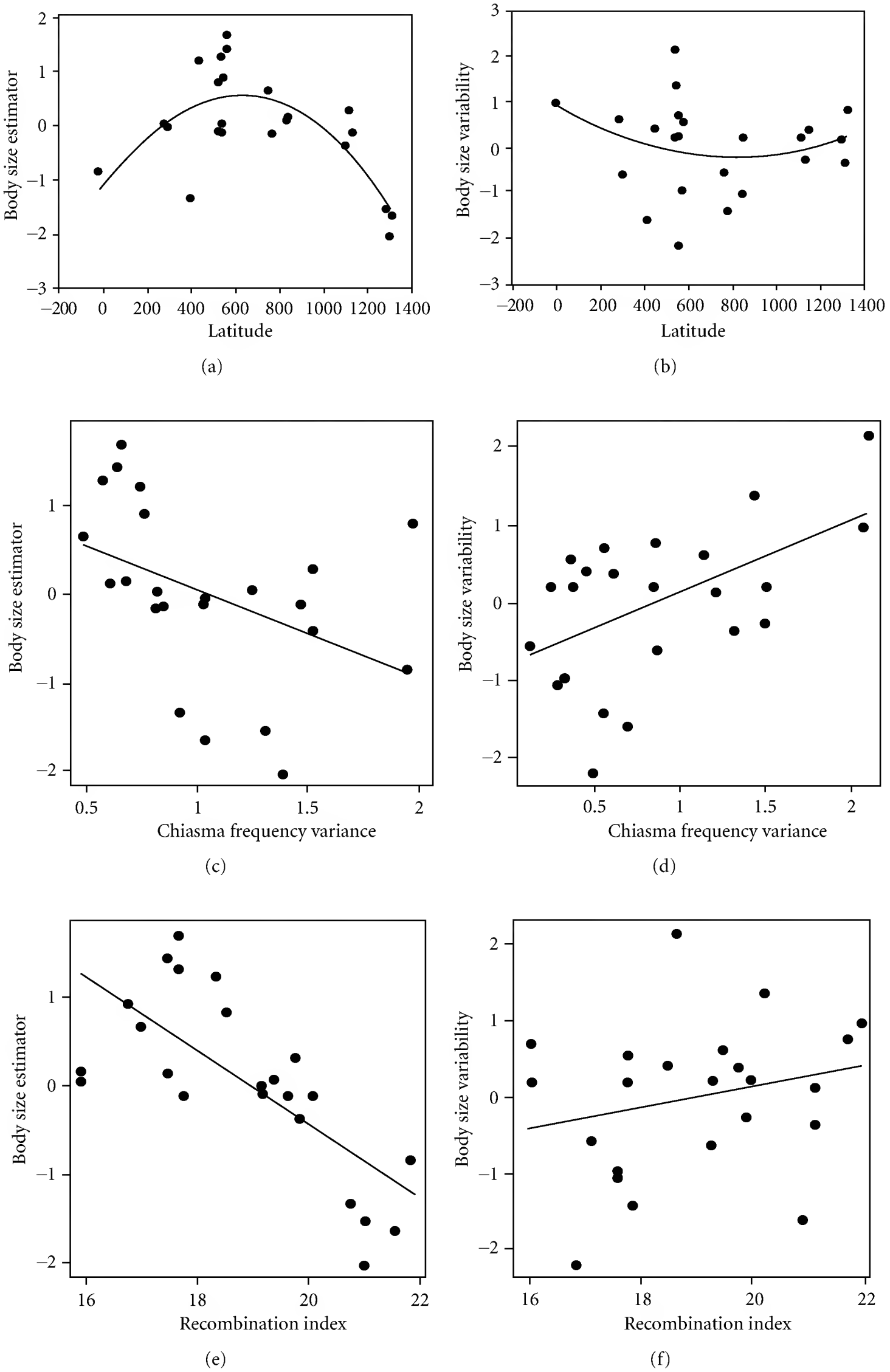

FIGURE 1: Regressions of body size and body size variability of *D. pratensis* on latitude and two estimators of genetic recombination. Body size and its variability are represented by the first and second principal components of a PCA analysis of six linear morphometric measurements and their coefficients of variation. PC1 showed high loadings for the six measurements and PC2 for the six CVs. (a) Distribution of body size along the studied gradient. Marginal populations show smaller body sizes than central ones. (b) Distribution of body size coefficients of variation along the studied gradient. Marginal populations show higher variability than central ones. (c) and (d) Regression of body size and body size variability on between-cell variance of mean chiasma frequency. Body size decreases but its variability increases with higher chiasma variance. (e) and (f) Regression of body size and body size variability on an *ad hoc* recombination index. Body size decreases but its variability increases with higher recombination frequency.

The "Northern" race, widely distributed in central Argentina, polymorphic for fusions L1 and L6 (L1/L6) and L3 and L4 (L3/L4), contacts a geographically restricted "southern" race, polymorphic for fusions L1 and L2 (L1/L2), L3/L4, and L5 and L6 (L5/L6). Complex Rb heterozygotes with reduced fertility occur at this mosaic hybrid zone [51] and chromosome frequencies change abruptly over rather short distances and altitudes (ca. <1,000 m and <500 m, resp.) with fusions L1/L2 and L5/L6 occurring more frequently at higher altitudes [51, 59].

Miño et al. [59] investigated morphometric (total body length, pronotum height and length, left third femur length, left third tibia length, and tegmina length), chromosomal, and molecular (genetic) variation in males and females of *Dichroplus pratensis*, at a microspatial scale. A microspatial altitudinal gradient was studied: samples were taken from the base to the top of Cerro Ceferino Hill, a hill of about ~456 masl. Both male and female grasshoppers showed extensive morphometric variation at a microspatial scale. Highly significant differences were observed between samples and sexes, as indicated by GLM (General Linear Model), with all six morphometric characters separately measured in both sexes from each sample as dependent variables [59]. Specifically for body length, significant differences were observed among grasshoppers from the hill base (~440 masl) and the hill top (~650 masl). Insects from the hill base were smaller (mean/CV body length in males = 21.73 mm/1.2; mean/CV body length in females = 23.86 mm/1.04) than those from the hill top (mean/CV body length in males = 23.12 mm/1.08; mean/CV body length in females = 24 mm/1.19). In Miño et al. [59], body length was significantly positively correlated with altitude in males. Furthermore, a PCA performed to investigate the relationship between body size and altitude, revealed that the first PC, a size estimator, showed the highest loadings for the majority of traits. Also within the Cerro Ceferino hill the trend for male body size was significant and positive. However, no significant correlations between altitude and body size were apparent for females despite a slightly increasing trend.

An additional dimension of intraspecific morphometric variation was analyzed in Miño et al. [59], and sexual size dimorphism was also present in *D. pratensis* samples from a microspatial altitudinal gradient. Sexual size dimorphism was female-biased for all traits in most samples. However, there was no significant relationship between SSD and altitude (ALT) although third tibia length was significantly correlated with ALT in an inverse function. Also, in male grasshoppers from Cerro Ceferino, body length increased significantly and linearly with mean fusion frequency, with frequency of fusions L5/L6 and L3/L4. No trend was statistically significant in females. The body size pattern observed at a microspatial scale in *D. pratensis* [59] differentiates from that observed at a large geographic scale where size shows an inverse correlation with altitude and latitude [10, 12]. It was proposed that in Sierra de la Ventana the body size trend is a likely consequence of habitat segregation of two forms well adapted to contrasting microhabitats within the hybrid zone; this zone, although environmentally heterogeneous, only represents a very small fraction of the species total geographic range and environmental variability.

In this study [59] it was also shown that chromosomal variation of insects was also correlated to microgeographic location: in the Cerro Ceferino Hill, the four fusions characteristic of the hybrid zone varied widely in the sampled grasshoppers, with mean frequency values (F) ranging from 2.5 to 3.0. Fusion L1/L6 was only recorded at the hill base. The frequencies of L1/L2 and L5/L6 increased towards the top of the hill reaching fixation in most samples; fusion L3/L4, showed high frequencies in all samples. Mean fusion frequency and frequencies of fusions L1/L2, L3/L4, and L5/L6 were positively correlated with altitude.

Molecular variation in *D. pratensis* from the Cerro Ceferino, a microspatial altitudinal gradient within the Sierra de la Ventana hybrid zone, was also assessed by Miño et al. [59] using RAPD primers. Significant differences were found in mean heterozygosity values among samples from the hill base to the top, samples from the slope being the more genetically variable. Moreover, samples from the hill base and top were significantly differentiated genetically (as revealed by Wright's 1951 F_{ST}; see Table 5 in [59]).

In conclusion, data of this study [59] revealed a pattern of morphological variation and genetic differentiation within very short distances in *D. pratensis* populations from Sierra de la Ventana hybrid zone. It was proposed that the observed pattern reflects local adaptation at a very small geographical altitudinal gradient, favored by differential adaptation of chromosomal hybrids (genotype combinations) that vary in fitness to heterogeneous abiotic and biotic conditions.

3. Discussion and Conclusions

The results reviewed in this paper have shown that two neotropical melanopline species, *Dichroplus pratensis* and *D. vittatus*, with largely overlapping but usually not locally sympatric geographic distributions, follow the converse to Bergmann's rule. These inverted patterns cannot be attributed to thermoregulatory responses but to interaction with abiotic environmental factors such as seasonality that shorten the time available for development, growth, and reproduction and others that control primary productivity and access to resources. Allen's rule was also not verified but a converse pattern or absence of pattern indicating that, again, thermoregulation is not involved in the proportion of protruding body parts. The observed trends are probably a byproduct of the converse Bergmannian pattern and allometric growth. The countergradient body size variation also indirectly affects Rensch's rule but in opposite ways in both species so that *D. vittatus* follows the rule while *D. pratensis* inverts it. It is important to note that the relationship between body size and abiotic factors produces in both cases a central-marginal size pattern, which in both species involves also an increase of size variability towards the margins. Furthermore, in *D. pratensis* the pattern is closely followed by a complex polymorphic chromosomal system that regulates genetic recombination probably as a selective response to increasing unpredictability of the environment

towards the margins of the distribution. Most of these patterns are repeated at a microspatial scale in *D. pratensis*.

However, it is possible that the effect of abiotic factors on body size and life history characteristics follows different paths in different species. A brief survey of the orthopteran literature in this respect suggests the former but also highlights some common points. In the bushcricket *Poecilimon thessalicus* (Phaneropterinae), collected at three mountain ranges in eastern Greece, it has recently been demonstrated that individuals of populations from the eastern slopes were consistently larger than those from the western slopes. Since these size differences cannot be attributed to a large geographic distribution (less than 1 degree latitude and longitude although altitude varied between 400 and 1,800 masl), no large temperature differences are expected to exert profound effects on body size. Thus, the most probable explanation of this size variation is that, in the dryer western slopes, growing season is shorter thus producing smaller individuals [62].

Another study involving the flightless bushcricket *Pholidoptera frivaldskyi* analysed three extremely isolated populations of this species. This bushcricket is endangered and inhabits fragments of mountainous areas (550–1800 masl in elevation) at the Carpathian Mountains and montane areas in Bulgaria, Serbia, Bosnia, and Macedonia. However, it has not been recorded out of Slovakia for more than 40 y. Despite their isolation, all three populations did not show consistent differences in body size apart from the intrapopulational ones. This may indicate a similar environmental effect of ambient conditions on the phenotypic plasticity of the populations and a genetic uniformity aided by the small size of the populations and a relatively recent origin before fragmentation [63].

In a study of species composition and body size of Tettigoniid species in Atlantic coast salt marshes on *Spartina alterniflora* (Poaceae) communities (latitudinal range, 13.19°), Fabriciusová et al. [63] showed converse Bergmannian patterns for two species, *Orchelimum fidicinium* and *Conocephalus spartinae*. *O. fidicinium*, the largest species, dominated the tettigoniid community at low latitudes and *C. spartinae,* the smallest species, at high latitudes. Furthermore, both species showed a converse Bergmannian pattern at the intraspecific level, individuals being progressively smaller towards higher latitudes. According to the authors several factors might explain this shift in dominance and size trends, including changes in climate, plant phenology, and plant zonation patterns.

Altitudinal body size clines have been less explored in Orthoptera. However, recently, Ciplak et al. [64] have shown converse Bergmannian clines for a grasshopper (*Oedipoda miniata*) and a katydid (*Poecilimon birandi*) along a 2,000 m altitudinal gradient in Anatolia (Turkey). Although the authors did not explore the relationship between body size and abiotic factors, they found that, in both species, larger *O. miniata* individuals were found at sites of higher densities of the species and lower sizes where grasshopper diversity was higher (thus suggesting that interspecific competition could play a role in determining body size). Nevertheless, they suggested that sites of high density are the most ecologically favourable (central), which possibly depend on a combination of abiotic factors that maintain an environment supportive of larger body sizes.

It is thus clear that most well-studied Orthoptera follow the converse to Bergmann's rule and that the most probable explanation for these trends is not thermoregulatory but has to do with increasing seasonality, availability of resources, and growth and developmental time [10, 12, 42–45, 65, 66].

The study of abiotic factors that influence the distribution of geographic body size of animals is thus relevant from several points of view. The confluence of climatic and ecological factors affects so many life history characteristics that knowledge about the trade-offs between the biology of organisms and the environment is essential for a true comprehension of the evolutionary history of species and higher taxa as well as the impact of ongoing and prospective climatic change on their geographic distribution. In the case of Melanopline grasshoppers, and especially those of the *maculipennis* group treated in this paper, biotic and abiotic factors and body size variation may be correlated with complex chromosome polymorphisms. As we demonstrated in *D. pratensis* marginal populations occupy ecologically suboptimal environments in southern margins (Patagonia) and in high altitude in the sub-Andean populations at more than 2,400 m above sea level in the northwest. In these populations the morphological (and genetic) variability increases with the decrease in body size. The release of genetic variability due to high recombination would favor adaptation of natural populations to harsh environments in marginal regions. Although *D. vittatus* is not chromosomally polymorphic, it exhibits the same body size trends of *D. pratensis* (except for sexual size dimorphism) within the same general geographic area. Harsh abiotic and biotic conditions in marginal areas and increasing seasonality determine the shortening of the time available for growth and development, thus allowing for lower body sizes as well as small, low-density, sparsely distributed populations. Marginal areas are zones of continuous extinction and recolonisation according to changing climatic conditions. This is of relevance in the context of current climatic change because increasing temperature may allow the expansion of these species, which are serious crop pests in some parts of their ranges, allowing for the transformation of previous marginal areas into more favorable ones. It is thus of utmost importance to recognize abiotic and ecological body size predictors that may help understand future range expansions and prospective outbreaks.

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Research Article

Unusual Ant Hosts of the Socially Parasitic Ant *Anergates atratulus* (Schenck, 1852) (Hymenoptera, Formicidae)

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The extreme inquiline ant *Anergates atratulus* (Schenck, 1852) (Hymenoptera, Formicidae) was collected in ant nests of *Tetramorium moravicum* Kratochvil, 1941 in Bulgaria and of *T. chefketi* Forel, 1911 in Bulgaria and Turkey. The reported ant hosts belong to the *Tetramorium chefketi* species complex in contrast with the typical hosts from *Tetramorium caespitum/impurum* complex. This finding confirms the assumption that a broader range of host species for the socially parasitic species *A. atratulus* may be expected. Present data on the new host species expand knowledge about biology of this rare ant species, included in the IUCN Red List of threatened species.

1. Introduction

Anergates atratulus (Schenck, 1852) is a rare workerless socially parasitic ant from the Palaearctic region, which has even been introduced together with its host in North America [1]. This extreme inquiline is represented only by female and pupoid type male individuals, whose morphology and anatomy indicate a highly specialized level of parasitism [2]. The body of males is depigmentated, the cuticle is thin, the petiole and postpetiole are widely connected, and degenerate mandibles, palps, and antennae are observed. Female wing venation is reduced and the occipital region is narrowed [3]. Mature females are typically physogastric and found in queenless host nests [2, 3].

Although *A. atratulus* was reported previously mainly in *Tetramorium caespitum* (Linnaeus, 1758) and *T. impurum* (Förster, 1850) [4–7] nests within the *Tetramorium caespitum/impurum* complex, it was also recorded from Sicily (Italy) from a nontypical low altitude (300 m) in a nest of *T. diomedeum* Emery, 1908, which is a member of the *Tetramorium ferox* complex [8]. Future clarification of the complicated taxonomic composition of the *Tetramorium caespitum/impurum* complex will probably enlarge the number of known host ant species parasitized by *A. atratulus* [5].

The only report about *T. chefketi* Forel, 1911 as a host of *A. atratulus* was given by Schulz and Sanetra [9] as an amendment of the identified material published by Heinze [10] from Tavşanli (Turkey, Kütahya district). *Tetramorium moravicum* Kratochvil, 1941 was also mentioned in Sanetra and Buschinger [5] as a possible host of *A. atratulus*, but without any additional data and references.

In Bulgaria, *A. atratulus* was reported to parasitize *T. caespitum* nests in several mountains—the Western Balkan Range, Vitosha, Osogovska, Rhodopes, and the Black Sea coast [11, 12], but no specific habitats and collecting data for this species were given in these studies.

The first data on the presence of *A. atratulus* in Turkey was given by Heinze [10] who reported parasitized *T. chefketi* nests in Anatolia [9]. Following the record of Çamlitepe and Aktaç [13] from the European part of Turkey without mentioning the host species, Aktaç et al. [14] recorded *A. atratulus* in a *T. caespitum* nest in the same region (Kofçaz-Ahmetler Village, Kirklareli district). More recently, Schulz and Sanetra [9] found this species in a *T. caespitum* nest in Erciyes Mountain, Kayseri district in Anatolia.

The present study identifies known localities of *A. atratulus* in Bulgaria and Turkey from nests of two nontypical ant host species: *T. chefketi* and *T. moravicum*. The new data

broaden the knowledge about the biology of this extremely rare ant species, included in the IUCN Red List of threatened species [15].

2. Results

2.1. *Anergates atratulus (Schenck, 1852)*

2.1.1. Studied Material from Bulgaria. Eastern Rhodopes, Krumovgrad district, near Golyama Chinka Village (41°24′28″ N 25°34′43″ E), 430 m, 21.07.2009, leg. A. Lapeva-Gjonova, 1 gyne in a nest of *Tetramorium chefketi*; Konyavska Mountain, Kyustendil district, the main road to the TV tower (42°21′54″ N 22°49′39″ E), 1170 m, 22.09.2009, leg. A. Lapeva-Gjonova, 3 gynes in a nest of *Tetramorium moravicum*.

The first habitat is in the Eastern Rhodopes (South Bulgaria)—a mountain with relatively low altitude and increased Mediterranean climate influence. The collecting locality was situated in an open dry area close to an oak and pine forest. One alate *A. atratulus* gyne was collected from a *T. chefketi* nest under a stone. The second habitat was in the Konyavska Mountain (West Bulgaria), where three alate gynes were collected from a *T. moravicum* nest, also under a stone, on talus in the beech forest belt.

2.1.2. Studied Material from Turkey. Sündiken Mountain, Eskişehir district, Mihalicçik-Yalimkaya Village (39°58′ 31″ N 31°14′05″ E), 1198 m, 10.08.2010, leg. K. Kiran and V. Aksoy, 1 gyne in a *T. chefketi* nest; Bozok Plateu, Yozgat district, Büyükincirli Village (39°38′17″ N 34°55′08″ E), 1049 m, leg. K. Kiran and V. Aksoy, 1 gyne in a *T. chefketi* nest.

The first habitat was in the northwest of Central Anatolia in an old forest zone, almost more than 100 years old, occupied by *Pinus nigra* Arn. trees. The slope of the area was nearly 30% and the underlying stones were very few and small. The second locality was a broad river bank with *Salix* and *Populus* trees in the steppe zone of northeast Central Anatolia. Both gynes were collected from nests in the ground without stones on them.

No gynes of the host *Tetramorium* species were found in any of the investigated nests.

3. Discussion

Tetramorium chefketi and *T. moravicum* from the *Tetramorium chefketi* species complex are nontypical hosts of the socially parasitic ant *A. atratulus*. This study provides the first specific data about the presence of *A. atratulus* in a nest of *T. moravicum*, a xerophilous species inhabiting sunny, dry, and open places with low and scarce herb vegetation [16]. This ant host species is distributed in the Western Palaearctic from Southern France to the Caucasus [17].

The present record from Bulgaria represents the first report of *A. atratulus* parasitizing a *T. chefketi* nest in Europe since Heinze [10] and the redetermination of the host species *T. caespitum* given by Schulz and Sanetra [9] from the Anatolian part of Turkey. In Bulgaria, however, the habitat of the host is situated at a much lower altitude (430 m) than in Tavşanli (1000 m) in Turkey. *Tetramorium chefketi* is a species distributed from the eastern part of Southern Europe to Kyrgyzstan [17].

Host queens were not found in any of the parasite-occupied nests, supporting previous data about *A. atratulus*'s exploitation of already orphaned *Tetramorium* colonies [2, 3].

Our results confirm the previous suggestion that *A. atratulus* parasitizes a wide range of ant host species of the genus *Tetramorium*. Although *A. atratulus* usually exploits *T. caespitum*, a common Palaearctic ant species, this inquiline species is very rare and is included in the IUCN Red List of threatened species. Conservation measures are aimed to protect host ant species and the habitats they occupy. *Tetramorium chefketi* and *T. moravicum* inhabit mainly, as in the case of *T. caespitum*, open, sun-exposed, and dry areas. Future investigations will clarify whether *T. chefketi* and *T. moravicum* are normally local hosts of *A. atratulus* or not. However, data from these two host *Tetramorium* species might have been rare simply due to their limited geographical distributions compared to *T. caespitum*. Present results will be useful for local-scale needs in conservation management of native xerophylous places and for examining the evolution of specific social parasite-host relationships.

Acknowledgments

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Research Article

Effect of Population Density on Timing of Oviposition and Brood Size Reduction in the Burying Beetle *Nicrophorus pustulatus* Herschel (Coleoptera: Silphidae)

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Burying beetles (*Nicrophorus* spp.) bury small carcasses to feed their larvae. Carcasses are a limited, high-quality resource and contests over carcasses become more frequent with increasing population density. Successful beetles kill eggs and larvae present on carcass. In response, females should accelerate oviposition, while offspring development should increase to minimize mortality. Both value of a carcass and frequency of contests decrease as larvae develop. If overproduction of offspring is an insurance against high mortality, females should reduce brood size as carcass value declines. Testing our predictions, we reared female burying beetles, *Nicrophorus pustulatus*, at high and low densities and compared oviposition and brood reduction. High-density females delayed oviposition, suggesting that high population density imposes nutritional and/or physiological stress. Females responded to the physiological constraints and the potentially high mortality rates of eggs and newly hatched larvae by lengthening oviposition period and changing brood reduction rate.

1. Introduction

In response to environmental conditions, females can adjust their reproductive phenotype by modifying the number and size of offspring [1–6]. As population density increases, females often produce fewer and larger offspring [1, 3–8]. This is commonly observed when females compete for access to resources, and increased population density leads to more frequent and intense contests over these resources [1, 3, 6]. Adjustment of offspring number and, to a smaller degree, adjustment of offspring size, can occur during every stage of reproduction [9–11]. Females can regulate the number and size of eggs they produce [2, 12] and actively destroy eggs [11]. In animals with extensive parental care, reduction of offspring number can also occur after hatching or birth of offspring [9, 10]. Typically, females cause the death of an offspring indirectly by feeding the most competitive offspring preferentially and/or ignoring aggressive fatal interactions among competing siblings [10]. In some species, filial infanticide has been observed as the means of brood reduction [9, 10].

Burying beetles (*Nicrophorus* spp.) provide extensive parental care and are known to adjust brood size primarily through filial infanticide [13–15]. Burying beetles use small dead vertebrates (e.g., mice and birds) as food resource for their offspring. Severe contests over the ephemeral resources are usually won by the larger beetles. A carcass, once discovered by a pair of burying beetles or a single female, is quickly buried. While burying the carcass, the beetles remove fur or feathers, deposit oral and anal secretions, and work the carcass into a ball. Within 12 to 48 h after discovery of the carcass, the female lays eggs in the soil surrounding the carcass [13–15]. Clutch size is substantially larger than the number of larvae dispersing [14]. Females lay larger clutches when carcass size increases [16]. After the larvae have hatched, they crawl to the carcass where they are fed by the parents with regurgitated carcass for about three days. Afterwards, the larvae feed by themselves. The male generally

deserts the brood when the larvae are feeding by themselves, while the female stays with the larvae until the larvae have consumed the carcass and are dispersing to pupate in the soil [13–15].

Despite being buried, the carcass can still be discovered by other burying beetles [14, 15, 17]. Successful intruders kill all eggs and larvae encountered [18]. Takeovers occur most frequently between burial of the carcass and hatching of the larvae [19]. Thereafter, takeovers quickly become less likely because the resource value of the carcass decreases as it is consumed, and levels off at very low values when the larvae are about two-to three-days old. [18]. Assuming that overproduction of eggs is an insurance against high mortality rates of eggs and newly hatched larvae [20], we predict that female burying beetles will start adjusting brood size through infanticide after the larvae started hatching and both the value of the carcass and the likelihood of takeovers are declining rapidly, and finish adjusting brood size when the two- to three-day-old larvae have reduced the value of the carcass for other burying beetles. Changes in population density may influence the temporal pattern of offspring number reduction. With higher population density, we expect that intrusions become more likely and with it the possibility of partial infanticide in a failed take-over attempt. To be able to compensate potential loss of eggs and larvae, high-density females should therefore wait longer to reduce their broods.

Based on life history theory [21], we assume that a high probability of takeovers exerts strong selection on minimizing the duration of the developmental stage(s) with very high offspring mortality rates. At high population density, females should thus accelerate oviposition as well as egg and larval development, which can be influenced by females though transmission of nongenetic developmental resources [22, 23]. Consequently, larvae would hatch earlier and develop faster, thus consuming the carcass sooner causing a steeper decline in the value of the carcass. The final brood size of high-density females, however, should be lower than that of low-density females. To maximize fitness, females experiencing high population density with frequent contests over resources won by large individuals, should produce fewer, but larger, offspring than low-density females [24].

To test our hypotheses, we reared female *N. pustulatus* at high- and low densities and compared timing of oviposition and brood reduction as well as offspring development and size between high- and low-density females. In particular, we addressed the following questions: (1) do high-density females lay eggs earlier than low-density females?, (2) do eggs and larvae of high-density females develop more rapidly?, (3) do high-density females adjust brood size later than low-density females, and (4) will they have fewer, but larger, larvae dispersing?

2. Material and Methods

Burying beetles *N. pustulatus* used in this study, came from a laboratory colony established in 2002 with 92 pairs of beetles caught in the Research Forest of Berea College, KY, USA. The beetles of this laboratory colony are kept individually in containers (15 cm × 10 cm × 5 cm) filled 2 cm deep with humid peat at a 15L:9D photoperiod and 22 ± 1°C. The beetles are fed a pea-sized amount of canned cat food (Science Diet, Hill's Pet Nutrition Inc., Topeka, KS, USA) twice weekly.

This study was carried out in summer 2006. To investigate the effect of population density on timing of oviposition, offspring development, and brood size reduction as well as offspring size, we assigned newly emerged females from the laboratory colony at random to a high-density (i.e., four females per container) or a low-density treatment (i.e., one female per container). Container size (15 cm × 10 cm × 5 cm) was the same for both treatments. All containers were filled 2 cm deep with moist peat. Twice weekly, we placed a pea-sized amount of canned cat food (Science Diet, Hill's Pet Nutrition Inc., Topeka, KS, USA) in all containers with low-density females, while containers with high-density females received four times the amount of canned cat food.

When the females were between 28 and 58 days old and sexually mature, the females were assigned at random to offspring developmental stages at which the trial would be terminated. At termination, offspring number and size as well as time elapsed to reach the particular developmental stage were determined. The offspring developmental stages included: $E_{1.5}$ (first laid eggs were 1.5 days old), $L_{0.5}$, L_1, $L_{1.5}$, L_2, $L_{2.5}$, L_3 (oldest larvae were 0.5, 1, 1.5, 2, 2.5, or 3 days old), and $L_{\text{dispersal}}$ (larvae had left the brood cavity and were dispersing). Females assigned to the different offspring developmental stages and density treatments did not differ in age (developmental stage of offspring: $F_{7,281} = 1.84$, $P = 0.08$; female density treatment: $F_{1,281} = 2.40$, $P = 0.12$). Once assigned to an offspring developmental stage, females were transferred to a new container (15 cm × 10 cm × 5 cm) filled 2 cm deep with moist peat and mated with a randomly chosen, nonsibling male from the colony. A total of 320 matings were set up: 20 matings for each combination of female density and offspring developmental stage. The following day, the male was removed, and a previously frozen mouse (mean ± SD: 23.7 ± 2.0 g; range: 18.0 to 27.9 g) was placed into the container with the female. Average mouse weight did not differ between high- and low-density females ($F_{1,281} = 0.18$, $P = 0.67$) and between the different developmental stages ($F_{7,281} = 0.38$, $P = 0.91$). To simulate conditions underground, containers with mice were kept in a dark room at 22 ± 1°C. Containers were checked twice daily for eggs and newly hatched larvae. When the offspring had reached the designated developmental stage, the container was searched for eggs, and the larvae were removed from the carcass. For all developmental stages, eggs were counted, but egg size was only determined for the stage $E_{1.5}$. From each container of stage $E_{1.5}$, the length of a subsample of 10 eggs was measured using a dissecting scope with an ocular micrometer. The larvae of each container were counted, and the brood weight was measured. Larval weight for each brood was calculated as brood weight divided by the number of larvae. Offspring number for each brood was determined as the sum of all eggs and all larvae found in a container. Time elapsed until oviposition was determined as

the number of half-days between the time when the mouse was added to the container (i.e., earliest time when females could discover the carcass), and the first observation of eggs in the container. Egg development was defined as number of half-days between the first observation of eggs and the first observation of newly hatched larvae. Larval development was calculated as number of half-days between the first observation of newly hatched larvae and the point in time when larvae were dispersing.

2.1. Statistical Analysis. Time elapsed until oviposition, egg development, and larval development were analyzed with the GENMOD procedure in SAS (SAS Institute Inc., Cary, NC, USA). Poisson distribution and the log function were chosen as error distribution and link function, respectively. Female density was the main effect in the model and mouse weight was included as covariate. Offspring number and number of eggs and larvae were also analyzed with the GENMOD procedure using Poisson distribution and the log function as error distribution and link function, respectively. We included in the models as main factors female density and developmental stage of offspring and the interaction of the two main effects. Mouse weight was used as covariate. Egg length and larval weight were analyzed with the GLM procedure in SAS. The model for egg length included female density as main effect and mouse weight as covariate. The model for larval weight contained as main factors female density and developmental stage of offspring and the interaction of the two main effects. Mouse weight was used as covariate. Larval mass was natural log-transformed before analysis to achieve normality. Not all of the original 340 matings were successful; therefore, only 297 broods were included in the data analyses.

3. Results

3.1. Duration of Development. High-density females began to lay eggs later than low-density females ($\chi^2 = 6.57$, d.f. $= 1$, $N = 297$, $P = 0.01$; Figure 1). The duration of egg and larval development did not differ between high- and low-density females (eggs: $\chi^2 = 0.02$, d.f. $= 1$, $N = 260$, and $P = 0.89$; larvae: $\chi^2 = 0.17$, d.f. $= 1$, $N = 40$, and $P = 0.68$; Figure 1). Mouse weight had neither an effect on the start of oviposition ($\chi^2 = 0.05$, d.f. $= 1$, $P = 0.83$) nor on the duration of egg or larval development (eggs: $\chi^2 = 0.15$, d.f. $= 1$, and $P = 0.70$; larvae: $\chi^2 = 0.01$, d.f. $= 1$, and $P = 0.93$).

3.2. Number of Offspring. The total number of offspring was significantly affected by the developmental stage of the offspring ($\chi^2 = 1778.18$, d.f. $= 7$, $N = 297$, and $P < 0.0001$). Offspring number was largest at the egg stage and declined continuously until the first-hatched larvae were two days old. From then on, offspring number changed little (Figure 2(a)). Both high-density and low-density females showed the same pattern in the change of offspring number in relation to the developmental stage of the offspring (interaction between density and developmental stage: $\chi^2 = 11.47$, d.f. $= 7$, $N = 297$, and $P = 0.12$). However, high-density females had significantly fewer offspring than low-density females ($\chi^2 = 10.47$, d.f. $= 1$, $N = 297$, and $P = 0.001$; Figure 2(a)). Offspring number increased as mouse weight increased ($\chi^2 = 24.15$, d.f. $= 1$, $N = 297$, $P < 0.0001$, and slope $= 0.03$).

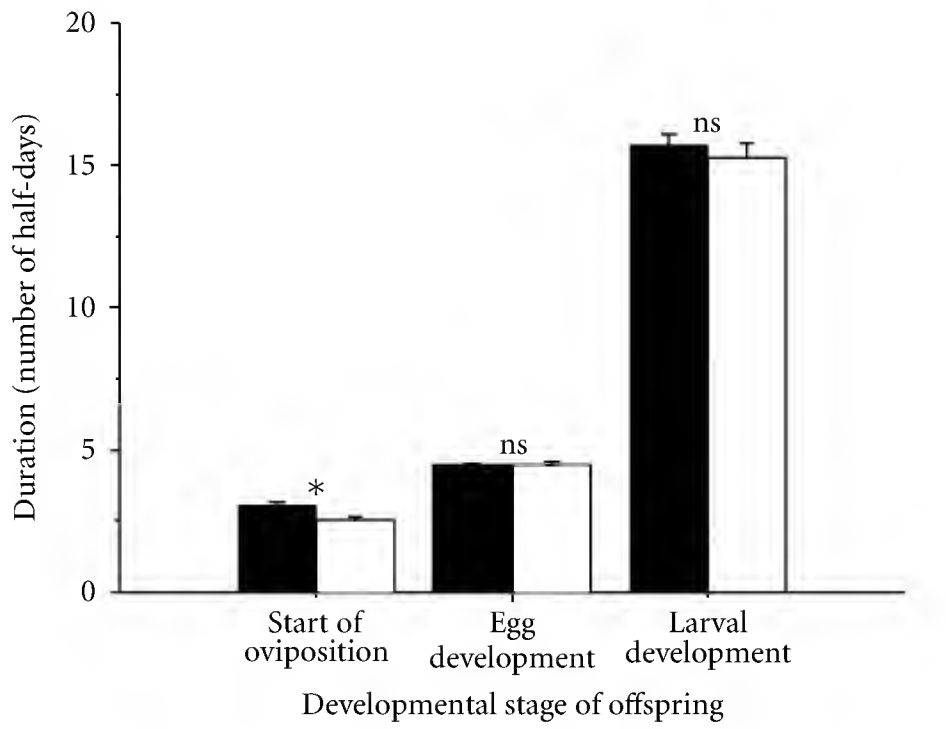

Figure 1: Effect of density on time elapsed until the first egg was laid (i.e., start of oviposition) as well as egg and larval development. Black bars: high-density females; white bars: low-density females. Means and SE are shown. $^*P = 0.01$; ns: not significant.

The number of eggs present after the first larvae had hatched, depended on the developmental stage of the offspring ($\chi^2 = 335.70$, d.f. $= 3$, $N = 220$, and $P < 0.0001$; Figure 2(b)). However, in broods of high-density females, there were more eggs present and for a longer time than in broods of low-density females (Density: $\chi^2 = 7.08$, d.f. $= 1$, $N = 220$, and $P = 0.01$; interaction between density and developmental stage: $\chi^2 = 25.47$, d.f. $= 3$, $N = 220$, and $P < 0.0001$). The number of eggs present increased as mouse weight increased ($\chi^2 = 29.03$, d.f. $= 1$, $N = 220$, $P < 0.0001$, and slope $= 0.09$).

The number of larvae present on the carcass after the first larvae had hatched was significantly affected by the age of the larvae ($\chi^2 = 354.89$, d.f. $= 6$, $N = 260$, and $P < 0.0001$; Figure 2(c)). The pattern of change in larvae number with larval age differed between high- and low-density females (interaction between density and developmental stage: $\chi^2 = 14.82$, d.f. $= 6$, $N = 260$, and $P = 0.02$). On carcass of low-density females, the decline in larvae number began earlier, but leveled off at the same time as the number of larvae on carcass of high-density females (Figure 2(c)). Overall, high-density females had fewer larvae than low-density females ($\chi^2 = 24.46$, d.f. $= 1$, $N = 260$, and $P < 0.0001$). The number of larvae increased with increasing mouse weight ($\chi^2 = 10.37$, d.f. $= 1$, $N = 260$, $P = 0.001$, and slope $= 0.02$).

3.3. Offspring Size. Egg size (measured as egg length) did not differ between high- and low-density females ($F_{1,33} = 0.69$ and $P = 0.41$; Figure 3(a)). The size of larvae (measured as larval weight) changed significantly with larval age ($F_{6,245} = 224.45$, and $P < 0.0001$; Figure 3(b)). But the pattern of the larval weight increase with larval age did not differ between

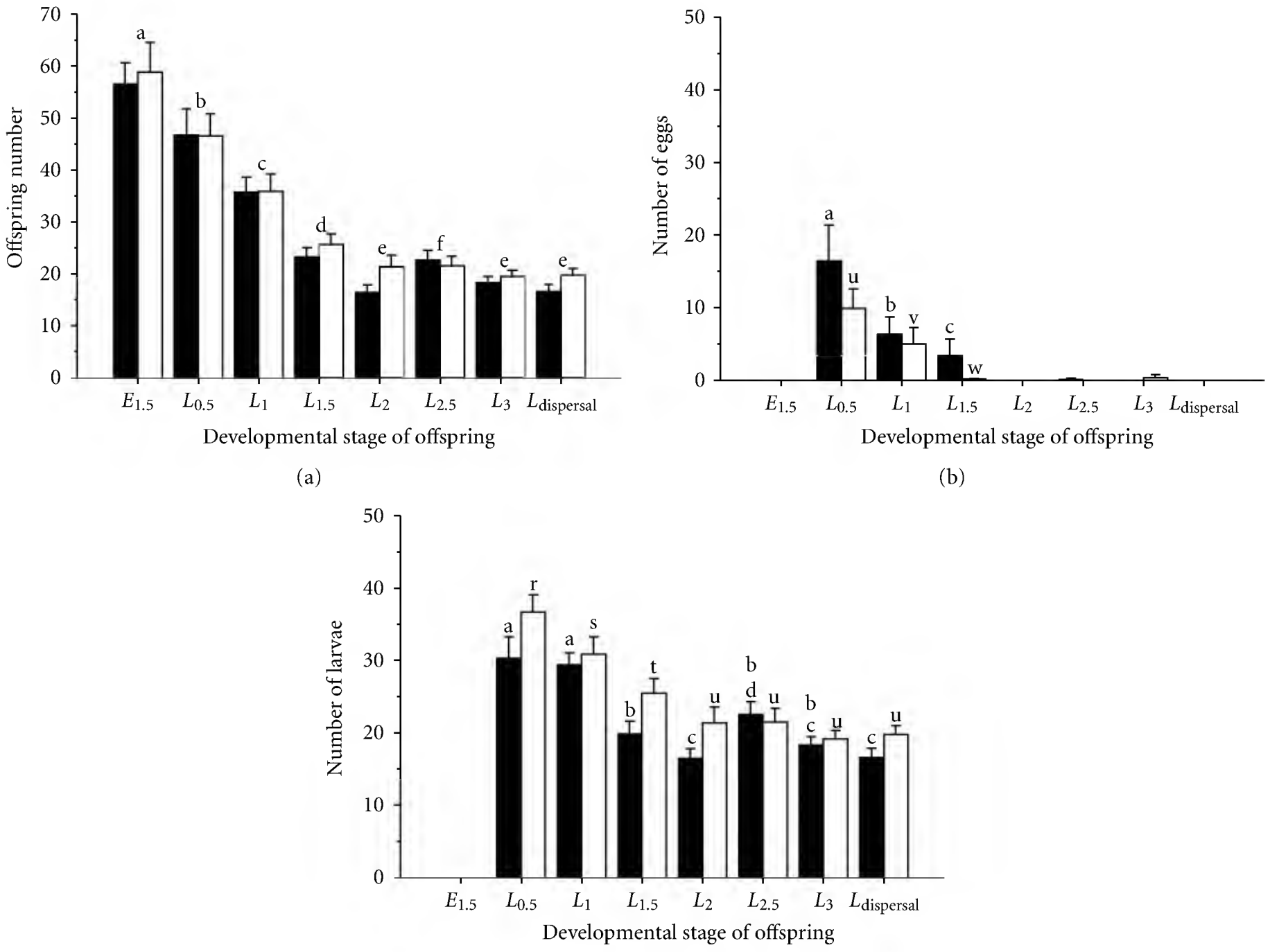

FIGURE 2: Total number of offspring (a), number of eggs present after first larvae had hatched (b), and number of larvae (c) in relation to developmental stage of offspring for high-density (black bars) and low-density females (white bars). $E_{1.5}$: first laid eggs were 1.5 days old; $L_{0.5}$, L_1, $L_{1.5}$, L_2, $L_{2.5}$, and L_3: oldest larvae were 0.5, 1, 1.5, 2, 2.5, or 3 days old; $L_{\text{dispersal}}$: larvae had left the brood cavity and were dispersing. Different letters above bars in (a) indicate difference at $P < 0.05$ between developmental stages for both female densities combined, while different letters above bars in (b) and (c) indicate difference at $P < 0.05$ between developmental stages within each female density treatment. Means and SE are shown.

high- and low-density females (interaction between density and developmental stage: $F_{6,245} = 1.96$ and $P = 0.07$). Larval weight did not differ between high- and low-density females ($F_{1,245} = 2.98$, $P = 0.09$). Mouse weight affected neither egg size ($F_{1,33} < 0.01$, $P = 0.95$) nor larval weight ($F_{1,245} = 0.03$, $P = 0.86$).

4. Discussion

As predicted, high-density females had overall fewer offspring (i.e., eggs and larvae) and, in particular, fewer larvae than low-density females, corroborating other studies on burying beetles [1, 6]. However, contrary to our prediction and Creighton's [1] study on *N. orbicollis*, offspring of high-density females were not larger than offspring of low-density females. This may be due to weaker selection on larval mass in *N. pustulatus* compared to *N. orbicollis*, as we have suggested previously [6]. Although *N. pustulatus* accept mice readily for reproduction in the laboratory and show the same behaviors as other burying beetles, a host shift to snake eggs has been observed [25, 26]. A clutch of snake eggs may provide breeding opportunities for more than one pair of beetles therefore reducing intensity of selection on body size.

Our experiment showed that the number of larvae on carcass of high-density females started to decline later than on carcass of low-density females, even though the number of eggs was declining for both high- and low-density females (Figures 2(b) and 2(c)). This suggests that high-density females began brood reduction at the same time as low-density females, but differed in the rate of brood reduction. After initiation of brood reduction, high-density females seemed to reduce their broods at a slower rate than low-density females, but soon after reduced their broods much faster (Figure 2(c)). Consequently, both high- and low-density females reached their final brood size when the larvae were two days old (Figure 2(c)). This pattern of brood reduction may be a bet-hedging strategy allowing high-density females, in case a failed takeover with partial

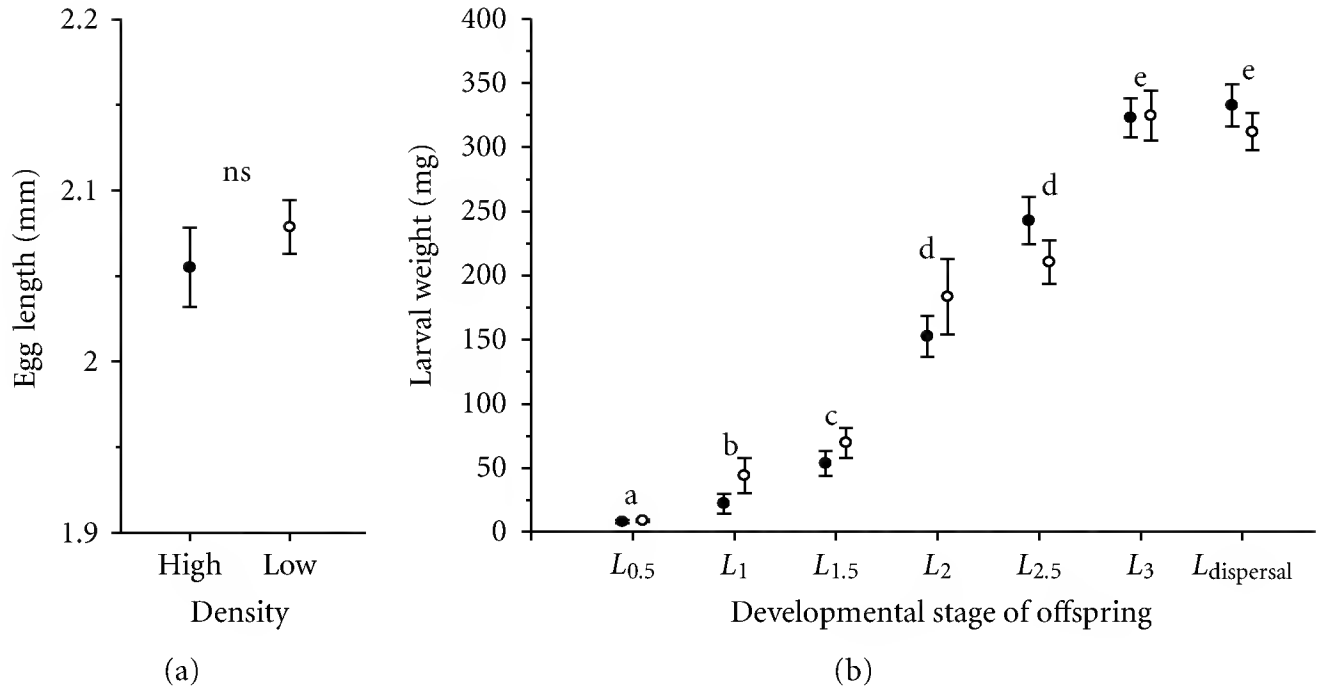

FIGURE 3: Egg size (measured as egg length) (a) and larval weight in relation to developmental stage of offspring (i.e., larval age) (b) for high (filled circles) and low-density females (open circles). $L_{0.5}$, L_1, $L_{1.5}$, L_2, $L_{2.5}$, and L_3: oldest larvae were 0.5, 1, 1.5, 2, 2.5, or 3 days old; $L_{dispersal}$: larvae had left the brood cavity and were dispersing. Different letters indicate difference at $P < 0.05$ between developmental stages for both female densities combined. Means and SE are shown.

infanticide occurs, to compensate the loss of offspring, while maximizing larval growth. Reducing the number of larvae as fast as possible may be beneficial because a larger brood size early in development can negatively affect the final size of larvae (Chris Effken and Claudia Rauter, unpublished data).

High-density females began to oviposit later and had more eggs and for a longer time than low-density females suggesting that high-density females oviposit not only later, but also over a longer time period and that larvae hatch more asynchronously. This oviposition pattern may have been caused by lower body condition of high-density females. Even though high-density females received the same amount of food per individual, behavioral interactions among high-density females may have lead to higher energy use and unequal access to food causing nutritional stress and lower body condition. In a similar experiment [6] where we maintained 1, 2, 4, or 6 female beetles in a container, we found that body weight of small females within high-density containers, decreased as density increased indicating nutritional stress. In the burying beetle *N. orbicollis* nutritional stress in form of low quality diet leads to slower ovarian development and delayed oviposition as well as to fewer and smaller eggs [27]. An alternative, but not mutually exclusive, physiological explanation for the delayed and extended oviposition by high-density females may be physiological stress in form of elevated stress hormone levels. Competitive interactions among conspecifics can cause physiological stress in form of elevated levels of stress hormones [28–32], which can adversely affect reproduction [32, 33].

Later oviposition and a longer oviposition period at high population density, may not only be the consequence of physiological stress, but also be an adaptive response to high population density with increased competition for carcass. Delayed oviposition of high-density females can have fitness benefits, if after an unsuccessful take-over attempt the losing, subdominant female stays close and lays eggs, usually earlier than the successfully defending, dominant female, near the carcass [34, 35]. Typically, the larvae of the subdominant female hatch earlier and are killed by the dominant female [35]. Larvae present on the carcass, before the female's own larvae are hatching, are killed by the female because burying beetles use temporal cues to distinguish between their own and unrelated larvae [35, 36]. Eggert and Müller [35] suggested that delayed oviposition by the dominant female in nontolerant breeding associations allows the dominant female to better discriminate between related and unrelated larvae, and preferentially kill unrelated larvae, thus increasing its fitness.

At high-density, an extended oviposition period and thus more asynchronous hatching of larvae may also be an adaptation to unpredictable and variable survival of eggs and newly hatched larvae when the take-over risk is high. Assuming that the value of a carcass decreases with decreasing carcass size similarly to the decrease in value observed in carcasses that are consumed by developing larvae [18], our results show the same patterns as findings in *N. vespilloides* [37]. With increasing carcass size, female *N. vespilloides* lay eggs in larger intervals resulting in more asynchronous hatching of larvae [37].

Our data suggest that high population density imposes nutritional and/or physiological stress on females causing delayed oviposition. However, females respond to the physiological constraints and the potentially high mortality rates of eggs and newly hatched larvae by lengthening the oviposition period and changing the brood reduction rate.

Acknowledgments

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Research Article

Observations of the Biology and Ecology of the Black-Winged Termite, *Odontotermes formosanus* Shiraki (Termitidae: Isoptera), in Camphor, *Cinnamomum camphora* (L.) (Lauraceae)

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Aspects of the biology and ecology of the black-winged termite, *Odontotermes formosanus* Shiraki, were examined in a grove of camphor trees, *Cinnamomum camphora* (L.), located at the Fruit and Tea Institute, Wuhan, China. Of the 90 trees examined, 91.1% had evidence of termite activity in the form of exposed mud tubes on the bark. There was no relationship between tree diameter and mud tube length. Mud tubes faced all cardinal directions; most (60%) trees had multiple tubes at all directions. However, if a tree only had one tube, 22.2% of those tubes faced the south. The majority (>99%) of mud tubes were found on the trunk of the tree. Approximately 35% of all mud tubes had termite activity. Spatial distribution of termite activity was estimated using camphor and fir stakes installed throughout the grove. Camphor stakes were preferred. Kriging revealed a clumped distribution of termite activity.

1. Introduction

The black-winged termite, *Odontotermes formosanus* Shiraki, is distributed throughout Southeast Asia including Burma, China, India, Japan, Thailand, and Vietnam where it is an economically important pest of crops, forests, and various wooden structures [1]. Although this species consumes wood and other cellulosic material, it does not directly use these for food. Rather, masticated cellulosic material is used to grow fungus gardens which are the termite food. Termite infestation may result in weakened trees and reduction of yield in fruit trees, or even death of tress, without proper prevention and management [1]. Foraging areas of this species range from 4.2 to 35 m; foraging territories are 13–367.9 m^2 [2]. Damage to camphor trees appears as areas of removed bark that may extend from the soil line and roots to the tree crown. These termites move up the tree by building mud tubes along the trunk and removing the bark beneath. In severe infestations, these termites can infest and hollow out branches resulting in severe limb drop especially during windy or icy weather. The mud tubes are thought to provide protection from predators such as ants and from the environment by allowing the creation of a dark and humid microclimate.

Camphor, *Cinnamomum camphora* (L.), is an aromatic tree in the laurel family (Lauraceae) that is native to Southeast Asia including southern China. It is an economically important species because it is used for construction and furniture making, as a spice in cooking, as incense, as a medicine, as an ornamental plant (pers. comm.), and as a repellent for several insect pests [3]. Extracts of camphor trees include several essential oils including camphor, linalool, and 1–8 cineole [4]. The latter two essential oils have toxic and repellent properties to a number of insects

FIGURE 1: Satellite image of Fruit and Tea Institute, Wuhan, Hubei, China. Top of the image is the north (Google Earth, version 6.0.3.2197, 2011).

including cockroaches [5, 6]. Interestingly, even with its toxic and repellent characteristics, camphor is a preferred indirect food plant of the black-winged termite [1].

The objectives of this study were to examine the distribution of black-winged termites in camphor groves and on camphor trees, determine if there is a relationship between tree size and the length of termite tubes, measure feeding preferences, and observe various aspects of termite tubing behavior.

2. Materials and Methods

This study was conducted at the Fruit and Tea Institute of the Hubei Academy of Agricultural Sciences, at Jin Shui Zha, Jiang Xia District, Wuhan, China, the Institute is situated on approximately 2.3 km^2 (230 hectares) at 30°17′53.74″N 114°08′29.76″E and an elevation of 55 m above sea level. It is located in a relatively rural area about 38 km from the urban areas of Wuhan. There are a number of brick and cement buildings, fields of tea, and orchards of kiwis, oranges, and pears. There are also several groves of camphor trees, *Cinnamomum camphora* (L.), situated between buildings (Figure 1). The camphor trees were planted in a regular grid pattern with almost equal distance between individual trees. A cursory inspection of the groves and nearby (ca. 100 m) wooden structures for the presence of tunnels and live termites and pieces of wood was conducted. Observations were made on 25-26 October 2009 with subsequent visits in March 2010 and July 2011.

Approximately one month before our observations (24–29 August 2009), pairs of fir, *Cunninghamia lanceolata* (Lambert) Hooker, and camphor wood stakes (5 by 3 by 40 cm) were installed 20 cm deep into the soil in the camphor groves (Figure 2). Fir was selected because it is a common wood species in the test area and stakes were readily available. Stakes were installed at 5 m intervals forming a uniform sampling grid. There were a total of 85 pairs of camphor-fir stakes installed in two groves (east and west). The percentage of stake locations with termite feeding, preferences in feeding between the types of wood, and the distribution of termite activity were recorded.

Measurements of termite infestations included visual inspection of 90 camphor trees located in the two adjacent groves of 45 trees each. Each tree was inspected for termite tubes and the location (trunk, roots, leaves, etc.), length (from the ground to the highest point on the trunk or branch), direction (cardinal direction), and presence of termites in each tube were recorded. The size of each tree was also estimated by measuring the circumference of the trunk 1.5 m above the ground with a tape measure. Presence of termites was determined by manually removing 5–10 cm section of each tube at several heights above the ground and noting the presence of termites.

The bark on a number of trees along the northernmost edge of each grove was covered with moss. When the moss-covered bark was removed it was evident that termites had formed a ca. 0.5 cm foraging space under the bark. There were no mud tubes; the moss-covered bark likely served the same protective functions as tubes. To determine the behaviors of these termites, sections of moss-covered bark (and active mud tubes) were removed and the areas were observed over a 2–4 h period.

Data are expressed as means ± standard errors, and differences were considered at $P \leq 0.05$. Regression and correlation analysis was used to determine the relationship between tree trunk diameters and termite tunnel length, analysis of variance (ANOVA) was used to determine differences in the orientation of termite tunnels on tree trunks, *t*-tests were used to compare wood preference, the length and areas of mud and moss-covered sections and repair times, and Kriging was used to estimate the distribution of termite activity in camphor groves. SigmaPlot 12.1 [7] was used for ANOVA, correlation, regression, and *t*-test analyses, and Surfer 10.0 [8] was used for Kriging.

3. Results

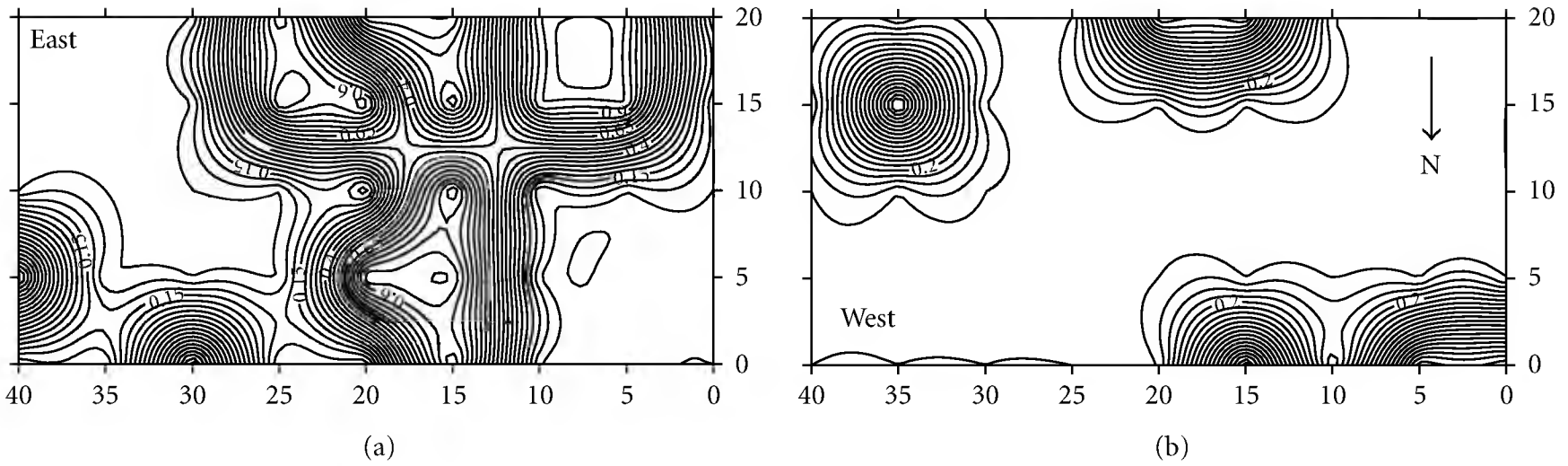
Of the 85 pairs of monitoring stakes, only 10 (11.8%) were infested by termites. There was no significant preference between the camphor (13.3%) and fir (8.9%) stakes (*t*-test; $P > 0.05$). The distribution of termite activity in each of the two camphor groves is illustrated in Figure 3. There are several concentrated areas of activity, but most stakes were not attacked. Termite activity was concentrated at both the northern and southern portions of both the eastern and western groves with relatively little activity in the center of the western grove and the southeaster and northwestern portions of the eastern grove. In contrast, virtually every tree (93.3%) had termite tubes indicating that the entire area of both groves was foraging territory of one or more termite colonies.

Camphor trunk circumference ranged from 42 to 183 cm with a mean of 109.73 ± 3.05 cm. Termite tube length ranged from 0 to 6 m with a mean of 3.31 ± 0.20 m. There was no significant relationship between camphor tree trunk circumference and the length (height) of termite tubes (regression; $P > 0.05$) (Figure 4).

Over 90% (91.1%) of the 90 camphor trees examined showed signs of current or prior infestation as determined by the presence of mud tubes. There was no obvious directional preference by these termites for the location of their tubes on tree trunks. Mud tubes were found facing all four cardinal

(a) (b)

Figure 2: Grove of camphor trees with pairs of sampling stakes: (left) pair of sampling stakes.



(a) (b)

Figure 3: Distribution of termite activity as measured by attacked stakes. (a) The eastern grove, (b) western grove. Increasing contour values indicate increasing probability of termite activity.

directions on 60% of the infested trees. Of those trees having only one tube, 11.1% of the tubes were formed on the north facing side of a tree compared with 22.2% formed on the south facing side (Figure 5), these proportions were not significantly different (ANOVA; $P > 0.05$). All mud tubes were broken open to determine termite activity. Only 34.4% of tubes were active, and there was no significant directional preference of the active tubes (ANOVA; $P > 0.05$).

A total of four mud tubes and four moss-covered areas were selected and the mud or moss removed to reveal active termites. There was no difference in the mean length of the areas removed (ca. 5.6 cm); however, the moss-covered areas (2.25 ± 0.26 cm) were significantly wider (ca. 2.2 times) than mud tubes (1.18 ± 0.10 cm) (t-test; $t = 3.3806$, df $= 6$; $P = 0.0089$). All exposed areas were repaired by the termites within 1 h. Mud tubes (18.23 ± 3.40 min) were repaired significantly faster than moss-covered areas (41.30 ± 5.51 min) (t-test; $t = 3.5656$, df $= 6$; $P = 0.0118$).

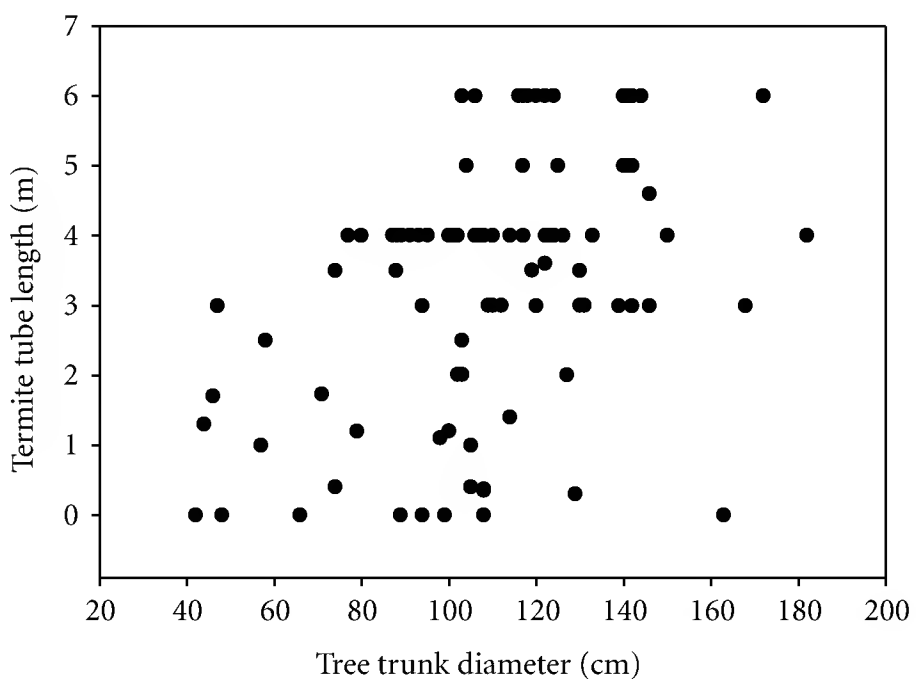


Figure 4: Relationship between tree trunk diameter measured 1.5 m above the ground and maximum termite tube length on the tree trunk.

4. Discussion

Black-winged termites were distributed throughout both adjacent groves of camphor trees. Inspection of nearby (within 100 m) trees, small wooden structures, and even relatively small (<2 cm diameter) sticks revealed the presence of these termites. Nearly all camphor trees (93.3%) in both groves had been attacked by termites as evidenced by the presence of mud tubes on the bark. The size and therefore age [3] of camphor trees were not related to termite attack. A variety of studies have shown that the concentration and composition of protective compounds, such as essential oils, in plants change with age. Also, as trees age and grow, they increase in height and circumference. There was no relationship between tree size and length of termite tubes

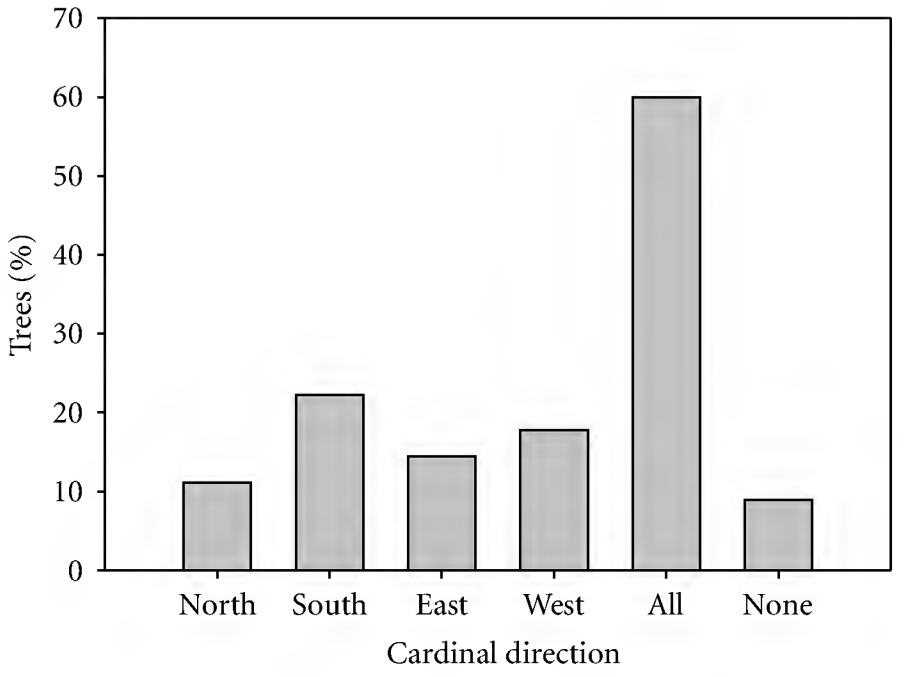


Figure 5: Percentage of trees with termite tubes oriented in each direction. All indicate that termite tubes were present in all four directions.

(Figure 4) indicating a probable lack of effective change in protective compounds with camphor tree age, or black-winged termites are not affected by these compounds.

Even though there was evidence of almost complete infestation of trees, only 11.8% of the pairs of monitoring stakes were infested. It is possible that the approximately one-month period between installation of the stakes and inspection was not sufficient for the termites to locate the stakes. It is more likely, however, that the termites had sufficient wood on the camphor trees and only attacked the stakes if a subterranean foraging tunnel directly contacted them. The condition of the wood stakes may also affect feeding activity and it is possible that the stakes had to age and decay to become as attractive as bark. Decomposing wood has greater concentrations of sucrose and more associated yeasts than sound wood [9]. In addition, fungi that are associated with wood decay are often consumed by termites [10], and these fungi are rich in urea [11]. Laboratory feeding preference studies that compare black-wing termite feeding on camphor and fir are clearly indicated. Many factors including wood density, presence of protective compounds, and concentration of glucose affect black-winged termite feeding preferences [12]. In a recent study with the black-winged termite, Kasseney et al. [12] found that solid wood consumption was inversely correlated with wood density and positively correlated with glucose concentration. Although camphor had moderately dense wood, it also had the greatest concentration of glucose among the wood species tested [12]. Perhaps feeding on bark allows these termites to avoid the dense solid wood while feeding on more glucose-rich cellulose. Unfortunately, the Kasseney et al. [12] study did not use fir as one of the wood choices. Interestingly, there was no preference between camphor and fir wood stakes in this study. However, in a similar field study about 0.5 km from the camphor groves, black-winged termites showed a decided (2 : 1) preference for camphor over fir wood stakes when the stakes were installed in a kiwi field (unpublished). Camphor is probably a preferred wood, and there is sufficient camphor in the camphor groves to interfere with relatively small camphor stakes.

The spatial analysis of the termite activity (Figure 3) indicated several areas of greater activity in both the east and west camphor groves. These areas of greater activity tended to be nearer to the northern and southern borders of the groves rather than in the center of the groves. Since many termites, and other insects, are known to follow structural guidelines in their foraging patterns [13, 14], it is possible that the greater activity along the northern and southern borders is due to the presence of cement sidewalks and brick walls that enclose the camphor groves. It is also possible that the greater activity in certain areas is a result of greater termite density in those areas or closer proximity to a primary nest. There was no correlation between termite activity in stakes and activity on trees.

The majority (60%) of trees had termite tubes facing all cardinal directions (Figure 5). If trees had only one termite tube, there was no preference for direction. Cardinal direction and therefore light and temperature exposure could affect the distribution of termite mud tubes on trees. Exposure to increased temperature could cause the conditions in tubes on one side of a tree, such as the side facing south, to become too hot for foraging workers. Increasing heat could cause an increase in desiccation or reach the critical thermal maximum. Water and temperature relations have not been studied in this species, but information on these aspects of termite physiology could help explain their micro- and macrodistribution patterns.

Black-winged termites usually construct mud or soil tubes when they are foraging above ground and exposed to the environment. Hundreds of meters of mud and soil tubes were observed in this study, most on camphor tree trunks and branches. These termites will also forage in other protected structures such as moss- and bark-covered voids. These voids ranged in size from 1 cm in width to >10 cm and could extend >1 m in length and were about 0.5 cm in height. The surfaces of these voids were very smooth and did not contain mud or soil. When a section of moss-covered bark was removed to expose the void, termite workers immediately began to seal the exposed areas by bringing soil and mud and depositing it along the exposed area. Rather than resealing the entire exposed area, the termites rapidly constructed a mud tube that provided an enclosed foraging corridor. Termites required about twice the time to repair exposed moss-covered bark foraging areas as they did to repair similar sized damage to mud tubes. It is likely that mud tubes could be repaired more quickly because there were mud tubes close to the broken area and termites removed some of this mud to repair the break. Termites foraging under moss-covered bark probably did not have ready access to mud or soil and had to transport it from the ground up to the broken area. The rapid repair of all broken foraging tubes and areas indicates the importance of these structures to the biology of the black-winged termite and the size of their colonies.

In conclusion, the black-winged termite is an important pest of camphor trees particularly in dense groves. Most trees in an area will be attacked, but sampling studies that rely

on wooden stakes may require extended periods to yield results. This termite builds mud tubes on all age and size trees and shows no preference for the direction of these tubes. Further studies on the physiological ecology of this species will provide insight into foraging and above-ground tubing activities. Additional studies on the distribution patterns of this species will aid control strategies by accurately providing locations for insecticidal bait placements.

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Research Article

Association of Climatic Factors on Population Dynamics of Leaf Roller, *Diaphania pulverulentalis* Hampson (Lepidoptera: Pyralidae) in Mulberry Plantations of Sericulture Seed Farm

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The production of quality mulberry leaf and subsequent production of quality silk is hampered due to the incidence of various insect pests. The present study analyses the population dynamics of *Diaphania pulverulentalis* (leaf roller), a serious pest of mulberry in a sericulture seed farm. The results indicated that maximum population buildup of the pest was recorded during rainy season. High humidity coinciding with low temperature because of southwest and northeast monsoon was conducive for breeding and multiplication of the pest. Correlation studies revealed that there was a significant negative correlation between increase in temperature and pest infestation. All other weather factors recorded from the study location have a positive correlation with incidence of the pest. The regression model developed also supported the relationship between the pest population buildup and weather factors.

1. Introduction

The existence and prosperity of sericulture industry depends upon the production of quality silk. For production of quality cocoon and silk, silkworm larva should feed with quality mulberry leaves, which is the exclusive food plant of the *Bombyx mori* L. The process of mulberry leaf production is suffering due to the attack of various insect and noninsect pests. Being, a perennial blooming and high biomass producing plant with luxuriant growth under irrigated condition of recommended package and practices often leading to the breeding and multiplication of various pests. This condition leads to rapid pest proliferation, which resulted qualitative and quantitative loss of mulberry plants and ultimately low productivity in sericulture.

Diaphania pulverulentalis is one of the devastating pests of mulberry in southern states, namely, Andhra Pradesh, Karnataka, and Tamil Nadu. The pest reported from different locations of India (Nagaland [1]; Jammu [2]; Kashmir [3]; Punjab [4]) and the incidence reported from sericulture countries like China and Japan [5]. The early stages, larvae of the pest inhabits apical succulent portion of the shoot and leads to its destruction, resulting in stunted growth thereby affecting considerable decline in leaf yield about 12.8% with an average incidence of 21.77% [6, 7]. The incidence of pest occurs during June–February months and causing severe damage to young plantation and it affects severe loss in tender chawki leaves, which is very much essential for young age silkworm larvae. The infestation and population buildup of a pest is greatly affected by weather parameters like temperature, relative humidity, rainfall, and so forth. Climatic factors also have a dominating influence on the survival, development, and reproductive capacity of insect pests. In recent years, many pests and diseases have been reported to be the major limiting factors affecting the production of mulberry leaves due to intensive cultivation practices and indiscriminate use of nitrogenous fertilizers and pesticides. There is a change in the insect pest scenario

in mulberry due to changes in climate and agro ecosystem. Besides, the above practices, the use of high-yielding varieties and monoculture also invited pest problems and minor pests have become major ones. Several workers studied the seasonal incidence of the pest and higher infestation was reported during October to February months in Krishnagiri [8, 9] and October to December in Salem areas of Tamil Nadu [10]. Since an understanding of the population dynamics of the pest species is vital for evolving appropriate and timely management strategies, the study was conducted to estimate the seasonal population variations of *D. pulverulentalis* in relation to climatic conditions. The present study was taken up in mulberry plantations of a Sericulture Basic Seed Farm about the population dynamics of leaf roller and seasonal incidence of the pest. The study results may bring out appropriate ecological requirements, particularly weather factors like temperature, relative humidity, and rainfall, that play a vital role in multiplication and distribution of insect pests, and these factors will give momentum to research on pest management strategies.

2. Methodology

2.1. Study Location. The study area P3 Basic Seed Farm is located in Mysore, Karnataka, India and it was situated at 12° 18′N 76°39′E and has an average altitude of 770 meters (2,526 ft). It is in the southern region of the state of Karnataka and spreads across an area of 128.42 km^2. The summer season is from March to middle of June, followed by the monsoon season from the middle of June to October and the winter season from November to mid-February. The highest temperature recorded in Mysore was 38.5°c (101°F) and in winter, temperatures as low as 9.6°C (49°F). The average annual rainfall received was 798.2 mm. The parental silkworm rearing at basic seed farm has to be organized in a manner to ensure that silkworm rearing is free from disease menace. Besides, these various cocoon characters and fecundity, have to be according to the norms fixed for each race. The mulberry garden has to be managed with due care and right input has to be added to produce healthy and succulent leaf.

2.2. Study Material. Diaphania pulverulentalis is a major pest of mulberry and it belongs to family Pyralidae. It was reported as a major pest of many agricultural and horticultural crops and lay about 80–150 eggs on tender apical leaf buds of mulberry and hatching generally take place within 2-3 days. The larval stage, which causes severe damage to apical portion of the plant and the period, completes 8–12 days. The pupation takes place in the soil and the period takes 7–9 days. The adult longevities of 7–12 days and 9–14 days were recorded for male and female respectively. The target area of the leaf roller is the apical portion of the mulberry shoot. The young caterpillar binds the leaflets together with silky secretion, settles inside, and devours the soft tissues of the leaf surface and so the pest is popularly called as leaf roller or leaf webber. The web protects the larvae from natural enemies and even spraying of insecticide and killing the target pest become difficult. Late instar caterpillars feeds, on tender leaves and cause severe damage.

2.3. Host Plant. Mulberry (*Morus* sp.) is exclusive food for economically important silkworm (*Bombyx mori* L.), which is cultivated in tropical and temperate countries of the world. In India, it is cultivated mostly in the tropical region, evergreen throughout the year. Due to continuous crop improvement in the field of mulberry breeding and genetics, many new mulberry varieties have been evolved, much better than the local varieties in respect of quality and quantity of leaf produced. At the same time, these improvements paved ways for their susceptibility to be attacked by pests and diseases.

2.4. Sampling and Statistical Analysis. The mulberry garden of the basic seed farm was divided into six sub plots, and pruning schedule was adjusted to conduct six silkworm crops annually. Luxuriant growth and availability of different qualities such as tender, medium, and coarse leaves are assured throughout the year. For observations of pest incidence, ten plants from each subplots were selected at random. Thus, 60 plants were observed every week for a period of three years. The incidence of *D. pulverulentalis* was observed and the number of insects on each selected plant was counted and the total pest infestation was calculated. The data were tabulated in different months and the experiment was continued for three years (2008-2009, 2009-2010 and 2010-2011) and leaf roller infestation for each month was calculated. The weather factors like maximum temperature, minimum temperature, maximum humidity, minimum humidity, and rainfall data were recorded from the study location every day and month wise data were tabulated. The influence of weather factors on population density of leaf roller was analyzed by a simple correlation study and coefficients were worked out for a period of three years. In order to investigate the simultaneous influence of the climatic factors on pest incidence, a multiple linear regression analysis was accomplished.

3. Results and Discussion

3.1. Population Dynamics. The percentage of infestation varied in different months of the year, with a seasonal influence and a difference in the insect population in each year was observed. The three-year observation for the incidence of leaf roller indicated that during the year 2008-2009, mild infestation was started in the month of April (0.56%) and an increase was recorded on subsequent months and after the onset of monsoon rain, the infestation was further intensified. The larval establishment severe damage was found during the months of June–October and the peak infestation was observed in September (39.56%). After October infestation was gradually declined, and again a slight increase was recorded during December (17.86%). This may be due to the unexpected cyclonic rainfall received, which resulted increase in relative atmospheric humidity and a further population buildup of the pest. During January, the infestation was lowest (4.3%) and subsequent two

Table 1: Leaf roller infestation during different months (percentage).

Years	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan	Feb.	Mar.	Range of infestation
2008-09	0.56	12.45	32.56	38.56	31.78	39.56	38.45	12.78	17.86	04.3	0	0	0.56–39.56
2009-10	0.36	16.45	32.66	39.46	35.78	40.16	39.35	11.78	06.76	02.3	0	0	0.36–40.16
2010-11	6.56	12.15	31.26	39.23	31.12	38.56	37.45	14.78	28.86	05.2	0	0	05.2–39.23
Average	2.49	13.68	32.20	39.08	32.89	39.43	38.42	13.11	17.83	03.93	0	0	2.49–39.43

Table 2: Association of climatic factors with leaf roller infestation during 3 years.

Year	Maximum temperature	Minimum temperature	Maximum humidity	Minimum humidity	Rainfall
2008-09	−0.473**	0.328*	0.594**	0.584**	0.396*
2009-10	−0.548**	0.347*	0.741**	0.835**	0.378*
2010-11	−0.666**	0.411**	0.566**	0.802**	0.410*
Pooled	−0.562**	0.362*	0.633**	0.740**	0.395*

** Significant at 1% level; * Significant at 5% level.

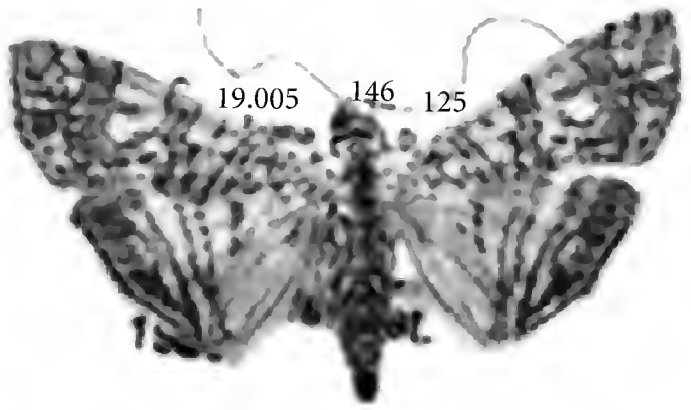
months (February and March) infestation was zero. High temperature and low humidity prevailing during February–March in the study location created an unfavorable ecological condition for the development and population buildup of leaf roller. Studied the infestation of leaf roller in dry areas of Chamaraj Nagar, Karnataka (India) and found that the infestation was recorded maximum during rainy season and least in summer [11]. However, some workers reported infestation was high during January [12, 13]. During 2009-2010 almost the same line of observations was made and the infestation was started at a low level during April (0.36%) and it was recorded slightly higher in succeeding month (16.45%) when compared with the previous year (Table 1). This may be due to the early showers received during the end of April. The trend was followed similar to the previous year and the infestation was increased during subsequent months until the commencement of the winter season. The peak infestation was recorded during September (40.16%) and from November onwards infestation was marginally reduced. There was a rapid buildup of the pest population in the middle of each year beginning with June and ending in December. The influences of climatic factors on the incidence pest during different months are summarized in Figure 1. The peak relative humidity that occurred during August–September months influenced the highest incidence of the pest. The gradual decline in the population of leaf roller in January onwards was in consonance with the drop in the relative humidity.

During the year 2010-2011 the infestation of the leaf roller was started little early in April (Table 1) and higher infestation was recorded when compared with previous years (6.56%). This is also may due to early rain fall received at the end of March and as a result a favorable climatic condition was created for population buildup of leaf roller in the field. Similar, to previous two years the population was recorded peak during June–October and highest infestation was recorded during the month of July (39.23%). After October, during the beginning of the winter season there was a decline in population and again a slight increase was observed during the month of December (28.86%) due to the unexpected cyclonic rainfall.

3.2. Correlation between Incidence of Pest Population and Abiotic Factors. The infestation of leaf roller showed a great sensibility to weather variations occurring over the period studied. A significant and negative correlation was observed between the percentage of leaf roller infestation and maximum temperature recorded from the study location ($r = -0.473$ in 2008-2009, $r = -0.548$ in 2009-2010, and $r = -0.666$ in 2010-2011). The study results were in accordance with the results of earlier workers [12]. The correlation coefficient data indicated that when the temperature rises up during the hottest months of February–April the infestation was recorded very low or nil (Table 2). However, a positive correlation was recorded between the infestation and minimum temperature. Similarly, correlation studies were worked out between infestation of leaf roller and maximum humidity, minimum humidity, and rainfall recorded during different months for a period of three years. All correlation coefficient (r) data showed a positive correlation between the two variables. Significant ($P \leq 0.1$) correlation was observed between maximum humidity and leaf roller infestation (0.594 in 2008-2009, 0.741 in 2009-2010, and 0.566 in 2010-2011). The analysis also revealed a positive and highly significant correlation between leaf roller infestation and minimum humidity (0.584 in 2008-2009, 0.835 in 2009-2010, and 0.802 in 2010-2011). The results indicated that weather factors have greatest importance upon the population fluctuation of leaf roller. Quite a few workers reported similar results from Tamil Nadu climatic conditions [13, 14]. The rainfall data of the study location also significantly correlated with leaf roller infestation (0.396 in 2008-2009, 0.378 in 2009-2010, and 0.410 in 2010-2011). The results of the analysis showed that due to continuous rain (June–October) naturally increased relative atmospheric humidity as well as percentage of soil moisture. It created a suitable condition for the luxuriant growth of mulberry and

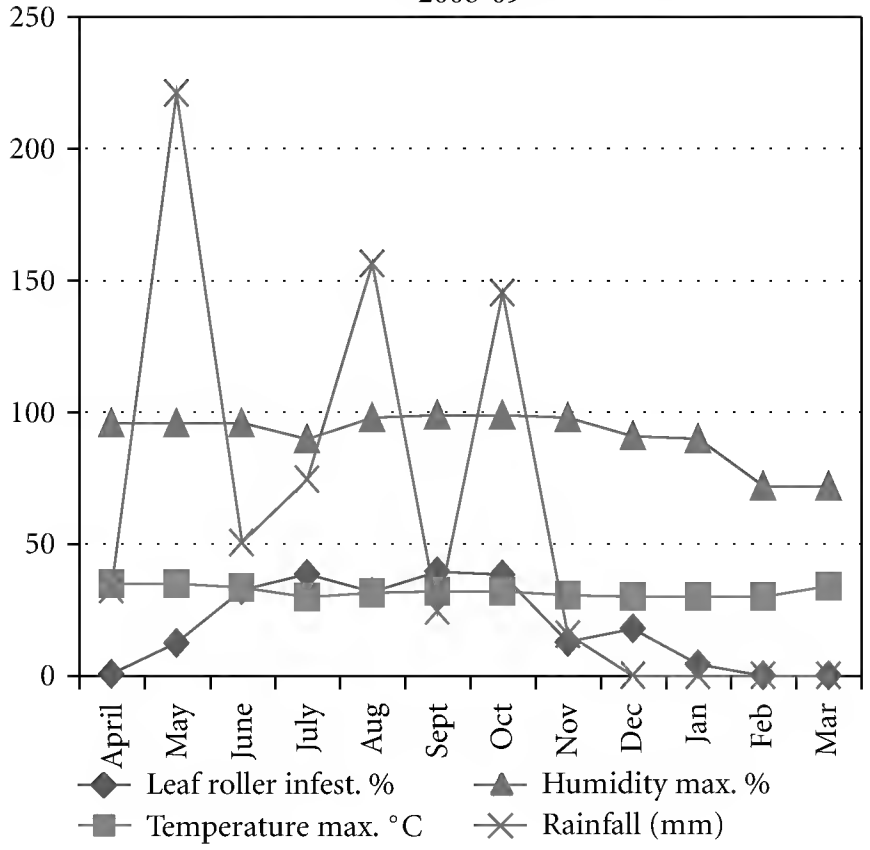
Larva of leaf roller

(a)

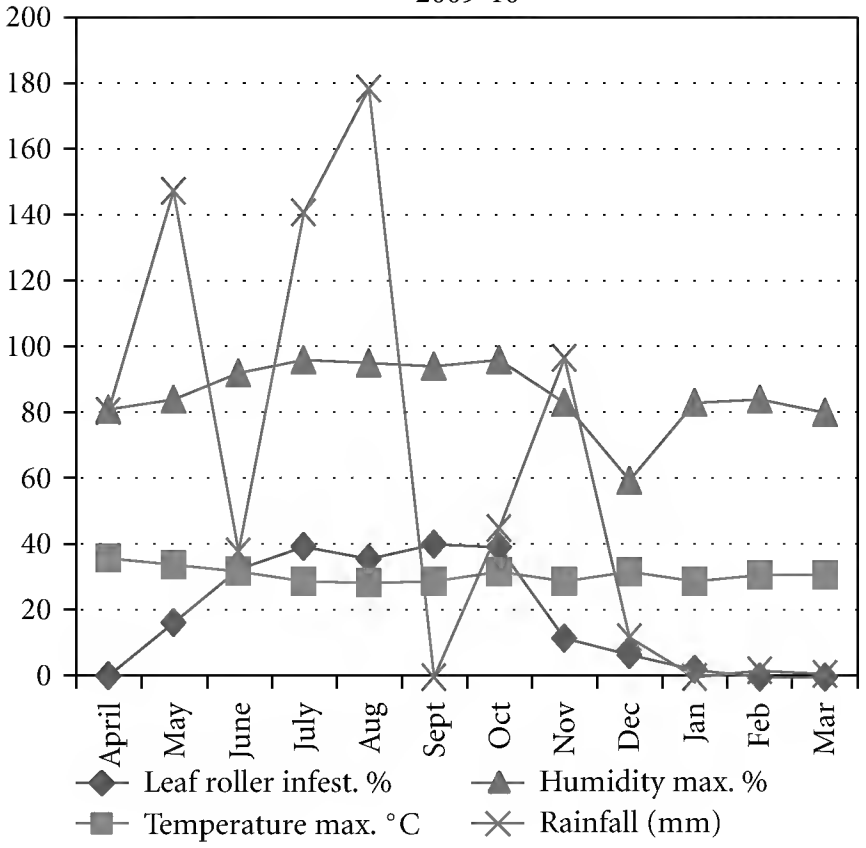


Adult moth of leaf roller

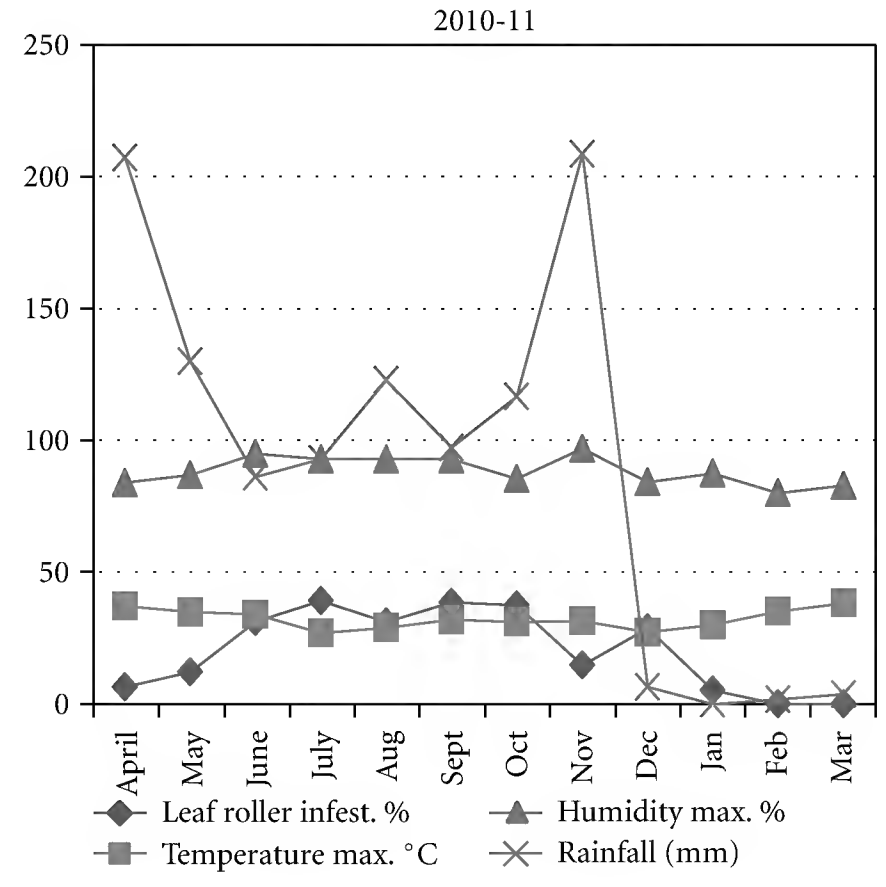
(b)



(c)



(d)



(e)

Figure 1: Association leaf roller (*Diaphania pulverulentalis*) infestation and abiotic factors of the study location (3-years period).

Table 3: Regression model developed for Leaf roller infestation.

Years	Maximum temperature ($X1$)	Minimum temperature ($X2$)	Maximum humidity ($X3$)	Minimum humidity ($X4$)	Rainfall ($X5$)
2008-09	$Y = -64.291+1.415X1$ $R^2 = 0.171^*$	$Y = 17.160 - 0.107X1$ $R^2 = 0.190^*$	$Y = 72.168 - 0.998X3$ $R^2 = 0.350^*$	$Y = 0.257 - 0.363X4$ $R^2 = 0.281^*$	$Y = 15.765 - 0.123X5$ $R^2 = 0.003$
2009-10	$Y = -99.537+2.605X1.$ $R^2 = 0.120^*$	$Y = 17.370 - 2.012X2$ $R^2 = 0.122^*$	$Y = 87.908 - 1.245X3$ $R^2 = 0.541^{**}$	$Y = -49.670 - 1.458X4$ $R^2 = 0.694^{**}$	$Y = 12.546 - 0.101X5$ $R^2 = 0.146$
2010-11	$Y = -110.883 + 2.798X1.$ $R^2 = 0.441^{**}$	$Y = 06.104 - 1.492X2$ $R^2 = 0.173^*$	$Y = 122.281 - 1.661X3$ $R^2 = 0.324^*$	$Y = 12.542 - 0.321X4$ $R^2 = 0.641^{**}$	$Y = 14.431 - 0.165X5$ $R^2 = 0.055$

** Significant at 1% level; * Significant at 5% level.

availability of more nutritious and succulent leaves naturally favored a population buildup of leaf roller. Same results were observed and concluded the peak leaf roller infestation occurs both in southwest and northeast monsoon period, and showed that rainfall, and humidity were conducive for the multiplication of the pest [15]. However, maximum infestation was reported during the winter season in Kerala, (India) climatic condition [16]. These reports make it clear that there was a linear relationship of decreasing atmospheric temperature and increasing relative humidity with increased pest incidence. The significant correlation found between the leaf roller infestation and abiotic factors definitively help to develop a predictive model, by which the outbreak of this pest could be known in advance, so that timely control measures can be taken up to curtain the problem. The climate change can affect the response of insect pests to the host plants, although it is difficult to predict the impact of climate changes on various insect pests; the overall response is dependent on the impact of climate change on the insect, plant host and natural enemy relationship.

3.3. Regression Model. Linear regression analysis revealed that measured environmental variables have significant effects on leaf roller pest densities ($P < 0.05$) confirming results of correlation analysis. The importance of relative humidity and pest incidence for explaining significant portions of the independent variable for densities of leaf roller is also emphasized. The stepwise regression analysis constructed to investigate the abiotic factors contributed the most to the variance of the leaf roller population (Table 3). Regression analysis showed that minimum humidity recorded from the study location significantly contributed to the population variation of the pest (28% in 2008-2009, 69% in 2009-2010, and 64% in 2010-2011). Similarly, maximum humidity also significantly and positively contributed for variations in the population buildup (35% in 2008-2009, 54% in 2009-2010, and 32% in 2010-2011). Analysis also showed that the maximum temperature significantly and negatively contributed to the variation of leaf roller population (17%, 12%, and 44% for 2008-2009, 2009-2010 and 2010-2011 resp.). The optimum regression model indicated the strong influence of maximum humidity and minimum humidity on variation in pest population in mulberry plantation. The forecast model can be used to predict the initiation and "red alert" season of the pest attack. This serves as a scale for the sericulturist to adopt effective crop protection measures at the appropriate time.

Timing of the insect pest appearance varies, depending on differences in temperature throughout the years, which makes the pest's forecasting and management difficult. One way to promote our understanding of the phrenology of *Diaphania pulverulentalis* is to develop a population dynamics model that explicitly incorporates temperature-dependent development. Indeed, models for temperature-dependent development of insect pests have been widely used as decision-support tools to improve the efficiency of pest management.

Forecasting the peak abundance of pest and diseases in advance helps in timely management of crop pests. The correlation and multiple regression analysis clearly showed the importance of weather factors in the pest incidence. Among the models regression can be used for forecasting the pest and also these models can be utilized in agroadvisories after validating with individual seasonal data.

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Research Article

Declining Bark Beetle Densities (*Ips typographus*, Coleoptera: Scolytinae) from Infested Norway Spruce Stands and Possible Implications for Management

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The eight-toothed spruce bark beetle (*Ips typographus*) is the most serious insect pest in Central European forests. During the past two decades, extreme meteorological events and subsequent beetle infestations have killed millions of cubic meters of standing spruce trees. Not all the infested stands could be cleared in time, and priorities in management had to be set. Natural or man-made buffer zones of about 500 meters in width are frequently defined to separate differently managed stands in Central Europe. While the buffer zones seem to be effective in most of the cases, their impact has not been studied in detail. Beetle densities were therefore assessed in three case studies using pheromone traps along transects, leading from infested stands into spruce-free buffer zones. The results of the trap catches allow an estimation of the buffer zone influence on densities and the dispersal of *Ips typographus*. Beetle densities were found to decrease rapidly with increasing distance from the infested spruce stands. The trap catches were below high-risk thresholds within a few hundred meters of the infested stands. The decrease in catches was more pronounced in open land and in an urban area than in a broadleaf stand. Designed buffer zones of 500 m width without spruce can therefore very probably help to reduce densities of spreading beetles.

1. Introduction

The eight-toothed or European spruce bark beetle (*Ips typographus* L.) is, from an economic and ecological point of view, one of the most serious forest pests in Europe [1, 2]. It mainly attacks Norway spruce (*Picea abies* (L.) Karst.). Poorly textured, even-aged, pure stands of spruce are particularly vulnerable. Suddenly exposed spruce on the edges of stands are highly attractive to bark beetles. The pest outbreaks, such as after windstorms or droughts, are likely to trigger dieback of host trees on a large scale within several years. This happened for example after the years 1983 and 1984, when heavy thunderstorms swept over the Bavarian National Park, resulting in an abundance of uncleared wind-felled trees. The following bark beetle outbreak spreads rapidly to the surrounding stands due to favourable weather conditions. Managed spruce stands bordering the park were also affected [3]. In wide parts of Central Europe, the infestations in the years after the storm "Lothar" in 1999 and again after the hot and dry summer of 2003 were similarly impressive. In Switzerland, the spruce bark beetle killed a volume corresponding to 40% of the spruce increment that grew in the period from 1999 to 2005 [4].

The mass attacks posed a great challenge for the forest services and forest owners. Logistic problems were often the reason why conventional control measures could not be organized in time. A certain amount of wind-felled spruces and infestation spots had to remain uncleared. That is why forest owners and the local authorities have to set management priorities according to what functions the forest has and what type of landscape is involved [5]. A considerably high level of *Ips typographus* infestation can often be observed in disturbed and unmanaged spruce stands such as those in protected areas [6], which leads to a greater beetle pressure on neighbouring managed stands. If managed stands border unmanaged ones, the edges of

cleared infestation spots often become reinfested with bark beetles, due to a high beetle pressure from the uncleared spots. A clever choice of natural spruce-free borders like mountains, meadows, villages, lakes, or broadleaf stands help to minimize such buffer-zone problems between managed and unmanaged spruce stands.

Because the distance of *I. typographus*' active dispersal flights is believed to vary within a few hundred meters, a buffer zone of 500 meters between uncleared infested stands and managed forests is recommended in forest practice. If a spruce-free belt cannot be selected, intense monitoring takes place within the buffer zone and beetle attacked trees will be felled and removed or debarked. Within such a zone, no infested spruce is tolerated.

The safety distance of 500 meters is based on the experience of field foresters and the results of several studies. The GIS-based study of Wichmann and Ravn [7] showed that the short distance dispersal of beetles is less than 500 meters since at a distance of more than 500 meters an area with wind-thrown or infested trees has no significant influence on a beetle population. Similar findings were obtained in the Bavarian National Park. Despite heavy infestation pressure, Heurich et al. [3] found that a reinfestation in the peripheral zone could be limited to a distance of 500 meters. Becker and Schröter [8] showed that standing attacks markedly decreased 500 meters away from the primary infestation spot. On the other hand, Duelli et al. [9, 10], who investigated the migrational behaviour of the spruce bark beetle and their flight patterns outside forests or outside spruce stands in situations with a low beetle impact, did not observe such a clear decrease. In contrast to these dispersal studies in areas with a low beetle density, in the present study, we analyzed distance-dependent beetle captures emanating from stands with high beetle densities and infestations on standing spruce. In particular, we wanted to clarify whether the postulated minimal width of a buffer zone of 500 meters is reasonable and if it can be recommended for control strategies in Switzerland. What is relevant in this case is that the impact decreases sufficiently within this distance to reduce the infestation risk for neighbouring stands. So the goal was not to ascertain the origin of all the captured beetles, but rather to find out whether there is a distance-dependent decrease in beetle pressure emanating from known infestation spots.

2. Materials and Methods

2.1. Study Areas. For our study, we focused particularly on forest districts with a high beetle infestation in the previous year, which had resulted in considerable compulsory fellings, infestations spots, and beetle catches in pheromone traps. The research areas were chosen to include Norway spruce stands bordering large open areas or pure broadleaf stands. No experiments within spruce stands were conducted because it would not have been possible to specify how many of the beetles caught in the traps spread from the initial infestation spots without marking and releasing beetles.

All transects started at spots or stands that had been infested by *Ips typographus* in the previous year. In all three study areas, the *I. typographus* populations were bivoltine as the altitude varies between 400 and 700 m a.s.l. The preliminary transect of 2006 led to a city (Figure 1(a)) and the 2008 transects led to an open area (Figure 1(b)), and a broadleaf stand (Figure 1(c)). The transect in the city of Zurich (8°33′51.56″/47°23′4.35″) was approximately 2,000 meters long. It started on the Zurichberg (Figure 1(a)) in a mixed stand with several uncleared infestation spots within a few hundred meters and then went southwest towards the city centre (main station). It crossed an urban area with green spaces and gardens, where some scattered Norway spruce occur as ornamentals.

In Oberbüren (9°10′36.24″/47°26′23.74″) in Canton St. Gallen, transect b was nearly 1,000-meter long (Figure 1(b)). It led from west to east with an uphill slope of just 3%, away from a formerly infested Norway spruce forest edge to an open area (9°13′44.1″/47°26′3.06″). The traps were set up along the talus of a motorway several dozen meters away from traffic. The Norway spruce stand had scattered infestations in the previous years on an area of nearly 20 hectares.

The 1,320-meter-long transect c at Renedaal near Reutenen (9°2′47.65″/47°39″8.32″) in Canton Thurgau (Figure 1(c)) led northwest with a downward slope of 7% into a broadleaf stand (9°2′0.52″/47°39′34.87″) of about 45 hectares. This transect started in a recently cleared infestation spot within a mixed stand of roughly 2.5 hectares containing Norway spruce. The transect went through an almost pure broadleaf stand (mainly European beech, *Fagus sylvatica*) with only a few single spruce trees, silver firs (*Abies alba*), and Scots pines (*Pinus sylvestris*), all without any bark beetle infestations. The second half of transect c followed a forest road.

2.2. Arrangement of the Traps. Four to seven pairs of black slit traps were set per transect. The lure in all traps was Pheroprax of BASF, a synthetic aggregation pheromone, based on (S)-*cis*-verbenol and methylbutenol. The traps were arranged pairwise to average out the influences of the microlocations and eventual lure irregularities. The trap arrangement in Zurich was somewhat different from the others. Only four pairs of traps were used. The distance between the single traps within a pair varied in this case between 10 and 90 meters, with the traps often set up on buildings (roofs, terraces) to prevent vandalism.

In the two 2008 experiments, a pair of traps was positioned at each sampling point with the upper end of the trap at breast height (1.3 meter). The trap pairs had a gap of approximately 10 meters between the single traps. The front side of the trap was arranged so as to have as much antemeridian and midday sun as possible. Furthermore, care was taken to place the traps at homogeneous microlocations to ensure catch conditions were as uniform as possible.

The preliminary experiment in Zurich (transect a) was performed in a shortened period from June 19 to August 15, 2006. It did not cover the whole flight activity of the overwintered *Ips typographus* generation. The traps in transects b and c were installed on April 23 and 24, 2008, and immediately baited with the attractants. The traps were last emptied and then removed on September 11, 2008. Exactly

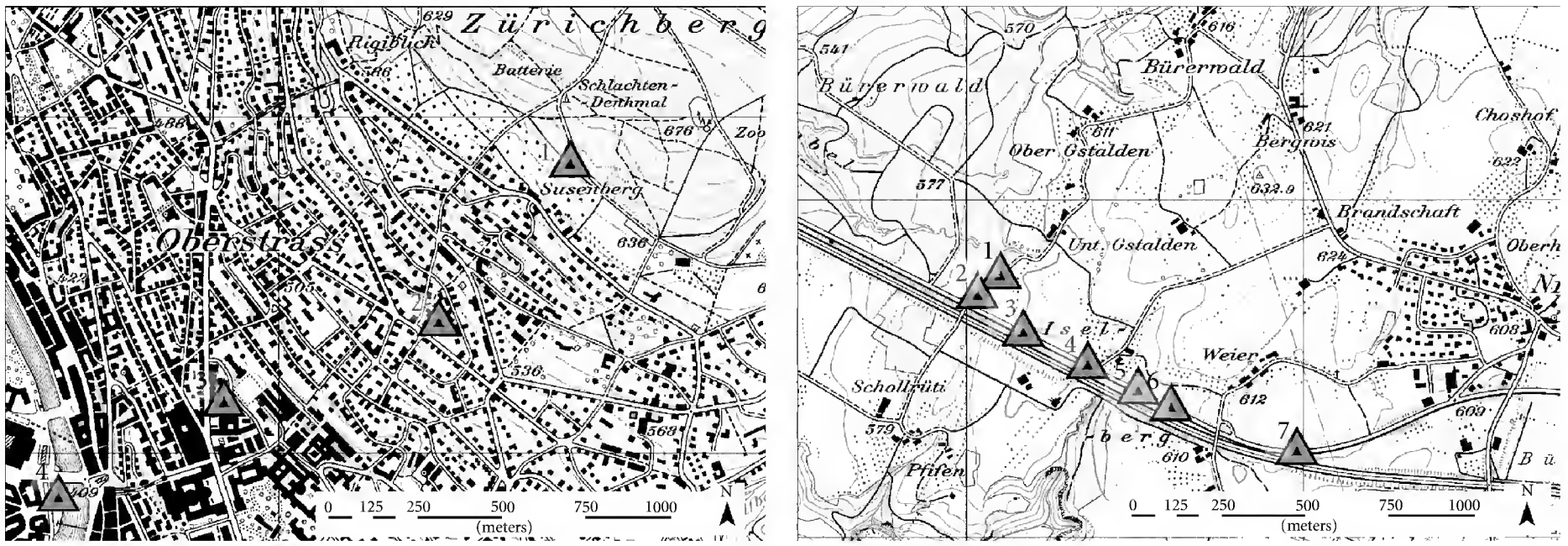


FIGURE 1: The design of the trap transects in (a) the city, (b) the open land, and (c) the broadleaf stand. All transects start in stands with infestations spots. (Maps: VECTOR 25, swisstopo (JA100118), 2010.)

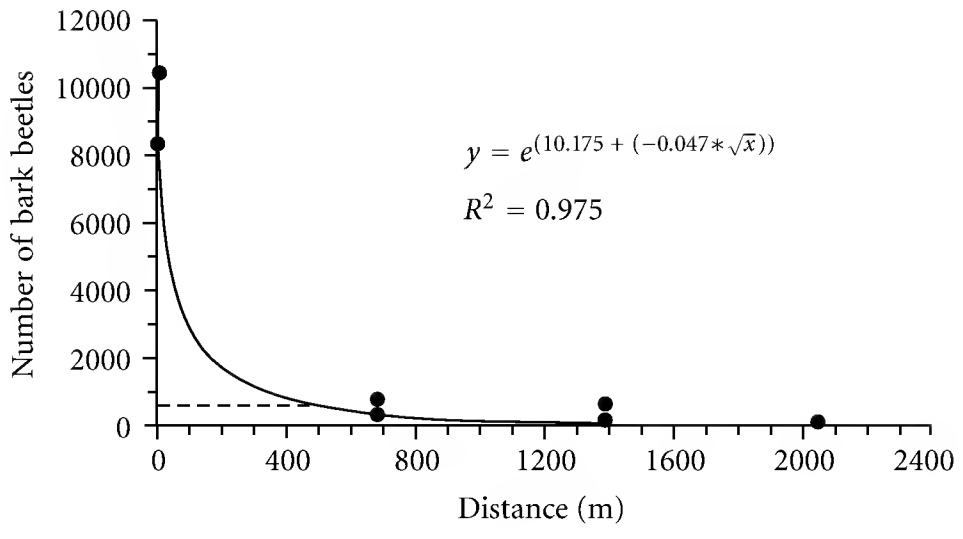
every 14 days, the traps were inspected, emptied, and the beetles counted.

2.3. Data Analysis. The data were plotted with the program DataDesk (Version 6.3, Data Description, Inc., Ithaca, NY), and a curve was fitted to the points. For the "fall-off of density with distance," the equation of the type $y = a + bx$ was applied, or rather an improvement on it: $y = e(a + bxc)$, according to Southwood [11], where x is the number of beetles and y is distance from the infestations. The curve with the best fit was that of Hawkes [12] of the type $y = e(a + b\sqrt{x})$. Duelli et al. [10] used this formula to represent the spread of freshly emerged beetles. This equation assumes that the lengths of individual moves are not haphazard and random [11]. The coefficient of determination R^2 was calculated to describe the correlation. For the whole analysis, the sample pairs were not pooled. Instead, the single traps within the pairs were used.

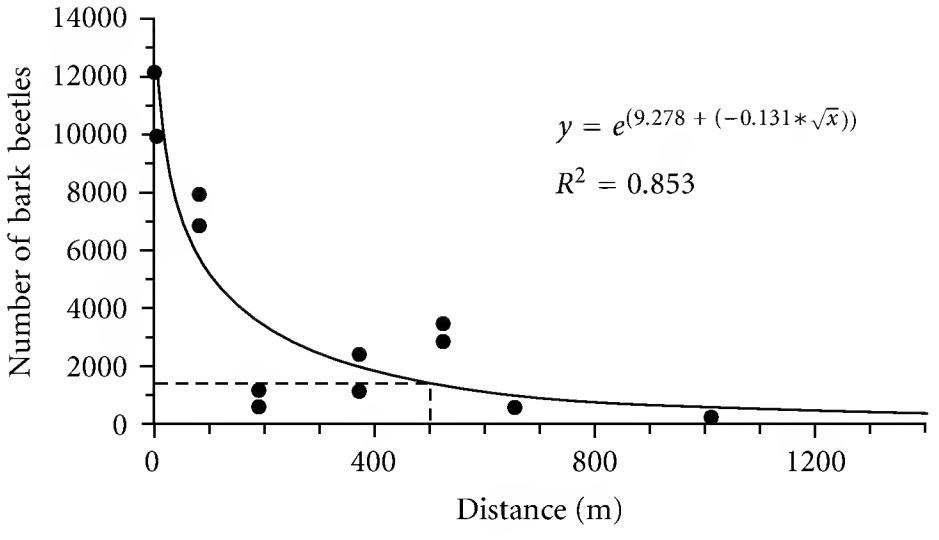
3. Results

3.1. Number of Beetles Caught. All pheromone traps caught at least a few specimens of *Ips typographus*, and the catches decreased with the distance to the infested stands (Figure 2). The average number of the catches per trap and transect ranged between 2,500 beetles in the city (transect a), 3,600 beetles in the open area (transect b), and 11,300 beetles in the broadleaf stand (transect c). Because all transects lead away from former infested stands or spots, it makes more sense to compare the trap catches in or near the infestation area only. This means the catches at the starting points of the transects are higher and demonstrate a considerable infestation level. At the starting point of transect c in the broadleaf stand, the catches (25,300) are about twice as high as at the starting point of transect b in the open area (11,200). The catches at the starting point of the city transect a (9,400) are below this value, but they were caught during a shorter trapping period that excluded the first flight in spring, which is when a high flight density of beetles is usual [13].

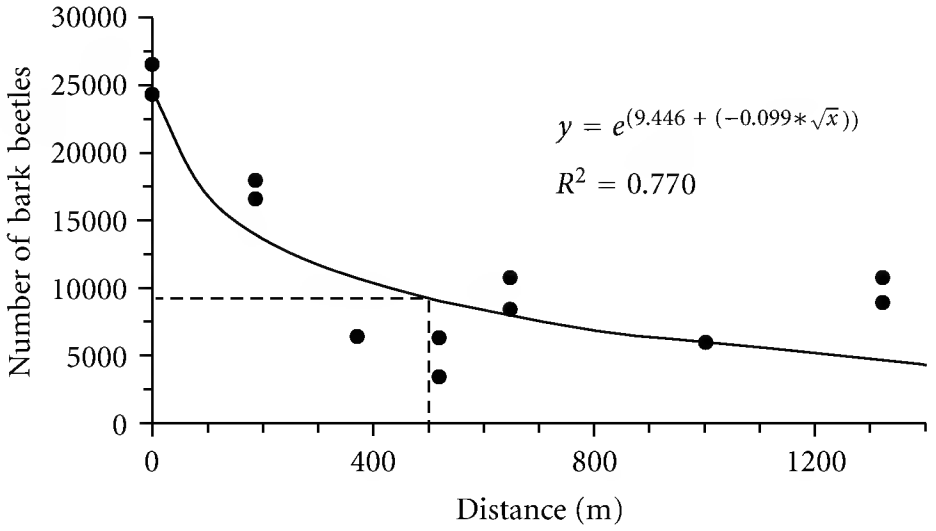
3.2. Beetle Densities in the City, Open Land, and Mixed Deciduous Stand. Along all transects, a clear decrease in the numbers of bark beetles individuals was monitored (Figure 2). In the city of Zurich, along transect a, the calculated curve of the catches declines quickly: after 40 meters, the beetle density was only half that in the forest and after 680 meters



(a)



(b)



(c)

FIGURE 2: Trap catches of *Ips typographus* in the city (a), in the open land (b), and in the broadleaf stand (c), starting from infested spruce. The calculated beetle density 500 meters from the infestation spot is marked.

only 4%. At a distance of 500 meters, it was calculated that 572 beetles would have been caught per trap, that is, only about 7% of the initial trap catches in the forest (Figure 2(a) and Table 1). In this preliminary study in 2006, the fast decrease in beetle density was possibly a consequence of having no traps between the infested stand with the first trap pair and the second trap pair already 680 meters away. Therefore, a goal of the other two case studies from 2008 was to fill in these gaps by including results from traps set closer to the infested stands.

The numbers of catches in the open area (transect b) declined also rather fast. The density dropped after just 200 meters to only one-fourth (Figure 2(b)). A second tiny peak at about 500 meters distance from the infestation point was probably due to a small and isolated spruce group bordering the open area to the south. This could have been a source of irregularity, which resulted in an increased number of catches at this trapping point. According to the applied regression formula by Hawkes [12], we estimated that 1,383 beetles would have been caught at a distance of 500 meters.

After the first 400 meters, the catches in the broadleaf stand (transect c) reached only one-fifth of the number of catches of the first trap pair. But at a distance of 500 meters, we calculated there would have been still 9,173 beetles. Towards the end of the transect, the catches even out to a level of about 7,500 beetles (Figure 2(c)). It can be assumed that this reflects the basic flight density level in this 45 hectare broadleaf stand that bordered forests with infested spruce. The population density seems to have been much higher in the broadleaf stand (c) than in the open area (b).

TABLE 1: Calculated values for the catches (beetles per trap) in (a) the city, (b) the open land, and (c) in the broadleaf stand transect.

	(a) City	(b) Open land	(c) Broadleaf stand
Catches at 500 m (n)	572	1,383	9,173
Catches at 500 m (%)	7%	12%	36%
50% catches at	40 m	70 m	250 m
5% catches at	570 m	990 m	4,160 m

The differences between the trap catches in the three investigation areas are shown in Figure 2. Whereas the number of beetles caught per trap in the broadleaf stand was more than twice that in the open land and in the city, the "broadleaf" curve does not decrease as fast as the other two. The beetle density in the deciduous stand was at least twice as high as in the open land or the city.

In the open area, a marked decline was observed after just 200 meters. The basic population evened out to a low level of about 1,000 beetles per trap. In the broadleaf stand, the gradient also declined rapidly but evened out to a considerable level of 7,500 beetles per trap. The population density halved at approximately 250 meters, much further than in the open land or in the city. At a distance of

500 meters, the catches still added up to 36% that at the beginning of the transect.

4. Discussion

4.1. Beetle Pressure and Frequency of Ips typographus. Altogether, a minimum of 61 beetles per trap were caught over the whole period in the city and a maximum of 26,405 beetles per trap at the beginning of the broadleaf stand transect. These figures indicate that *I. typographus* was present in all the studied areas and support the conclusion of Piel et al. [14], who claimed that this species is able to spread over large areas, even though the host tree is relatively rare. According to Duelli et al. [9], who studied the beetle flight in a low impact situation, considerably more beetles can be found in the forest than outside. Sanders [15] used pheromone traps to demonstrate that bark beetles pass through broadleaf forests in their dispersal flight during the latent phase. Gugerli et al. [16] found that the basic beetle population, and consequently also the mass reproduction, does not differ much genetically in space. This may indicate that there is a constant and significant gene exchange by migrating beetles.

It should be remembered that trap catches do not necessarily represent the actual population density, flight activity, and the migration of the spruce bark beetle, as described by Zolubas and Byers [17]. Zumr [18] and Duelli et al. [10] performed recapture experiments and found that, besides the released beetles, many other feral individuals were trapped. It is always difficult to interpret trap catches without considering the attractiveness of the host trees and the potential breeding material. Nevertheless, the above experiments and the present research show that the spread and area-wide distribution of *Ips typographus* are impressive. It would be possible to build up new mass attacks nearly anywhere in the forest if suitable breeding material is present and the weather conditions are favourable.

Under our study conditions, however, the population quickly thinned within 300 to 400 meters of an infestation spot (Figure 2), particularly outside the forest. Since beetles have very little chance of breeding in the conditions along the chosen transects, the numbers of beetles caught along the transects reflect the impact of the beetle populations rather well.

It was striking that three times more beetles were caught in the broadleaf stand than in the open area. There are several possible reasons for the relatively high number of catches in the deciduous forest. First, beetles probably prefer to swarm in forests. As already mentioned, Duelli et al. [9] found considerably more beetles in the forest than outside. In Norway, Botterweg [19] observed that *I. typographus* spreads homogenously throughout forested areas. Second, we were not able to find an absolutely pure deciduous forest with an isolated infected spruce stand nearby. Hence, the stand with a very few sporadically scattered spruce trees had to be accepted for the experiment, and these could have influenced the number of catches slightly, even though the trees were in good sanitary condition. Healthy spruce trees are not in strong competition with pheromone traps, and it can be assumed that the traps in our study were highly attractive and thus reflected the spreading behaviour of *I. typographus*. On the open land transect, a small strip of woodland may have interfered with the traps at 512 m distance. The small stand, which lies south of the traps across the motorway, contains some Norway spruce. It cannot be excluded that some of the bark beetles caught originated or were influenced by this stand.

The results from study-site a in the city should, however, be treated with caution, as the second trap pair was 680 meters away from the infestation spot and no data were collected between the first and the second trap pair.

The research areas were chosen to include infested Norway spruce stands that bordered directly onto an adequate open or urban area and onto a preferably pure broadleaf stand. We consciously did not choose transects within spruce stands because, without releasing marked beetles, it would not have been possible to identify the proportion of the trap captures that did not originate from the initial infestation spots. To simulate high beetle pressure artificially, several hundred thousand marked beetles would be necessary, which would pose an impractical logistical challenge. It is not, in fact, clear that all individuals caught in the traps have a common origin. Nevertheless, on all transects, the population thinned quickly within only a few hundred meters, regardless of the origin of the beetles.

4.2. Buffer Zones. Up to now, forest managers have tended to use an empirically derived rule that specifies that a combat and/or buffer zone of 500 meters around an infested stand prevents a substantial invasion into the adjacent forests [20–22]. Byers [13] maintained that a border area of 500 meters width is justifiable under epidemic conditions. This rule has often been applied in Central Europe but until now has never actually been tested experimentally. On the basis of a GIS analysis, Wichmann and Ravn [7] concluded that epidemical attacks only spread out across short distances of less than 500 meters. The present study of high beetle impact indicates that a clear decline in the beetle population takes place within the first 300 meters away from the infestation spot. It then evens out to a level that does not pose an imminent danger to surrounding Norway spruce stands. If the buffer zone is an open area, the population may even decline considerably within the first 200 meters. The findings of our study indicate therefore that open areas may provide very suitable border zones between managed and unmanaged stands and help lower high beetle pressure from a control-free infested stand.

Infestations of living spruce trees are likely to occur in Switzerland when there are over 12,000 beetles caught per pheromone trap and year as mentioned by Forster and Meier [23]. Above this threshold, there is a considerable risk that the spruce bark beetle will locally spread and attack new trees. Various authors have reported similar thresholds: in Swedish spruce forests between 10,000 [24] and 15,000 [25] beetles per trap and year and between 8,000 [26] and 10,000 [27] beetles per trap and year in Northern Italy. Thus, the estimate of 12,000 beetles for Swiss spruce forests seems reasonable. In most of the cases, the beetles spreading from infestation spots will very likely thin out within the first 300 meters without spruce and densities will drop under the above threshold. This means that a buffer zone 500 meters in

width [13] is probably on the safe side. This study therefore confirms the hypothesis that the bark beetle density declines considerably within the first 500 meters of a spruce free buffer zone (Table 1). For all these reasons, such a buffer zone can be recommended.

Whether such buffer zones inhibit a further enlargement of beetle-infested stands should be always considered critically. If the host tree resistance is low, new trees will be successfully colonized by a smaller quantity of migrating beetles. However, it seems possible to considerably reduce the spread of bark beetles through implementing forest protection strategies and well-timed and consistent control measures, at least where conditions are similar to those at the sites we studied.

However, some beetles can be transported by wind much further, even though there are usually no active and directed immigration flights over long distances. The population in such cases thins out quickly, and a new breeding locality is chosen more or less random. To spread geographically, the beetles have to build up first a new population at a distant infestation spot.

4.3. Consequences for Bark Beetle Management. The findings of this study should be used in bark beetle management practices, especially under difficult conditions where there is a need for buffer zones between managed and unmanaged spruce stands. What is crucial is that management priorities should be defined on a large enough scale on the basis of well-defined landscape compartments. Compartments for the interventions should be chosen to be at least 100 hectares in size. At the same time, natural area borders (mountains, meadows, villages, lakes) 500 meters in width will also help to improve the effectiveness of selected buffer zones [28]. Setting such spatial priorities in bark beetle management and clearly defining and separating areas can reduce the spread of beetle infestations and infestation pressure.

The present study does not refer to buffer zones between managed and unmanaged, differently infested spruce stands that directly border each other. Experience has shown that under such conditions, stand edges of cleared spots within selected buffer zones are often reinfested. In the worst case, infestations may continue until all spruce trees in the zone have been attacked and subsequently removed. Under such high-risk conditions, the flight behaviour and beetle pressure of *Ips typographus* may be different. This still needs to be studied in detail.

Acknowledgments

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Research Article

First Record of the European Rusted Flea Beetle, *Neocrepidodera ferruginea* (Scopoli, 1763), in North America (Coleoptera: Chrysomelidae: Galerucinae: Alticini)

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The European rusted flea beetle *Neocrepidodera ferruginea* (Scopoli, 1763) is reported for the first time from Québec and Ontario, Canada. It was likely introduced into southern Ontario at an international port on the Great Lakes in early 1970s, or possibly earlier in the 1960s. However, the exact location and date of introduction could not be precisely determined. The flea beetle has since dispersed northeastwards and reached Aylmer, north of Ottawa River, in Québec, by 2003. This is about 375 km from Niagara Falls, where the oldest known specimens were collected in 1977. In 2009, various wild habitats and cultivated areas of Aylmer were surveyed. The host plants of the larvae could not be determined, but adults were swept from many plant species including various weeds and cultivated grasses: *Alopecurus pratense* (meadow foxtail), *Dactylis glomerata* (orchard-grass), *Festuca rubra* (red fescue-grass), and *Poa pratensis* (Kentucky blue-grass). Adults were also collected from flowers of several weeds: *Aster* sp. (undetermined species), *Aster novae-angliae* (New England aster), *Ambrosia artemisiifolia* (small ragweed), *Echium vulgare* (viper's bugloss), *Nasturtium officinale* (water cress), *Melilotus alba* (white sweet-clover), *Hypericum perforatum* (common St. John's-wort), *Lythrum salicaria* (purple loosestrife), *Ranunculus acris* (buttercup), and *Solidago* spp. (goldenrods). Since larvae are known to develop inside the roots and central stems of cereals, this new alien species represents a threat to Canadian agriculture, particularly if it reaches the Prairies in western Canada, where cereals represent a considerable part of their economy. *European rusted flea beetle* and *Altise ferrugineuse européenne* are suggested for the English and French common names of this flea beetle, respectively.

1. Introduction

Spectacular and large (20–35 mm) alien insects such as the *Asian longhorn beetle*, *Anoplophora glabripennis* (Motschulsky, 1853), cannot establish themselves unnoticed due to their large size, showy appearance, and the considerable damage they cause to trees. Although much smaller (7–14 mm), the *Emerald ash borer*, *Agrilus planipennis* Fairmaire, 1888, was quickly identified by its bright metallic green color and evident damage to ornamental ash (*Fraxinus* spp.) trees. In contrast, decades may pass before small species are detected, and it may take even longer if they are not associated with economically important plants of horticultural, agricultural, or forestry value. The European flea beetle, *Neocrepidodera ferruginea* [1], is such a species. Its small size (3-4 mm) and rusty color contributed to hide it within the vegetation. In addition, the lack of regular insect monitoring in agricultural lands of eastern Canada or of surveys in natural habitats also explain in part why it has been thriving unnoticed for over 30 years. Consequently, the present communication provides information on its identification, habitats, biology, host plants, economic importance, and history of introduction into North America.

2. Material and Methods

The specimens collected by the authors were obtained using the technique of LeSage [2].

The habitus photograph was taken using a digital Leica DFC-420 camera mounted on a Leica MZ-16 dissecting

microscope. The male aedeagus was photographed with an image processing system composed of a Nikon SMZ-1500 stereoscopic microscope coupled to a Nikon Digital camera DXM-1200F. The final image treatment was made with Photoshop Elements 7.0 software. The female genitalic features were dissected in 75% ethanol, preserved in terpineol, positioned on a microscope slide, and photographed with a Nikon Coolpix 950 digital camera attached to a Nikon Eclipse E-800 compound microscope.

3. Nomenclature

Originally described as *Chrysomela ferruginea* by Scopoli [1], this species was transferred to the flea beetle genus *Altica* by Geoffroy [3], but in the eighteenth century, the "genus" concept was so broad that it included all contemporary flea beetles. In the second edition of the catalogue of the beetles preserved in the collection of count Dejean, Chevrolat [4] split the large genus *Altica* into several new genera including *Crepidodera* under which he listed *C. ferruginea*, but did not designate a type species for the genus. The generic description itself appeared seven years later in the universal dictionary of d'Orbigny [5] mainly based on a transverse furrow on the pronotum and elytral punctures arranged in rows. Foudras [6] fragmented the original *Crepidodera* into six genera using new taxonomic characters and designated *Chrysomela nitidula* Linnaeus, 1758 as the type species of *Chalcoides*, one of his new genera. According to Biondi [7], Maulik [8] was not aware of this designation and designated the same species (*nitidula*) as the type species of *Chalcoides* Foudras with the result that species previously placed in *Crepidodera sensu* Foudras had to be transferred to *Asiorestia* created by Jakobson [9]. For the following sixty years, the species *ferruginea* was cited by authors in this genus. Exhaustive lists of references can be found in Biondi [10] and Gruev and Döberl [11, 12], and a detailed history of *Asiorestia* in Biondi [7]. The last nomenclatorial change happened when Konstantinov and Vandenberg [13] established the synonymy of *Asiorestia* Jakobson, 1926 with the genus *Neocrepidodera* created by Heikertinger [14] to include in a new generic unit, a Siberian flea beetle, *Ochrosis sibirica*, described by Pic [15], and the Japanese *Crepidodera recticollis* described by Jacoby [16]. Consequently, the official current name of the species treated here is *Neocrepidodera ferruginea* [1].

The early French entomologist Geoffroy [3] and Fourcroy [17] referred to *Neocrepidodera ferruginea* as the "*Altise fauve à stries*" on the basis of its elytral punctures arranged in rows, whereas Olivier [18] and du Chatenet [19] named it "*Altise ferrugineuse*" based on the typical reddish brown color of this flea beetle. In England, Shaw [20] and Jones and Jones [21] reported it as the "*Wheat flea beetle*." Davies [22] used a more specific name: the "*Wheat stem flea beetle*."

Adults are polyphagous and larvae attack several cultivated cereals [10, 19, 23–25]. Consequently, it might be confusing, even erroneous, to assign a popular name on the basis of a single host plant. On the other hand, the body color is typical and unusual, at least among the North American flea beetle fauna, although such rusted color is also found in other species of the Old World. For these reasons, we follow Olivier [18] and suggest "*European rusted flea beetle*" and "*Altise ferrugineuse européenne*," respectively for the English and French common names of *N. ferruginea*. We have added a reference to the origin of the beetle to indicate that it is an alien species for North America.

Figure 1: *Neocrepidodera ferruginea*, dorsal habitus. Body length, 3.5 mm, excluding antennae.

4. Identification

Several recent publications are available for the identification of *Neocrepidodera* at generic level. In North America, Riley et al. [26] provided the most recent synthesis of the North American leaf beetles including a key to *Neocrepidodera* and all other flea beetle genera of this continent. In the Old World, the handbook of Konstantinov and Vandenberg [13], and the keys of Warchalowski [27], are excellent tools for the identification of the Palaearctic fauna. The European and Mediterranean fauna were treated by Warchalowski [28], the fauna of Britain and Ireland by Cox [24], and the flea beetles of France by Doguet [29]. Species in the *N. ferruginea* group were recently revisited by Baselga and Novoa [30] including the illustration of their spermatheca and styli (vaginal palpi) for all treated species.

The characters most often used in generic keys are antennae 11-segmented, antennal calli raised and oval, pronotum transverse, narrowly explanate laterally, pronotal antebasal transverse impression present as well as a pair of lateral longitudinal furrows, elytral punctures generally arranged in regular rows, no hairs on elytra, procoxal cavities closed behind, and metatarsal claws not strongly inflated.

At the specific level, the diagnostic characters of *Neocrepidodera ferruginea* are as follows. Length of adult, 2.6–3.7 mm. Color uniformly yellowish brown to rusty brown (Figure 1). Frontal carina broad and low, frontal tubercles triangular, and not delineated posteriorly by a groove. Ocular groove behind eyes present. Frons and vertex smooth. Pronotum transverse, laterally margined; anterior angles large, protruding, bearing large seta; microsculpture finer on disk, coarser at basal groove; basal transverse groove

FIGURE 2: *Neocrepidodera ferruginea*, male aedeagus: (a) lateral view; (b) ventral view.

moderately deep, lateral longitudinal furrows deep. Elytra tapering at apex; elytral punctures large, deep, in regular rows, sometimes partially duplicated or disordered near suture; humeral callus well developed. Species fully winged. In the male, first tarsal segment of front and median legs markedly broadened (Figure 1). Sides of male aedeagus subparallel in dorsal view with apex triangular, slightly nipple-shaped in middle (Figure 2(b)); tip slightly upright in lateral view (Figure 2(a)); ventral median membranous portion almost reaching base. Female spermatheca elongate, slightly ovoid at base, spermathecal pump cylindrical and bent at 90°, spermathecal duct not coiled, bent at 45° to the median axis of the spermathecal pump (Figure 3(a)). Styli moderately short and thick, fused at base, tapered and pointed at apex (Figure 3(b)).

The body color of the native North American *Neocrepidodera robusta* (Leconte, 1874) is deep red, whereas *N. ferruginea* is brownish yellow (Figure 1). The antennomeres of the former are shorter and thicker than those of the latter. The elytra of the former are not tapered at the apex as they are in the latter. *Neocrepidodera robusta* has been reported from Québec to British Columbia and in Newfoundland [32, 33], but specimens are rare in collections examined. Those from Québec and Ontario, preserved in the CNC, were all collected in northern localities of these provinces and not in southern localities as is the case for *N. ferruginea*.

The larva of *Neocrepidodera ferruginea* was described in detail by Blunck [23] and included in keys by Oglobin and Medvedev [34], Medvedev and Zaitsev [35], and Steinhausen [36, 37]. These keys are largely based on the number, position, and chaetotaxy of the tergites. According to these authors, the larva of *Neocrepidodera ferruginea* is yellowish-white with dark-brown head, legs, and thoracic and abdominal tergites. The epicranial suture is Y-shaped, the coronal stem short, only one-third the length of the head. There are 6 setae on the frons and 10 on the vertex. One ocellum is present near the base of each antenna, but only

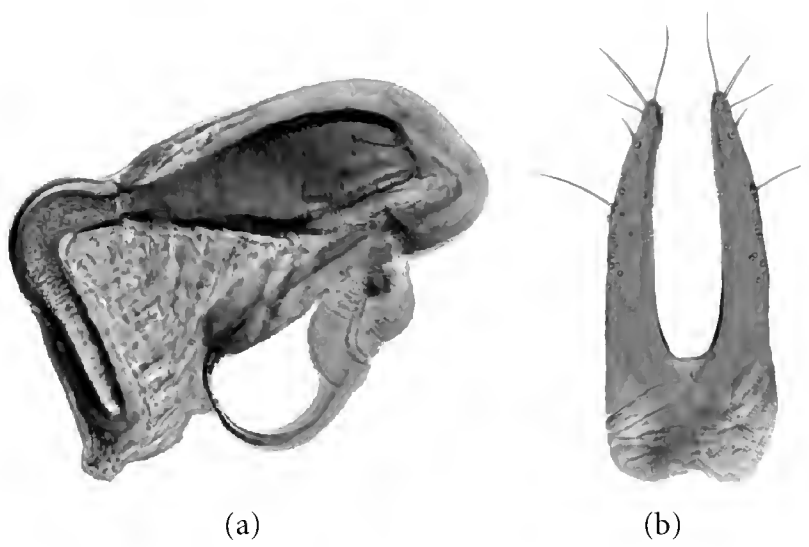

FIGURE 3: *Neocrepidodera ferruginea*, female genitalia: (a) spermatheca; (b) styli (vaginal palpi).

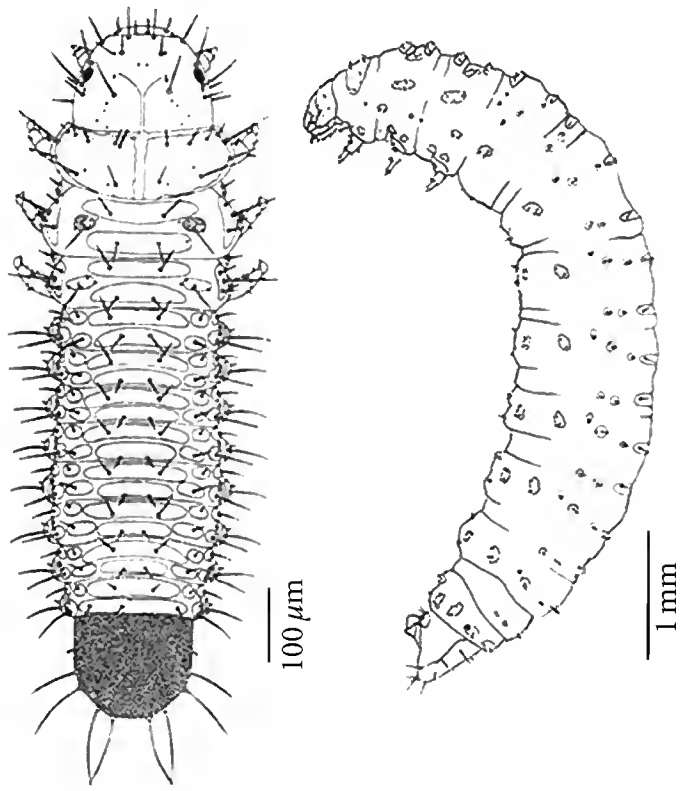

FIGURE 4: *Neocrepidodera ferruginea*, habitus of larva: (a) dorsal view; (b) lateral view. Modified from Blunck [23].

in the first instar larvae. The antennae are 2-segmented. The maxillary palpi are 3-segmented and the labial are palpi 2-segmented. The pronotum bears 10 setae on its anterior margin, 6 on the posterior margin. The last (9th) abdominal tergite forms a plate bearing 8 marginal and 4 discal setae; the whole surface is covered with an alveolar microsculpturing (Figures 4(a) and 4(b)). The pupa is still undescribed. The egg was sketched by Blunck [23], and its rough hexagonal microsculpture was illustrated, but its distinctive fine microsculpture was not shown (Figure 5).

5. Introduction into North America

During the winter of 2008, a small yellowish flea beetle was found in a pitfall trap from an alvar (limestone plain) of Aylmer (Québec). It was an unusual flea beetle for the local fauna. Comparisons with European specimens of similar appearance exchanged with the late French entomologist Michel Bergeal [25] convinced us that this specimen was identical to the Palaearctic *N. ferruginea*. During the summer

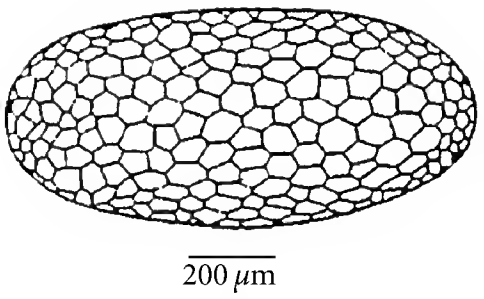

FIGURE 5: *Neocrepidodera ferruginea*, egg (after Blunck [23]).

of 2009, several natural and agricultural open habitats of Aylmer were surveyed in order to collect more specimens. The unsorted and unidentified holdings of the Canadian National Collection (CNC) were also examined, and eleven specimens were found: two were mixed with the miscellaneous Newfoundland flea beetles donated by Dave Larson (formerly at Memorial University, St. John's), nine were found in the unsorted beetles of the Carr's collection recently bequested to the CNC. Additional specimens received in the 1990s from S.A. Marshall (Guelph University), are also included in the present communication.

The date and place of these insect specimens are the most important clues to determining the history of introduction of this alien species. As concerning *Neocrepidodera ferruginea*, the oldest voucher specimens available were collected in 1977, but this species must have been thriving many years before this date. For instance, the lily leaf beetle, *Lilioceris lilii* [1] (Criocerinae), a bright red-bodied beetle with black head and appendages, was first detected in Montréal in 1943, but persisted at low levels for 30 years before expanding on the island of Montréal and making a noticeable jump to Ottawa by 1981 [38, 39]. It reached Nova Scotia in 1992, Toronto (Ontario) in 1993, and Portage la Prairie (Manitoba) in 2003 [40, 41]. Another pest, the cereal leaf beetle, *Oulema melanopus* (Linnaeus, 1758) (Criocerinae), is suspected to have been introduced into North America between 1947 and 1949, but was detected and positively identified only in 1962 from Berrien Co., in Michigan [42–44]. These case studies lead us to believe that *N. crepidodera* may have gone through a similar introduction and distribution pattern, survived at low numbers for many years, and only commenced long-range dispersal when high population levels were reached. The two 1977 specimens collected at Niagara Falls represent the beginning of the population expansion phase. This locality is probably not the original site of introduction which is more likely an international port located on one or the other Great Lakes.

6. Distribution

According to the *Catalogue of Palaearctic Coleoptera* of Löbl and Smetana [31], the distribution of *Neocrepidodera ferruginea* includes the Azores, all of Europe, except Iceland, the European parts of Russia, Asia Minor, Iran, and Turkey (Figure 6). It could also be present in Armenia, but no published records are available, and this needs to be confirmed. However, according to Gruev and Döberl [11, 12], Corsica, Egypt, Morocco, Siberia, and West Turkestan are doubtful records.

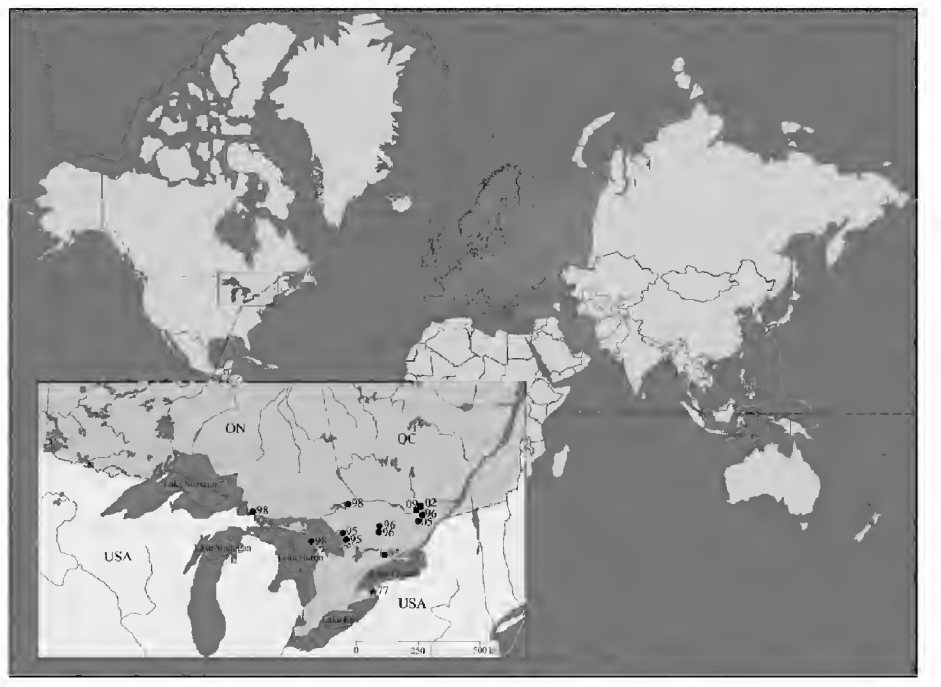

FIGURE 6: Old World distribution of *Neocrepidodera ferruginea* according to the countries listed in the catalogue of Löbl and Smetana [31]. Insert: distribution of *Neocrepidodera ferruginea* in North America. Star: Niagara Falls (Ontario), where the first specimens were collected. Solid circles: collection localities with collection years of 77 (1977) 95 (1995), 96 (1996), 98 (1998), 02 (2002), and 05 (=2005). Solid square: all collections in Aylmer (Québec) grouped together.

In North America, *N. ferruginea* is currently known from southern Ontario, and southwestern Québec on the north shore of the Ottawa River (inserted in Figure 6). The collection data of these new locations are as follows.

Material Examined. Canada: Québec: Gatineau/Aylmer (45°24′ N, 75°50′ W): Baillie street (north), 28.VII.2009, L. LeSage, border of Breckenridge stream on *Solidago* spp., grasses, and so forth, general sweeping [CBUM] 2; [CFIM] 2; [CNC] 3; Boucher Forest (north-east), north path, east-west, 16.VIII.2009, L. LeSage, on *Rhus radicans*, and so forth, general sweeping [CBUM] 1; [CNC] 1; Boucher Forest (west), 11.VIII.2009, L. LeSage, under Hydro-Québec electric line, on flowers of *Ambrosia* sp., *Solidago* spp., *Daucus* sp., *Melilotus* sp., general sweeping [CBUM] 3; [CFIM] 1; [CNC] 2; Boucher road (north), 8.VIII.2009, L. LeSage, in waste land on flowers of *Solidago* spp., *Melilotus* sp., *Daucus* sp., *Aster* spp, *Ambrosia* sp., and so forth, general sweeping, [CBUM] 1; [CNC]1; Boucher/Klock roads (north-east), close to transmission tower, 21.VII.2009, L. LeSage, waste land, on *Daucus* sp., *Solidago* spp., *Lythrum* sp., *Salix* sp., *Scirpus* sp., *Melilotus* sp., and so forth, general sweeping [CNC] 1; (north-west), 14.VIII.2009, L. LeSage, waste land, grasses, *Scirpus* sp., *Daucus* sp., *Solidago* spp., *Melilotus* sp., and so forth, general sweeping [CBUM] 1; [CNC] 1; Chagnon street (east), 18.VII.2010, V. Théberge, close to Hydro-Québec electricity line, on grass [CNC] 1; Cook road (north), 2.VII.2009, L. LeSage, ditch between two pastures on *Lythrum* sp., *Nasturtium* sp., *Ranunculus* sp., *Scirpus* sp., *Echium* sp., *Daucus* sp., and so forth general sweeping [CBUM] 1; [CFIM] 1; [CNC] 1; 16.VII.2009, L. LeSage, ditch between two fields on *Nasturtium*, and so forth, general sweeping [CNC] 1; 5.VIII.2009, L. LeSage, ditch between two fields on *Nasturtium officinale*, *Scirpus* sp., general sweeping

[CBUM] 2; [CFIM] 2; [CNC] 2; 5.VIII.2009, L. LeSage, pasture, *Echium vulgare* flowers, *Daucus carota*, grasses, and so forth, general sweeping [CBUM] 1; [CFIM] 1; [CNC] 1; west of Cook dump, 17.VII.2009, L. LeSage, *Daucus* sp., flowers, *Melilotus* sp., *Hypericum* sp., and so forth, general sweeping [CFIM] 1; [CNC] 1; Cook road (south), 21.VII.2009, L. LeSage, close to pound with *Equisetum* sp., *Daucus carota* flowers, *Solidago* spp., grasses, and so forth, general sweeping [CBUM] 2; [CNC] 1; 25.VII.2009, L. LeSage, fallow field on limestone, *Asclepias syriaca*, *Solidago* spp., *Hypericum* sp., grasses, and so forth, general sweeping [CBUM] 3; [CFIM] 1; [CNC] 2.; 25.VII.2009, L. LeSage, fallow field on limestone, *Asclepias* sp., *Apocynum* sp., *Solidago* spp., grasses, and so forth, general sweeping [CBUM] 1; [CFIM] 1; [CNC] 1; Pink road (south), near Museum of Nature, path north-south, 26.VII.2009, L. LeSage, abandoned field, *Lythrum salicaria*, grasses, and so forth, general sweeping [CBUM] 1; [CFIM] 1; [CNC] 1; Pink/Scholle roads, 25.VII-1.VIII.2006, L. LeSage, alvar, burned cedar forest of origin, on limestone, yellow pan trap [CNC] 1; 31.VII.2009, L. LeSage, alvar, *Daucus carota* flowers, *Solidago* spp., *Hypericum* sp., *Melilotus* sp., grasses, and so forth, general sweeping [CBUM] 2; [CFIM] 1; [CNC] 1; Principale street (north), around Golf La Croisée, 30.VII.2009, L. LeSage, *Daucus* sp. flowers, *Arctium* sp., *Solidago* spp., grasses, and so forth, general sweeping [CBUM] 4; [CFIM] 2; [CNC] 2; 30.VII.2009, L. LeSage, around a containment basin, *Daucus* sp., *Arctium* sp., *Solidago* spp., grasses, and so forth, general sweeping [CBUM] 8; [CFIM] 2; [CNC] 3; Simmons street (south), 7.VII.2007, L. LeSage, cleared space for future street, small stand of *Plantago major*, general sweeping [CNC] 1; 27.VIII.2009, L. LeSage, clearing in mixed forest, on limestone, grasses, *Solidago* spp., sweeping, LL03–39 [CBUM] 2; [CFIM] 1; [CNC]; Vanier/Pink roads (north), north Mountain View church, 29.VII.2009, L. LeSage, abandoned field, grasses, *Solidago* spp. flowers, *Asclepias* sp., and so forth, general sweeping [CBUM] 4; [CFIM] 1; [CNC] 6; Vernon street (north), tributary of Lac des Fées, 1.VIII.2003, L. LeSage, border of creek, *Lythrum salicaria*, sweeping, LL03-85 [CNC] 1. Gatineau Park (45°34′ N, 75°57′ W): 1 km south of Ramsay Lake, 19.VIII.2009, L. LeSage, forest path, toward [Eardley] Escarpment, general sweeping [CNC] 1. Ontario: Algoma District (48°00′ N, 84°00′ W): Sault Sainte-Marie, 29.VI.1998, R. Atkinson [CNC] 4; Bruce Co. (44°18′ N, 81°25′ W): Dunks Bay, 45.14 N; 81.38 W, 30.VII.1998, S.A., Marshall [CNC] 1; 20.VII.-1.IX.1999, S.A., Marshall, on shoreline, Malaise [CNC] 2; [SAMC] 3; 15.XI.1999-15.III.2000, S.A., Marshall, Lake edge, Malaise [SAMC] 1; 23–27.VII.2000, S.A., Marshall [CNC] 1; Dorcas Bay, VII.1998, S.A., Marshall, alvar [SAMC] 1; 16.VII-2.VIII.1999, S.A., Marshall, in pound behind dune, Malaise [SAMC] 1; Cameron Lake Road South, 20–29.VII.1999, S.A., Marshall, spring fen, pan trap [SAMC] 1; Georgian Bay, Fathom Five National Park, 25.VI-28.VII.1995, T. Woodcock & S. Marshall, Land Base Cedar Duff, pan trap [CNC] 1; [SMAC] 1; Hastings Co. (44°45′ N, 77°40′ W): Baptiste Lake, 10.VII.1995, B.F. & J.L. Carr [CNC] 3; Maynooth, 17.VII.1996, B.F. & J.L. Carr [CNC] 1; Cardiff, 22.VII.1996, B.F. & J.L. Carr [CNC] 1; Highway 69, 2 km north of Nobel, 23.VI.1996, B.F. & J.L. Carr [CNC] 1; Welland Co. (43°05′ N, 79°04′ W): Niagara Falls, 23.VII.1977, D. Larson [CNC] 2; Carleton Co. (45°15′ N, 75°45′ W): Stittsville, 5.VII.2005, Envirocontrol, Topsoil (large number) [CNC] 4. Nepean, 2.VII.1996, B.F. & J.L. Carr [CNC] 1; Nepean, 4.VII.1996, B.F. & J.L. Carr [CNC] 1; Parry Sound District (45°45′ N, 79°50′ W): Parry Sound, 12.VII.1995, B.F & J.L. Carr [CNC] 1.

7. Habitats

Neocrepidodera ferruginea has been reported from a large variety of habitats or sites. Medvedev and Zaitsev [35] stated that it was found on wet meadows, bogs, and cereal fields in Siberia and the Russian Far East. In France, Doguet [29] mentioned fallow fields, roadsides, cultivated areas, natural prairies, and so forth. In his guide of the leaf beetles of Europe, du Chatenet [19] reported it from fields, prairies, and fallows. In Britain and Ireland, Cox [24] listed woodland, downland, heathland, moorland, commons, arable land, set-aside, gardens, roadside verges, pits, quarries, sea walls, and dunes. In Québec (Aylmer), specimens were collected in alvars, abandoned fields, pastures, vacant lots, ditches, edges of creek, limestone outcrops, and forest trails. According to the Watford Coleoptera Group [45], meadows and wet meadows are the biotopes of this flea beetle in Britain.

8. Biology

Little is known on the biology of *Neocrepidodera ferruginea* in North American except for the habitats, where it was collected. In Europe, there has been few additional publications published since the pioneering work of Blunck [23]. According to this author, adults emerge in June in Germany, but earlier in warmer climates. They mate and lay eggs in summer and fall. Females deposit their eggs in the ground beside the seeds of potential host plants. The young larvae enter seedlings at the host's base and later excavate tunnels throughout their roots and central stems [46]. Larval development extends into the winter, but as temperature decreases, larvae leave their host plants, move deeper into the ground, and overwinter in the soil. They become active again the next spring as the top soil thaws after which they search for new host plants to complete their development. Pupation occurs underground in earthen cells and lasts 2-3 weeks. They become fully developed by the end of May or the beginning of June. There is only one generation per year (univoltine) in Germany.

9. Host Plants

Although dozens of "hosts" were reported for the adults of *Neocrepidodera ferruginea,* quite a few are probably no more than incidental presences of the beetle on such plants since larval development has been observed only in a few species of Poaceae. Blunck [23] tested over 40 potential hosts and estimated that half of them were markedly used or preferred by the adults in some 20 plant families (Table 1). Thistles (*Carduus* spp. and *Cirsium* spp.) are the most frequently cited hosts for adults: Bedel [47], Balachowsky and Mesnil [48],

Table 1: Most important lists of recorded host plants for *Neocrepidodera ferruginea*. The list of Blunck [23] includes only the first two categories.

Family	Latin names	Popular names	Blunck 1932 [23]	Biondi 1982 [51]	Doguet 1989	du Chatenet 2002 [19]	Cox 2007 [24]	LeSage/Savard 2011
Anacardiaceae	*Rhus radicans* L.	Poison ivy						X
Apiaceae	*Daucus carota* L.	Wild carrot						Flowers
Apocynaceae	*Apocynum androsaemifolium* L.	Spreading dogbane						X
Asclepiaceae	*Asclepias syriaca* L.	Milkweed						X
Asteraceae	(species not specified)	Composite family				X		X
Asteraceae	*Aster* spp.	Aster						Flowers
Asteraceae	*Aster novae-angliae* L.	New England aster						Flowers
Asteraceae	*Ambrosia artemisiifolia* L.	Small ragweed						Flowers
Asteraceae	*Artemisia vulgaris* L.	Mugwort	XX		X			
Asteraceae	*Carduus* sp.	Thistle			X			
Asteraceae	*Carduus acanthoides* L.	Plumeless nodding thistle		X				
Asteraceae	*Carduus nutans* L.	Musk thistle		X				
Asteraceae	*Centaurea acaulis* Willd.	Dwarf thistle	XX					
Asteraceae	*Centaurea cyanus* L.	Corn flower	XX		X			
Asteraceae	*Centaurea scabiosa* L.	Greater knapweed	XX	X	X			
Asteraceae	*Chrysanthemum leucanthemum* L.	Daisy	Xx				X	
Asteraceae	*Cirsium arvense* Scop.	Canada thistle		X				
Asteraceae	*Cirsium canum* Moench.	Queen Anne's thistle		X				
Asteraceae	*Cirsium lanceolatum* Hill.*	Spear thistle		X				
Asteraceae	*Cirsium oleraceum* L.	Cabbage thistle	XX		X			
Asteraceae	*Silybum marianum* Gaertn.	Milk thistle	XX		X			
Asteraceae	*Solidago* spp.	Goldenrod						Flowers
Asteraceae	*Taraxacum officinale* Weber	Dandelion	XX		X			
Boraginaceae	(species not specified)	Borage family				X		
Boraginaceae	*Borago officinalis* L.	Borage	XX				X	
Boraginaceae	*Echium vulgare* L.	Viper's bugloss						Flowers
Boraginaceae	*Symphytum officinale* L.	Comfrey		X				
Boraginaceae	*Symphytum* sp.	Comfrey	XX				X	
Brassicaceae	*Brassica napus* L.	Rape	X					
Brassicacae	*Brassica oleracea* L.	Cabbage	Xx				X	
Brassicacae	*Brassica rapa* L.	Wild turnip	Xx					
Brassicacae	*Nasturtium officinale* R. Br.	Water cress						Flowers
Cannabaceae	*Cannabis sativa* L.	Hemp			X	X		
Compositae	*Achillea millefolium* L.	Common yarrow	Xx				flowers	
Cyperaceae	*Scirpus* sp.	Bulrush						X
Equisetaceae	*Equisetum* sp.	Horsetail						X
Fabaceae	(species not specified)	Pulse family				X		
Fabaceae	*Melilotus alba Medik.*	White sweet-clover						Flowers
Fabaceae	*Onobrychis* sp.	Sanfoins ("sainfoins")			X			
Fabaceae	*Pisum sativum* L.	Peas					X	
Fabaceae	*Trifolium* sp.	Clover			X			
Fabaceae	*Vicia* sp.	Vetch			X			
Hypericaceae	*Hypericum perforatum* L.	Common St. John's-wort						Flowers

Table 1: Continued.

Family	Latin names	Popular names	Blunck 1932 [23]	Biondi 1982 [51]	Doguet 1989	du Chatenet 2002 [19]	Cox 2007 [24]	LeSage/Savard 2011
Lamiaceae	*Lamium* sp.	Dead-nettle					X	
Lythraceae	*Lythrum salicaria* L.	Purple loosestrife						Flowers
Poaceae	(species not specified)	Cultivated cereals	XX	X	X	X	X	
Poaceae	(species not specified)	Wild grasses	XX			X	X	X
Poaceae	*Avena sativa* L.	Oats	XX					
Poaceae	*Bromus* sp.	Brome grass			X			
Poaceae	*Cynosurus* sp.	Dog's tail			X			
Poaceae	*Dactylis* sp.	Orchard grass			X			
Poaceae	*Festuca* sp.	Fescue grass			X			
Poaceae	*Hordeum vulgare* L.	Barley	XX		(X)			
Poaceae	*Lolium* sp.	Ryegrass			X			
Poaceae	*Phalaris* sp.	Canary grass						
Poaceae	*Phleum pratense* L.	Timothy						X
Poaceae	*Poa* sp.	Bluegrass			X			
Poaceae	*Secale cereale* L.	Rye	XX					
Poaceae	*Triticum vulgare* Vill.**	Wheat	XX					
Polygonaceae	(species not specified)	Buckwheat family			X	X		
Polygonaceae	*Polygonum* sp.	Knotweed	XX				X	
Ranunculaceae	*Ranunculus acris* L.	Buttercup						Flowers
Salicaceae	*Salix* sp.	Willow						X
Urticaceae	*Urtica dioica* L.	Stinging nettle	XX	X				
Urticaceae	*Urtica* sp.	Nettle			X	X	X	

X: used
XX: strongly used
Xx: more or less used
(X): plant species doubtful
* *Cirsium lanceolatum* currently considered synonym of *Cirsium vulgare* (Savi) Ten. [58].
** *Triticum vulgare* currently considered synonym of *Triticum aestivum* L. [59].

and Doguet [29] in France; Lameere [49] in Belgium; Blunck [23] in Germany; Joy [50] in England; Ogloblin and Medvedev [34] in the European part of Ru

ssia of the past USSR, Medvedev and Zaitsev [35] in Siberia and the Russian Far East; Biondi [51], in Italy; Gruev and Tomov [52] in Bulgaria; Warchalowski [28] in Europe and Asia; Gök and Çilbiroğlu [53] in Turkey; Bukejs [54] in Latvia. Nettles (*Urtica* spp.), or more precisely the stinging nettle (*Urtica dioica* L.), are the next most frequently given hosts: Kutschera [55] for Europe; Allard [56] for Austria, Favre [57] for France; Joy [50] for England; Ogloblin and Medvedev [34] European part of USSR, Medvedev and Zaitsev [35] for Siberia and the Far East; Biondi [51] for Italy; Doguet [29] for France; du Chatenet [19] for Europe; Cox [24] for Britain and Ireland.

In addition to thistles and nettles, grasses are economically important hosts reported by many authors. According to Blunck [23], barley, rye, and wheat were frequently used. Doguet [29] listed blue-grass (*Poa* sp.), brome-grass (*Bromus* sp.), dog's tail (*Cynosurus* sp.), fescue-grass (*Festuca* sp.), orchard-grass (*Dactylis* sp.), and rye-grass (*Lolium* sp.), but did not specify a preference. du Chatenet [19] and Cox [24] referred to cultivated and wild grasses in general without mentioning a peculiar variety. During our 2009 survey four species appeared most common: Kentucky blue-grass (*Poa pratensis*), meadow foxtail (*Alopecurus pratensis*), orchard-grass (*Dactylis glomerata*), and red fescue-grass (*Festuca rubra*). Voucher specimens of these grasses are deposited in DAO herbarium in Ottawa.

Flowering weeds were also surveyed because they could easily be identified in pastures and fields while sweeping. The viper's bugloss, or blueweed, (*Echium vulgare*) cannot be missed with its numerous bright blue flowers forming a large ear-like cluster. These flowers seemed very attractive to adult *Neocrepidodera ferruginea*, and such attraction was reported by the early authors Fabricius [60] and Olivier [18]. Adults were also collected on the flowers of other plants: buttercup (*Ranunculus acris*), common St. John's-wort (*Hypericum perforatum*), purple loosestrife (*Lythrum salicaria*), small ragweed (*Ambrosia artemisiifolia*), various unspecified asters (*Aster* spp.), New England asters (*Aster novae-angliae*), various goldenrods (*Solidago* spp.), water cress (*Nasturtium officinale*), white sweet-clover (*Melilotus alba*), and wild carrot (*Daucus carota*) (Table 1). These plants are probably not

actual hosts, but alternative food sources for maturation feeding by adults. Such alternative feeding preference is noticeable and well known in corn leaf beetles (*Diabrotica* spp.). For instance, adults of the northern corn leaf beetle (*Diabrotica barberi,* Smith and Lawrence, 1967) migrate outside corn fields to feed on the pollen of various plants, preferably goldenrods (*Solidago* spp.), even though the larvae prefer corn. Such migration was observed in many corn fields located on both sides of Highway 20, between Montréal and Québec city in Québec [61]. According to Naranjo [62], adults stay in corn fields as long as the quality and the quantity of corn pollen is good and acceptable, but move outside when pollen is rare or of poor quality. Apparently, the adults of *N. ferruginea* behave in a similar manner, but more information is needed to confirm these preliminary observations.

10. Parasites

Unknown.

11. Predators

Unknown.

12. Economic Importance

Ahlberg [63] stated that following a dry spring, injury by flea beetles was unusually severe in Sweden in 1941. Barley and spring wheat, and to a lesser extent winter wheat and rye, were damaged by overwintering adults of *Phyllotreta vittula* (Redtenbacher, 1849). However, the larvae that occur low down in straw in late summer were not those of *P. vittula* as previously thought, but those of *Neocrepidodera ferruginea*, which was considered a serious pest in Sweden. Johansson [64] observed that the leaves of wheat which turned yellow and ceased to grow, contained larvae of *Chaetocnema aridula* Gyllenhal, 1827 and *C. hortensis* (Geoffroy 1785) and also *N. ferrugine.* However, he considered the latter of little importance, even though it may infest cereals and wild grasses.

In England, Gough [65] found *Neocrepidodera ferruginea* larvae in only nine fields during a survey of 136 fields of winter wheat, in Yorkshire, two-thirds of which were on light soils. Attacked wheat plants exhibited yellow central shoots, while the leaves remained green. Such symptoms were observed, in March, in only 3% of the fields. According to Jones and Jones [21], the normal host plants are thistles and related plants, but larvae may enter wheat plants by accident while searching for suitable hosts through the soil. Davies [22] reported extensive damage to spring oats, variety *Forward* in the Ruthin district of Denbighshire. Very few wireworms, or fruit fly larvae (*Oscinella* spp.; Diptera, Chloropidae) were found in oat stems, but larvae of *N. ferruginea* were numerous within the yellowed central shoots examined in the laboratory. In the field, damage appeared as large patches where plants had been considerably thinned.

In north-east Scotland, Shaw [20] reported damage to the winter wheat variety *Cappelle* and oats, in April. Areas with extensive thinning necessitated resowing. Where damage was significant, almost every damaged stem revealed a larva, but it was extremely difficult to find larvae inside oat stems, although some were found in the soil after much searching. The following year, Shaw and Osborne [66] reported extensive damage in a 20-acre field of the winter wheat variety *Cappelle* at Dirleton, east Lothian.

13. Discussion

Due to its small size, inconspicuous color, and still undetermined economic impact, *Neocrepidodera ferruginea* has remained unnoticed until now. On the basis of the available specimens, we believe that it was introduced in early 1970s, or even earlier in the 1960s, at an undetermined international port located on the Great Lakes [67]. The ports on Erie, ON or Huron Lake appear to be the most probable introduction sites since they are the closest to Niagara Falls, Ontario, Canada, where the first specimens were found. On the other hand, those on Lake Michigan or Lake Superior cannot be excluded if we suppose that *N. ferruginea* followed an introduction pattern similar to that of the cereal leaf beetle, which was probably introduced at an international port on Lake Michigan between 1947 and 1949, and positively identified only 15 years later, and has since been expanding eastward and westward [44].

In about 35 years, *Neocrepidodera ferruginea* moved eastward and reached the Ottawa Valley most likely by natural dispersal, a distance of about 375 km from Niagara Falls, where the first specimens were collected. Aylmer, on the north shore of the Ottawa River, is presently the northernmost known location of this flea beetle; however, we suspect that it has already established further north from this locality. In Ontario, it would be crucial to determine if it is moving westward towards the Canadian Prairies.

The attraction of *Neocrepidodera ferruginea* adults to the flowers of various plants is similar to that of the adults of the northern corn leaf beetle, which migrate outside corn fields to feed on the pollen of goldenrods and other weeds [68]. However, *N. ferruginea* do not have to migrate far outside fields since most weeds are ubiquitous and grow within fields in the company of cultivated grasses. Consequently, adults can easily move from one plant species to another and adjust their food preferences to the flowering sequence of weeds which varies with habitats and localities.

Abbreviations

CBUM: Centre sur la biodiversité de l'Université de Montréal, Montréal, QC, Canada H1X 2B2. S. LeTirant
CFIA: Canadian Food Inspection Agency
CFIM: Collections en Fiducie de l'Insectarium de Montréal, Insectarium de Montréal, Montréal, QC, Canada H1X 2B2. S. LeTirant
CNC: Canadian National Collection, Agriculture Canada, Ottawa, ON, Canada K1A 0C6. L. LeSage

DAO: Department of Agriculture Ottawa. Official acronym of the Herbarium of the Department, Central Experimental Farm, Ottawa. Amanda Ward
ECORC: Eastern Cereal and Oilseed Research Centre, Agriculture, Ottawa, ON, Canada K1A 0C6
SAMC: S.A. Marshall Private Collection, Department of Environmental Biology, University of Guelph, Guelph, ON, Canada N1G 2W1. S.A. Marshall.

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Research Article

2-methyl-3-buten-2-ol: A Pheromone Component of Conifer Bark Beetles Found in the Bark of Nonhost Deciduous Trees

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Volatiles from bark of aspen, *Populus tremula* L. and two species of birch: silver birch (*Betula pendula* Roth.) and common birch (*B. pubescens* Ehrh.), were collected by direct solvent extraction and aeration of both newly cut bark chips and undamaged stems in June 1998 and subjected to GC-MS analysis. The results showed the presence of 2-methyl-3-buten-2-ol (MB), one of the two principal aggregation pheromone components of the spruce bark beetle, *Ips typographus*, in bark extraction samples of all the three deciduous tree species tested. In addition, one more oxygenated hemiterpene, 3-methyl-3-buten-2-one, and (*E*)-3-penten-2-ol were also found in the bark extracts. Only trace amounts of MB were detected in some aeration samples of the fresh bark chips, and no MB was found from the aeration samples of undamaged stems at detectable levels. The occurrence of this compound was also confirmed in the bark of four exotic birch species: *B. albosinensis* Schneid., *B. ermanii* Cham., *B. jacquemontii* Spach, and *B. maximowicziana* Regel, but not yet in the European pines/spruces and the common yeasts. Our results raise major questions regarding the evolution, the tropospheric chemistry, and the ecological role of this hemiterpene alcohol. They also suggest that comparative studies on the biosynthetic pathways for MB in different sources would be of considerably evolutionary interest.

1. Introduction

Aspen, *Populus tremula* L. and two birch species, silver birch (*Betula pendula* Roth.) and common birch (*B. pubescens* Ehrh.), are the most common deciduous trees in Norway spruce forests of Scandinavia [1]. Studies showed that volatiles from leaves and bark of nonhost aspen/birch trees strongly inhibit pheromone attraction in spruce bark beetles, *Ips typographus* L. and *Pityogenes chalcographus* L. [2, 3]. In order to determine what kind of volatiles are responsible for the inhibition effect, volatiles from these nonhost trees were collected by headspace aerations and solvent extraction and analyzed by GC-MS [1, 4, 5]. Further electrophysiological and field bioassay studies showed that green leaf alcohols (GLVs) from leaves and bark and some specific compounds like *trans*-conophthorin, from bark of the nonhost deciduous trees, disrupt the secondary attraction response of sympatric coniferophagous bark beetles [4, 5]. Such inhibitory effects by angiosperm nonhost volatiles have been shown on many other conifer-inhabiting bark beetles throughout the world [3]. In the present paper, we report our finding of 2-methyl-3-buten-2-ol (here abbreviated as MB [6], while in atmospheric chemistry often as MBO), one of the principal aggregation pheromone components of *I. typographus*, in the bark of its nonhost trees *P. tremula*, *B. pendula*, and *B. pubescens*, which poses questions regarding the evolution and ecological role of this semiochemical in the natural habitat.

2. Materials and Methods

Volatiles from the nonhost bark were collected by direct extraction with diethyl ether and by aerations of both newly cut bark chips and undamaged stems in June 1998, Asa, Småland, Sweden. One cm^2 of bark cut into 2×5 mm pieces taken at 1.5 m height of standing trees from each species was extracted in 1 mL of diethyl ether. Fresh bark chips (size: 3×6 cm, with total area of ca. 1000 cm^2) from each individual

tree at breast height were aerated in the laboratory within 15–30 min after bark sampling. They were enclosed in a plastic-cooking bag (35 × 43 cm) with an activated charcoal filter tube at the air inlet. The volatiles in the bag were trapped on Porapak Q (30 mg, mesh 50–80 (Supelco), in Teflon tube: ID 3 mm × 35 mm) for 2 h at airflow rate of 300 mL/min and recovered by extraction with diethyl ether [1]. The same aeration setup was also used for the volatile collection from the undamaged stems at 1.3–1.7 m height for 1.5 h with battery-operated pumps. Air temperatures inside and outside of sampling bags were recorded during the aerations with a Min-Max reading thermometer. Additional fresh bark extraction samples of four exotic birch species, *B. albosinensis* Schneid., *B. ermanii* Cham., *B. jacquemontii* Spach, and *B. maximowicziana* Regel, using the same sampling approach as described previously for the three native Scandinavian species were taken from the Alnarp Botanical Garden, Skåne, Sweden in February 1999. All extracts were kept at −20°C before the GC-MS analysis. After collection, the bark samples were dried at 65°C for 72 h and weighed. The detailed information about the bark sampling is shown in Table 1.

The chemical analyses were made by a combined HP 5890 series II gas chromatography and HP 5972 mass selective detector (GC-MSD). The GC was equipped with a 25 m × 0.25 mm × 0.30 μm fused silica column, coated with CP-Wax no. 58 (FFAP CB) (Agilent Technologies). All samples were injected by a HP 7673 autoinjector (2 μL each). Helium was used as the carrier gas at an electronically controlled constant flow of 31 cm/s. The injector temperature was 200°C, and oven temperature was at 30°C for the first 3 min, then programmed to 200°C at 10°C/min, where it remained for 2 min.

Volatiles were identified by comparison of the retention indices and mass spectra with those of authentic compounds, with computerized data library, NBS75K, and with custom produced library (KE1995). Absolute amounts were obtained by comparison to the internal standard, the stabilizer, butylated hydroxytoluene (BHT) of diethyl ether.

3. Results

GC-MS analyses of bark solvent extracts clearly showed the presence of 2-methyl-3-buten-2-ol (MB) in the bark of all the three native Scandinavian deciduous tree species tested (Figure 1). This identification was proven by comparison of retention time and mass spectrum to the authentic compound and computer data libraries (NBS75K and KE1995) (Figure 1). The amounts of MB in the extracts were estimated ca 4.5, 2.5, and 10.7 μg/g dw, for *B. pendula*, *B. pubescens* and *P. tremula*, respectively (Table 2). In addition to the MB, one more oxygenated hemiterpene, 3-methyl-3-buten-2-one, and (*E*)-3-penten-2-ol were identified in the bark extracts, with their average amounts being lower than that of MB (Table 2). The occurrence of MB as a minor component was also confirmed in the bark samples of four exotic birch species: *B. albosinensis, B. ermanii, B. jacquemontii*, and *B. maximowicziana* by GC-MS.

GC-MS analyses of some aeration samples of the fresh bark chips did also indicate the presence of MB, but only in trace amounts which might be due to the major breakthrough of this highly volatile alcohol through the Porapak Q trap [7]. No MB was found in the aeration samples of undamaged stems at detectable levels.

4. Discussion

4.1. Insect Sources. MB was first identified as one of the principal aggregation pheromone components of spruce bark beetle, *Ips typographus* [8], and was also observed in emissions from the entrance holes made by this species on the trunks of both live [7] and cut [9] spruce trees [10], with emission rates per bore hole being significantly larger than the average content of hindgut. It has been reported as a pheromone component or male-specific compound of several other conifer bark beetles in Eurasia, *Ips* (*Orthotomicus*) *erosus* Woll. [11], *I. nitidus* Eggers [12], *I. shangrila* Cognato and Sun [13], *Pteleobius vittatus* (F.) [14], and *Pityogenes* spp. [15]. MB was found to be produced by females of *Ips amitinus* (Eichhoff) as well and seemed to be inhibitive [16]. It is reportedly an alarm pheromone of the European hornet, *Vespa crabro* L. [17].

4.2. Plant Sources. This is the first report on the presence of MB in bark of deciduous trees. In addition to the three major native angiosperm deciduous tree species (*P. tremula, B. pendula*, and *B. pubescens*), MB was also detected in bark samples (taken in February) of several exotic birch species, including *B. albosinensis, B. ermanii, B. jacquemontii* and *B. maximowicziana*. Thus, its natural occurrence in plants might be much more common than we ever realized. In fact, the emission of this isoprene alcohol from plants had been observed before; the orchid *Aerides lawrenceae* produces MB [18]. Interestingly, MB is also a hop constituent with sedative hypnotic activity [19] and formed from humulones and lupulones by reaction with OH radicals in the presence of atmospheric oxygen [20]. Further study suggested that the same reaction with OH radicals may occur *in vivo*. For instance, it induced the murine cytochrome P4503A and ethylmorphine N-demethylation (a functional marker for P4503A) in mice [21]. Both 2-methyl-3-buten-2-ol (MB) and 3-methyl-3-buten-2-one are parts of volatile composition from the headspace of five lima bean plants infested with two-spotted spider mites (*Tetranychus urticae* Koch) [22]. MB is also a fragrance ingredient used in cosmetics, fine fragrances, shampoos, toilet soaps, and other toiletries as well as in noncosmetic products such as household cleaners and detergents [23].

Zimmerman et al. (1991) noted the presence of MB in samples taken from enclosures placed around branches of Loblolly pine (*Pinus taeda* L.) [24]. Goldan et al. (1993) characterized the trace gas composition of ambient air in a small clearing in a predominantly lodgepole pine forest with a significant admixture of aspen and occasional Colorado blue spruce (3050 m elevation) in Colorado in June 1991 [25]. They found MB to be the dominant volatile organic compound (VOC), with a concentration 4–7 times higher than that of isoprene. Based on the fact that diurnal changes in

Table 1: Background information on bark samples of nonhost deciduous trees, Sweden.

Samples						Tree dimension			
Scientific name	Common name	Location	Date	Time	No. of trees	Height (m)	DBH (cm)	Dry weight or sampling area	Temperature (inside/outside) °C
Bark extractions of the key species									
B. pendula Roth	Silver Birch	Asa, Småland	1998-06-11	15:00–15:10	2	10–13	10–14	0.16–0.20 g	
B. pubescens Ehrh.	Downy Birch	Asa, Småland	1998-06-11	15:35–15:50	3	12–20	10–18	0.13–0.26 g	
P. tremula L.	Aspen	Asa, Småland	1998-06-11	15:10–15:25	3	8.5–9.0	8–10	0.13–0.20 g	
Bark extractions of additional birch species									
B. albosinensis var. *septentrionalis* Schneid.	Northern Chinese red birch	Alnarp, Skåne	2/24/1999	10:00–10:20	2	9–12	8–12	NA*	
*B. ermanii*Cham.	Erman's birch	Alnarp, Skåne	2/24/1999	10:21–10:40	2	8–10	8–11	NA	
B. jacquemontii Spach	White barked Himalayan Birch	Alnarp, Skåne	2/24/1999	10:41–10:55	2	8–14	7–12	NA	
B. maximowicziana Regel	Monarch Birch	Alnarp, Skåne	2/24/1999	11:10–10:25	2	15–20	15–22	NA	
Aerations of fresh bark chips									
B. pendula Roth	Silver Birch	Asa, Småland	1998-06-09	9:30–10:00	4	13–16	14–16	147–152 g	21.9–22.7/21–21.7
B. pubescens Ehrh.	Downy Birch	Asa, Småland	1998-06-08	17:00–17:30	4	15–20	24–30	140–210 g	22.8–23.4/22.5–23
P. tremula L.	Aspen	Asa, Småland	1998-06-09	9:30–10:00	4	12–17	11–20	106–194 g	21.9–22.7/21–21.7
Aerations of undamaged stems									
B. pendula Roth	Silver Birch	Asa, Småland	1998-06-29	12:50–14:20	2	15–20	18–20	0.22–0.24 m^2	17.5–31.4/15.5–21.3
B. pubescens Ehrh.	Downy Birch	Asa, Småland	1998-06-29	13:55–15:25	2	15–20	12–20	0.11–0.16 m^2	19.5–20.0/18.3–19.2
P. tremula L.	Aspen	Asa, Småland	1998-06-29	12:15–13:45	2	13–15	14–18	0.18–0.22 m^2	17.5–31.4/15.5–21.3

*No fresh/dry weights were measured, and the amounts of 232 MB in the samples were not quantified.

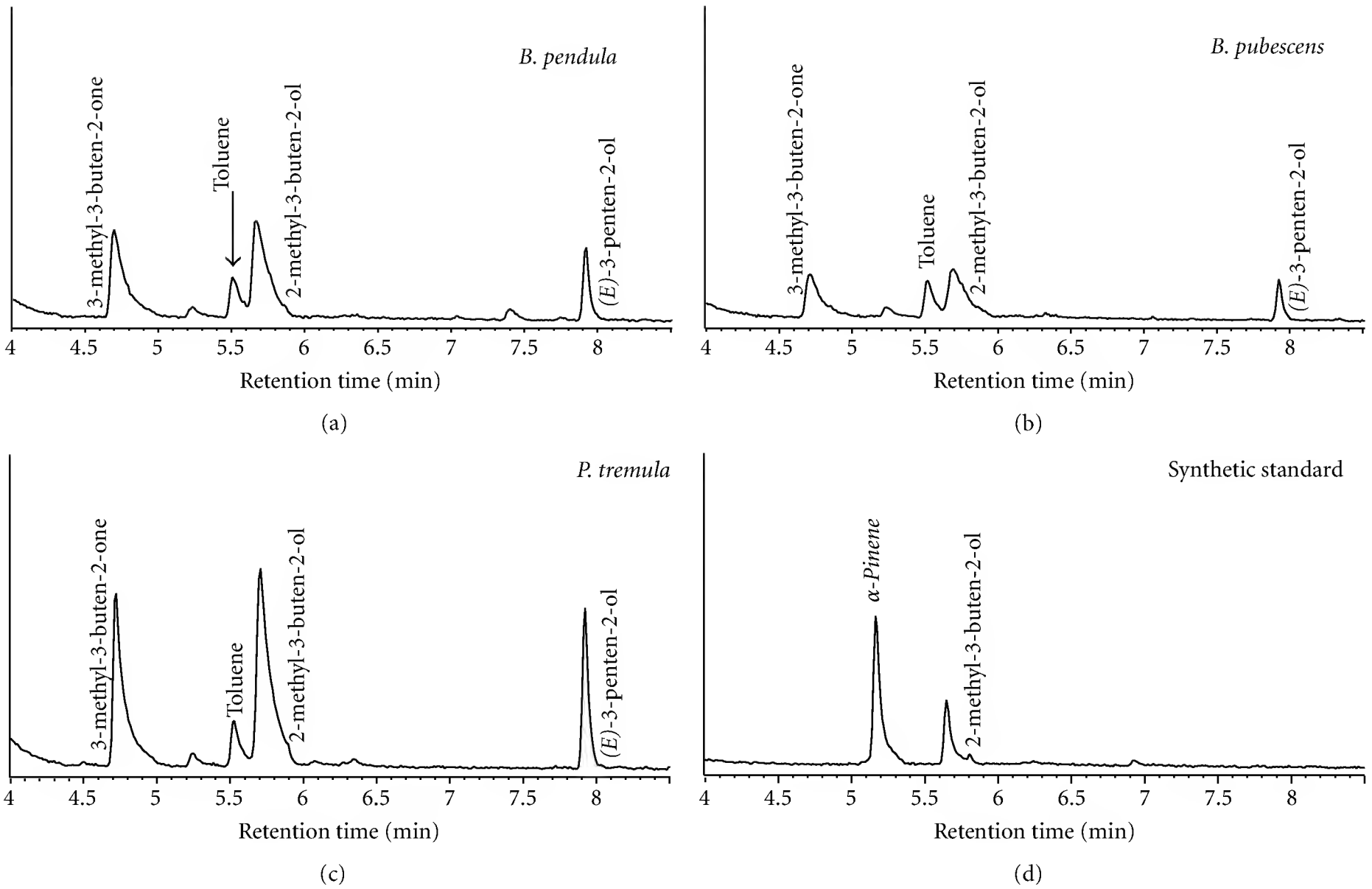

Figure 1: Gas chromatograms from bark extracts of *B. pendula, B. pubescens*, and *P. tremula,* and synthetic standard of 2-methyl-3-buten-2-ol (MB).

Table 2: Amounts of oxygenated hemiterpenes and (*E*)-3-penten-2-ol present in the bark extracts of deciduous trees, Asa, Sweden, June 11, 1998.

Compounds	Mean ± SD (μg/g DW bark)		
	B. pendula ($n = 2$)	*B. pubescens* ($n = 3$)	*P. tremula* ($n = 3$)
2-methyl-3-buten-2-ol (MB)	4.53 ± 1.37	2.51 ± 2.80	10.68±5.63
3-methyl-3-buten-2-one	3.83 ± 1.13	1.66 ± 2.06	7.42 ± 4.33
(*E*)-3-penten-2-ol	1.37 ± 0.46	0.61 ± 0.81	4.04 ± 2.02

ambient MB concentrations were very similar to those of isoprene, with known biogenic sources, and on the fact that MB concentrations did not correlate well with those of benzene, an indicator of anthropogenic source, they concluded that there was likely to be a large local biogenic source of MB, probably the lodgepole pine forest. Harley et al. (1998) successfully detected and measured the emission of MB from needles of several North American pine species, confirming MB as a biogenic VOC (BVOC) [26]. MB emissions from *Pinus ponderosa* were absent in the dark and strongly dependent on incident light, behaving similarly to net photosynthesis. The fact that MB emissions rapidly fall to near zero in darkness suggested that MB is being emitted immediately upon production, rather than stored in any specialized structures of pine needles. Their further screening study on 34 species of pines for MB emission in California showed that 11 species exhibited high emissions of MB (>5 μg C/g/h), and 6 emitted small but detectable amounts. All the emitting species are of North American origin, and most are restricted to western North America. Their results from both intact and severed branches indicated that MB emissions from pines may constitute a significant source of reactive carbon and a significant source to the atmosphere of acetone, a product of MB oxidation [26]. Interestingly, we find no MB records from European pines, and MB is not detected, based on its characteristic base ion $m/z = 71$, in the host tree *Picea abies* L. neither from bark extracts (C. Schiebe, unpubl.) nor emitted from foliage (M. Binyameen, unpubl.).

4.3. Microbial Sources. A plethora of short-chained BVOC is produced by yeasts and other microorganisms, including methyl butenol isomers [27, 28]. Somewhat surprisingly, the insect- and plant-produced 2-methyl-3-buten-2-ol is not reported in the microbial-related literatures, and is not found among VOCs analyzed with GC-MS from different types of cultured yeasts (M. Proffit, unpubl results).

4.4. Atmospheric Chemistry. Following the observation of MB emission in pine forest by Goldan et al. (1993) [25], several studies on the atmospheric chemistry of this BVOC alcohol have been actively carried out [29–35]. Harley et al. (1998) claimed that the major photochemical sink for MB

during daylight hours is assumed to be with OH^- [26]. The rate coefficients with similar values for the OH^- reaction with MB were reported by Rudich et al. (1995) and Ferronato et al. (1998), which suggested a relatively short atmospheric lifetime of ca. 2 hours [30, 34]. However, given typical atmospheric values of O_3 and NO_3, the rate constants for MB reaction with O_3 [32] or with NO_3 [33, 34] imply significantly longer MB lifetimes with respect to these destruction processes. Further reaction chamber experiments [29, 30] indicate that the MB–OH reaction leads to the production of acetone, glycol aldehyde, formaldehyde, and presumably 2-hydroxy-2-methylpropanal. The reaction with O_3 appears to yield the same major products, though in different proportions [29, 32]. Recently, Chan et al. (2009) suggested that photooxidation of MB might be a potential but minor source of secondary organic aerosol (SOA) [35]. Despite its structural similarity to isoprene, photooxidation of MB is not expected to make a significant contribution to SOA formation [35].

By using a model considering landscape average emission potential (μg C $g^{-1} h^{-1}$), total foliar density (g m^{-2}) (estimated by the available data on forest biomass and species composition), and emission activity factor, Harley et al. (1998) were able to compare the ambient concentrations of MB observed by Goldan et al. (1993) with their own enclosure rates of MB emission and found a reasonable agreement [25, 26]. Recent estimate of global MB emission is about 9.6 Tg per year [36, 37].

4.5. Potential Semiochemical Functions. MB seems to have multiple functions, including semiochemical, flavor, and pharmacological roles, and strong impact on atmospheric chemistry, which are dependent on its sources. The role of MB in the semiochemical system of *I. typographus* has been intensively studied. On the basis of dose-response curves from electroantennograms (EAGs), Dickens (1981) suggested that MB might act as a close-range/landing substance as it had a higher threshold (100 μg on filter paper) and very steep dose-response profile [38]. By using specially designed trap groups, Schlyter et al.(1987) clearly showed that MB does act as a close-range landing (or entering of trap holes) stimulus in the field [39]. However, our combined gas chromatographic-electroantennographic detection (GC-EAD) analysis of the bark extract samples of these three tree species showed no antennal responses by *I. typographus* to the existing MB (Zhang et al. unpubl.). It is mainly due to the fact that the amounts of MB in the extracts are much lower than the response threshold of *I. typographus* [38]. Furthermore, no MB was detected in the aeration samples of the undamaged stems, which might be caused by either the breakthrough of this compound through the Porapak Q trap or the minor amounts of release. Thus, the amounts of MB produced by the bark of nonhost birch or aspen, or emitted from the undamaged stems if any, most probably have no significant impact on the host selection behavior of *I. typographus* in the natural habitat. Goldan et al. (1993) doubted the source of MB from *I. typographus* and speculated an extra-insect source from host spruce trees [25]. In contrast, our GC-MS analyses of aeration samples of bark chips and cut branches with fresh needles of Norway spruce, *Picea abies*, did not find any MB at detectable levels [1]. In fact, no MB emitting trees had been discovered in Europe where the two bark beetles (*I. typographus* and *Ips* (*Orthotomicus*) *erosus*) use MB as parts of their pheromone systems prior to our current study.

Inclusion of MB in a trap consisting of a mixture of pheromone attractants for the spruce bark beetle, *Dendroctonus rufipennis*, was shown to reduce the number of *D. rufipennis* trapped, suggesting a possible antiattractant role of this alcohol [40]. However, MB exhibited no repellent properties when tested alone nor did it appear to have any effect on the aggregation response of two North American conifer bark beetles (*Ips paraconfusus* and *Dendroctonus brevicomis*) and their predators (Trogositidae and Cleridae) to their pheromones [41].

4.6. Biosynthesis. A biosynthetic study by Lanne et al. (1989), using radiolabeled precursors, clearly showed that MB is produced *de novo* by *I. typographus* through the mevalonic pathway [9]. The biosynthetic pathway in other insects (*Ips* (*Orthotomicus*) *erosus*, *I. nitidus* and *I. shangrila*, and *Vespa crabro*) and the Eurasian angiosperm trees (bark of birch and aspen) still remains unknown. However, the gene for MB synthase was recently identified from *Pinus sabiniana*, the MB producing pine species, and the protein encoded was functionally characterized by Gray et al. (2011) [42]. MB synthase is a bifunctional enzyme which produces both MB and isoprene in a ratio of ca. 90 : 1 [42] via dimethylallyl diphosphate (DMADP) [43]. Another oxygenated hemiterpene, 3-methyl-3-buten-2-one, was also found constantly from our bark extracts. It is not clear if this oxygenated hemiterpene is involved in the biosynthetic pathway of MB. Our results raise major questions regarding the evolution, tropospheric chemistry, and ecological role of this short, branched alcohol. They also suggest that comparative studies on the biosynthetic pathways for MB in different natural sources would be of considerable evolutionary interest.

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Research Article

Mesozoic Coleopteran Faunas from Argentina: Geological Context, Diversity, Taphonomic Observations, and Comparison with Other Fossil Insect Records

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The order Coleoptera is the most diversified group of the Class Insecta and is the largest group of the Animal Kingdom. This contribution reviews the Mesozoic insects and especially the coleopteran records from Argentina, based on bibliographical and unpublished materials (86 described species, 526 collected specimens). The material came from different geological units from the late Middle Triassic to the Late Triassic (Bermejo, Cuyo, and Malargüe basins) to the Middle-Late Jurassic and Early Cretaceous (Deseado Massif, Cañadón Asfalto, and San Luís Basin). The coleopteran record is composed of 29 described species with 262 collected specimens (isolated elytra) mainly represented by Triassic species and only four specimens recorded in Jurassic units, all of them currently unpublished. These fossil coleopterans provide fundamental information about the evolution of insects in the Southern Hemisphere and confirm the Triassic Argentinean insect deposits to be among the most important in the world.

1. Introduction

Continental invertebrate communities from the Mesozoic Era are represented principally by two phyla: Arthropoda and Mollusca. Arthropods constitute the most abundant and diverse fossil record in lacustrine sequences of Argentina with "conchostracans", insects and ostracods as most representative groups. The mollusks, represented by bivalve and occasionally gastropods, have a low diversity and restricted distribution [1].

The recent insects constitute the richest class in terms of species diversity with estimates ranging from 3 to 50 million species [2–5]. There are 1275 families of known insects in the fossil record and 967 presently existing, of which 70% are also known as fossils [6, 7]. The data from insect families/genera indicate that the group's growth model follows an exponential curve of evolution, contrary to the occurrence of new orders, which declines [8, 9].

The Coleoptera represents the most diverse order within the Class Insecta, both taxonomically and ecologically. In addition, it is the most specious group in the Animal Kingdom with the number of described modern species exceeding 350,000, representing about 40% of the known insect fauna [10]. This diversity is probably related to certain features that allow adult living in restricted niches while retaining their ability to fly [10].

The systematics and phylogeny of fossil beetles is a very complex area, with old and new proposals and numerous publications which surprisingly still not clear this question. Most studies use different methodologies and are based

mainly on adult morphology and rarely use immature stages of ontogenetic development. The basis of modern classification of fossil coleopterans was initially established by Crowson [10, 11].

The oldest record of the Coleoptera is from Paleozoic Era: Lower Permian deposits of the Wellington Formation from Oklahoma and Kansas (USA) [12, 13], Obora (Czech Republic) [14, 15], and the Chekarda, Ural region (Russia) [16].

Mesozoic beetles were much more common and diverse than Paleozoic; geological information shows that beetles have had a dominant record among the group of insects since early Jurassic [16]. However, further information still remains elusive as the Mesozoic beetles are less informative, because of isolate elytra, than those of the Paleozoic [11]. The Mesozoic associations consisted mainly of xylophagous forms and larval stages have been found in numerous localities, except in the Upper Cretaceous.

The aim of this paper is to present the record of abundance and diversity of Mesozoic beetles in Argentina. The information comes from a bibliographic compilation and study of materials found in recent paleontological expeditions conducted by our research group. At the same time, we provide information on the geology and age of localities where the material was collected and present a comparison with other Mesozoic groups of insects collected in Argentina and contemporary faunas from southern Hemisphere. Finally, it provides paleobiogeographic information and highlights the importance of beetles in understanding the evolution of the Mesozoic after the Permian Triassic extinction event.

Our analyses are based primarily on elytra in the Upper Triassic localities from Argentina, which provide a significant amount of information for Coleoptera and allow to have a vision of the composition of the assemblages about 252 million years ago. Cretaceous and Jurassic materials are mentioned in passing, and their study is in progress.

2. Previous Coleopteran Records

Contributions involving the study of beetles in Mesozoic continental sediments of Argentina began with Frenguelli [17, 18], who observed small curculionid elytra in shale samples from southwestern Mendoza Province (stratigraphical levels unknown) and other beetle elytra originating from various levels of the Ischigualasto Formation (probably levels of the Los Rastros Formation *sensu* Stipanicic and Bonaparte [19]), San Juan Province. Fossa-Mancini [20] reported the presence of galleries related to burrowing larvae of certain xylophagous beetles in silicified trunks from Upper Cretaceous of Patagonia. Feruglio [21] mentioned fossil beetles represented by silicified incomplete elytra, thorax, and abdomen remains, probably attributable to the Elateridae, from Laguna del Molino locality (Gran Bajo de San Julián) in Santa Cruz Province.

Subsequently, Genise [22] presented a description of different ichnofossils found in fossil trunks and fructifications from the Upper Cretaceous of Rio Negro Province, assigned to the probable activity of beetle and termites larvae. Further, Genise and Hazeldine [23] described insect traces in fossil wood from La Matilde Formation from the Jurassic Petrified Forest of Jaramillo in Santa Cruz Province, probably assigned to the activity of buprestid larvae. Martins-Neto and Gallego [24] disclosed the remains of beetles consisting of isolated elytra and body parts from the La Matilde Formation of Gran Bajo de San Julián in Santa Cruz Province, assigned mainly to Caraboidea. Gallego et al. [25] reported a second discovery of fossil beetles as *Argentinocupes* and *Argentinosyne* and other insects as blattids and hemipterans in the Bermejo Basin in San Juan Province and the first record from the upper Los Rastros Formation of this basin.

The Order Coleoptera was also treated in the works of Martins-Neto et al. [26–30] and Martins-Neto and Gallego [31], where new Triassic species assigned to Permosynidae, Schizocoleidae, Cupedidae, and Elateridae were described. The material (elytra) was collected in Ischichuca and Los Rastros Formations of the Bermejo Basin in La Rioja Province and Potrerillos and Cacheuta Formations, Cuyo Basin of Mendoza Province. Monferran et al. [32] mention a new locality with a record of Coleoptera: Estancia Fossati, Puesto Almada Member of the Cañadón Asfalto Formation, from Middle to Late Jurassic in age. Brauckmann et al. [33] describe two elytra of Permosynidae (*Ademosyne rosenfeldi* and *Ademosyne llantenesensis*) from the Llantenes Formation of the Malargüe Basin, Mendoza Province.

3. Material and Methods

Triassic specimens from the Cuyo Basin originate from three areas: (a) south of Cerro Cacheuta at the Puesto Miguez, Quebrada del Durazno and Agua de las Avispas localities, in Potrerillos and Cacheuta strata of the Upper Triassic; (b) north of Cerro Bayo at the Quebrada del Cerro de las Cabras locality in Cerro de las Cabras Formation of the Middle Triassic and Quebrada del Puente locality in Potrerillos Formation of the lower Upper Triassic; and (c) southeast of Cerro de los Colorados, Paramillos de Uspallata, in strata of the Cacheuta Formation (Upper Triassic).

In the Malargüe Basin, material was collected from the upper portion of the Llantenes section of the Llantenes Formation (Late Triassic). In the Bermejo Basin, the fossil insects come from Río Gualo, Picos Gemelos, Agua Escondida, Quebrada de Ischichuca Chica, and Chañares localities, belonging to Los Rastros (early Late Triassic) and Ischichuca (late Middle Triassic to early Upper Triassic) Formations, in La Rioja Province.

Jurassic beetles were collected from the La Matilde Formation of Middle to Late Jurassic ages, Laguna del Molino locality (Gran Bajo de San Julián), and from Estancia El Malacara locality (Bahía Laura), from Santa Cruz Province, and the Cañadón Asfalto Formation, of Middle to Late Jurassic age, from the Estancia Fossati locality, Chubut Province.

Cretaceous insects originate from the Anfiteatro de Ticó Formation, of the Baqueró Group, Bajo Grande, in Santa Cruz Province and La Cantera Formation, Gigante Group, in San Luis Province; both are from the Early Cretaceous.

It is important to emphasize that material collected in the Potrerillos-Cacheuta sequences during an expedition occur-

ring in April of 2010 was included in our analyses, as was Jurassic specimens from Chubut Province, collected during a 2009 fieldtrip and material from the Los Rastros Formation. This newly discovered material is important for understanding beetle evolution in Argentina during the Mesozoic because it is so diverse, abundant and well preserved.

The material cited in the literature is deposited in the paleontological collection with the acronyms PULR-I (Invertebrate Paleontological Collection, Universidad Nacional de La Rioja, La Rioja Province), CTES-PZ (Paleozoological Collection of the Universidad Nacional del Nordeste, Corrientes Province), MCNAM (Museo de Ciencias Naturales y Antropológicas "J. C. Moyano", Mendoza Province), MHIN-UNSL-GEO (Museo de Historia Natural de la Universidad Nacional de San Luis, San Luis Province), MLP (Museo de La Plata, Invertebrate Paleontology, La Plata, Buenos Aires Province), and CORD-PZ (Palaeozoological Collection, Universidad Nacional de Cordoba, Córdoba Province).

4. Mesozoic Insects Record from Argentina

Most information on the Mesozoic insect faunas of Argentina comes from the Triassic Period and, above all, from collections made of the Los Rastros Formation, Bermejo Basin (La Rioja), and Potrerillos Formation, Cuyo Basin (Mendoza). Both provide 90% of the total abundance of insects in Argentina, constituting one of the most important records of continental life developed in Gondwana. In addition, these basins are known for their large extent, exceptional outcrops, well-developed stratigraphy columns, and the wealth of their taphofloras and vertebrate faunas.

In Argentina, knowledge of Mesozoic insects has made remarkable strides in recent years, having as a background the works published by Wieland [34, 35], Tillyard [36], and Cabrera [37] on fossil insects from the Cacheuta and Potrerillos Formations of early Late Triassic age. So far, 86 species in 27 families of 12 orders have been described from 526 samples collected (Figure 1). The fossil insect fauna of Argentina comes from stratigraphic levels of continental sediments assigned to the interval between the upper Middle Triassic to Lower Cretaceous.

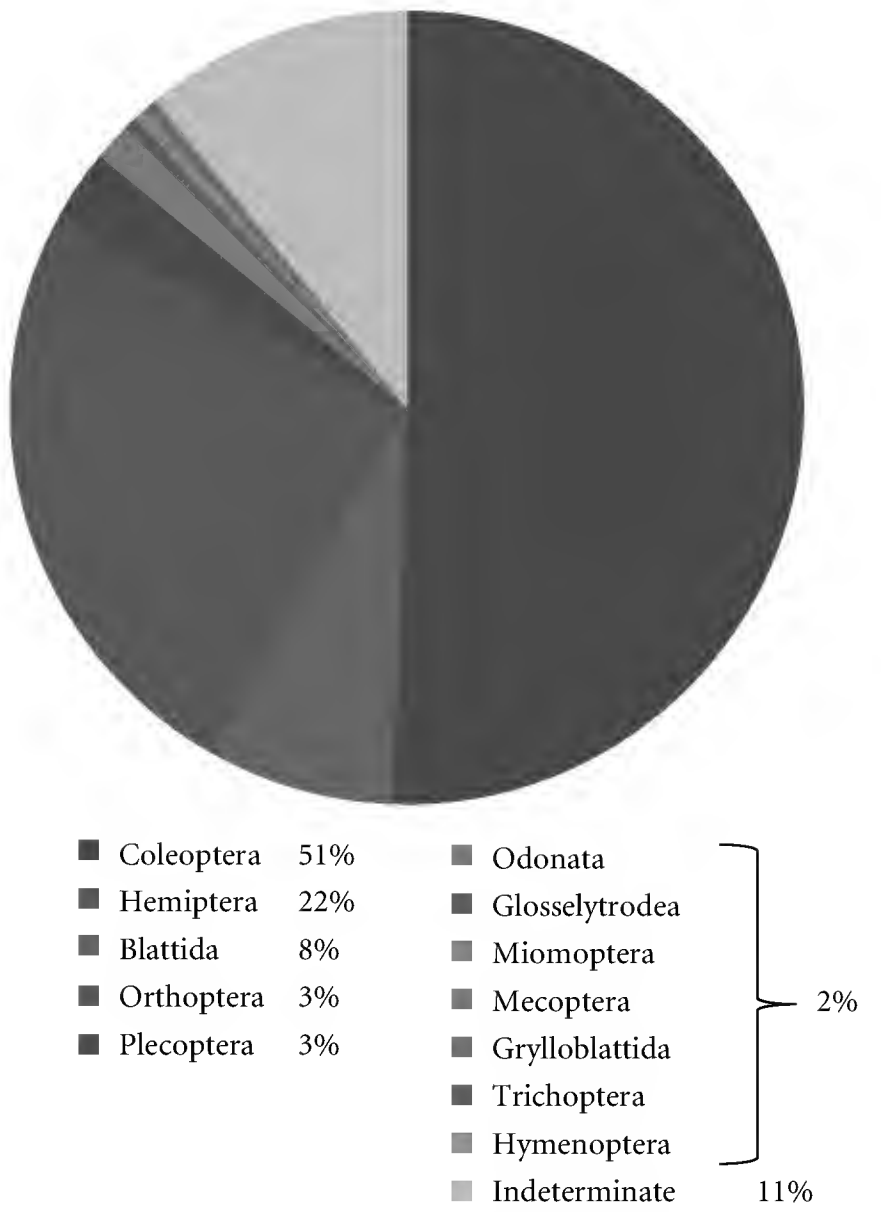

FIGURE 1: Pie-chart showing the abundance of Mesozoic specimens from Argentina, grouped by taxonomic order.

4.1. Insects Records and Geological Context (Figure 2)

4.1.1. Triassic. The fossiliferous potential of the Argentina Triassic units is highly significant, based on 510 collected specimens and 81 described species. The insect record comes from Los Rastros and Ischichuca Formations of the Bermejo basin, La Rioja Province; Potrerillos, Cacheuta, and Cerro de las Cabras Formations of the Cuyo Basin, Mendoza Province; and Llantenes Formation of the Malargüe Basin, southern Mendoza Province (Figure 3).

The Cuyo Basin of central western Argentina is composed of thick sedimentary sequences from the Middle to Late Triassic that constitute the Uspallata Group. These units have a rich and well-known *Dicroidium* flora, as well as a microflora and a fauna as invertebrates, fishes, and tetrapods that are interpreted as dwelling in fluvial-lacustrine systems. Insect collections of coleopterans, blattids, hemipterans, miomopterans, orthopterans, trichopterans, plecopterans, odonatans, and grylloblattids originate from the Cerro de las Cabras Formation (1 specimen), the Potrerillos Formation (21 described species and 229 collected specimens), and the Cacheuta Formation (2 described species and 27 specimens) [29, 30, 39–43].

The Malargüe Basin of southern Mendoza Province includes Choiyoi volcanic and overlain siliciclastic deposits. The insect fauna was collected from the upper section of the Llantenes Formation (Late Triassic) which is built of two coarsening-upward cycles reflecting a deltaic progradation of a fluvial into a lacustrine environment (lower part), succeeded by repeated progradation into a floodplain dominated environment (upper part; with insects, conchostracans, fish, and plants remains). The insect remains includes 2 coleopteran elytra and 1 mecopteran, and these news finds represent the youngest Triassic occurrence from Argentina and South American and the second youngest record from the Southern Hemisphere [33].

The Los Rastros and Ischichuca Formations (Agua de la Peña Group, La Rioja Province) are part of the Bermejo Basin (Ischigualasto-Villa Unión Basin), an extant basin located in northwestern Argentina. The Los Rastros Formation of early Late Triassic age is a lacustrine-deltaic that consists of several sedimentary cycles of black shales, siltstones, and sandstones. The succession is characterized by record of five plants, four invertebrates, and four vertebrates taphofacies [44]. The

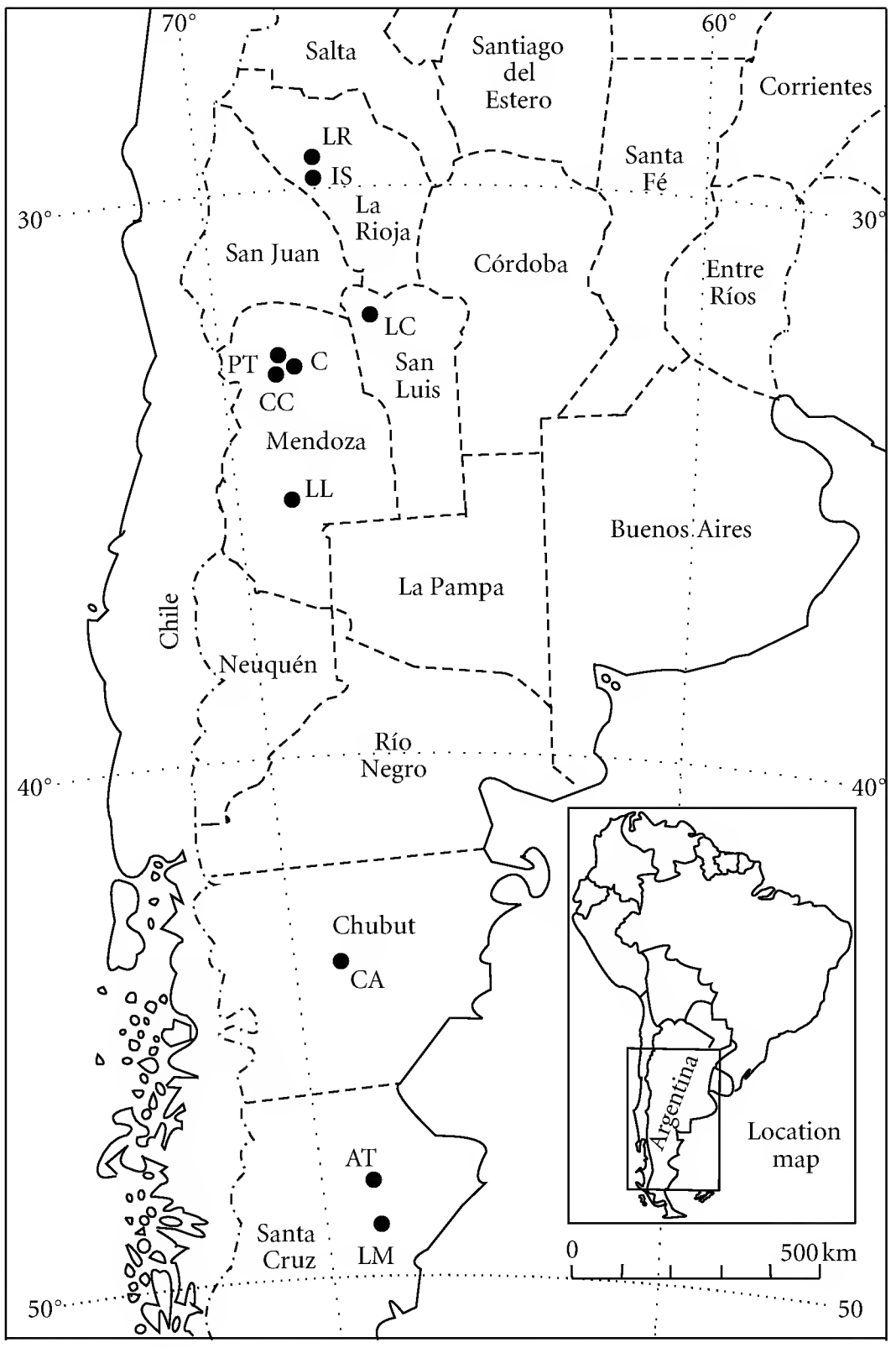

FIGURE 2: Map showing the Mesozoic units and outcrop areas approximately that carried the insect fauna mentioned in the text. AT: Anfiteatro de Ticó; C: Cacheuta; CA: Cañadón Asfalto; CC: Cerro de las Cabras; IS: Ischichuca; LC: La Cantera; LL: Llantenes; LM: La Matilde; LR: Los Rastros; PT: Potrerillos. (modified from Zavattieri [38]).

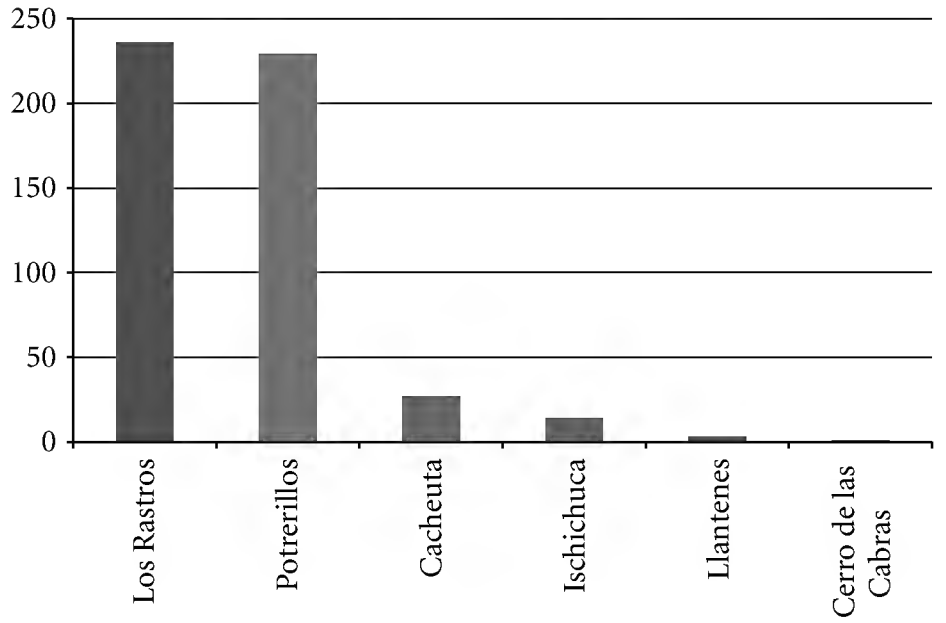

FIGURE 3: Bar graphic showing the abundance of specimens in different Triassic insect localities. (Note: included new undescribed materials).

insect record consists of 44 described insect species and 236 collected specimens and is comprised of coleopteran elytra and wing remains of blattids, plecopterans, miomopterans, orthopterans, glosselytrodeans, and odonatans [26, 27, 42, 45]

The Ischichuca Formation of late Middle Triassic to early Late Triassic age is predominantly composed of shallow and deep lacustrine facies with progradational deltaic successions, that starts to begin as fanglomerates with sandstones and tuffitic intercalations and continue with dark carbonaceous shales alternating with sandstones and pelitic and tuffaceous strata. In particular, insect remains are found in dark brown to olive-green claystones with abundant plant remains from the middle to the basal upper parts of the unit. This interval is interpreted as a shallow, partly saline lake (interpreted from a perennial playa lake association facies, L2 from Melchor [46, 47]) that ranges to a deep freshwater lake (deep freshwater lake association facies, L3 from Melchor [46, 47]).

The fossil insects, consisting of 11 described species and 14 specimens, comprise a blattid wing and coleopteran elytra [31, 41, 48].

4.1.2. Jurassic. The Jurassic insect fauna from Argentina (Table 1) is less well known than the Triassic one and also sparse when compared with other Jurassic localities of the world. This rarity is due to its restricted geographic distribution and low species diversity and is related to the low number of collections and studied localities.

The first contribution probably was made by Frenguelli [18] who mentioned and illustrated the "rare insect remains" of a paleohemipteran wing from the "Estancia El Malacara" from Bahia Laura, in Santa Cruz, Province. In the 1990s one of us (O. F. Gallego) and Rafael Martins-Neto restudied this specimen and concluded that it is probably not an insect. Later, Feruglio [21] reported the presence of silicified coleopteran remains from the Laguna del Molino locality of Gran Bajo de San Julian, in Santa Cruz Province) associated with plant fragments, bones, freshwater mollusks, and conchostracans. These previous contributions of insect reports were summarized by Gallego and Martins-Neto [49], Martins Neto and Gallego [24, 50], Monferran et al. [32, 51, 52], Genise et al. [53], and Gallego et al. [54] who reported new finds of insects from the orders Coleoptera (elytra and body remains), Heteroptera (fragmentary wings), and Trichoptera (wing fragments and larval cases) from both the La Matilde Formation of the Laguna del Molino locality and Cañadón Asfalto Formation of Cerro Cóndor and Estancia Fossati localities. These records are associated also with gastropods, bivalve mollusks, ostracods, conchostracans, plants, and fish remains. Other Jurassic insect records such as the presence of chironomid head capsules and larval cases [55] and mecopterans (Bittacidae) with complete bodies [56] come from Gan Gan locality of the Cañadón Asfalto Formation in Chubut Province.

The Jurassic fossil insect record from Argentina comes from two geological units: the La Matilde Formation (late Middle Jurassic, Santa Cruz Province) and the Cañadón Asfalto Formation of late Middle Jurassic to Late Jurassic age, Chubut Province.

The late Middle Jurassic La Matilde Formation comprises a volcaniclastic sequence that bears silicified woods (ferns and gymnosperms), a taphoflora, invertebrates, and tetrapods. The sediments show a typical continental sequence of a low-energy fluvial system, with lentic water bodies under reduced conditions within a floodplain environment, influenced by intensive volcanism evidenced by pyroclastic deposits [57].

The Cañadón Asfalto Formation of Middle-Late Jurassic age is a thick sedimentary sequence with volcanic intercalations that constitutes one of the most important nonmarine Jurassic records from South America. Two members can be distinguished, the lower one, the Las Chacritas Member, and the upper one, the Puesto Almada Member [58]. The insects were found in the upper member and particularly from the locality "Estancia La Sin Rumbo", an assemblage of "conchostracans", ostracods, bivalves, and caddisfly cases. This assemblage occurs in the upper part of a volcaniclastic lacustrine sequence consisting of yellowish tuffs and tuffites providing evidence of dry climatic conditions. The assemblage recorded from the Estancia Fossati locality came from three levels consisting of shales, limestones, and tuffs, associated with invertebrates such as bivalve mollusks, insect larval cases, ostracods, conchostracans), and fish scales. The Estancia Fossati faunas represent low-energy shallow freshwater environments within associated plant communities [54, 59].

Table 1: Mesozoic insects from Argentina.

Order	Triassic	Jurassic	Cretaceous	Mesozoic insects
Coleoptera	262	4		266
Hemiptera	104	4	6	114
Blattida	39		1	40
Orthoptera	16			16
Plecoptera	16			16
Odonata	7		1	8
Glosselytrodea	3			3
Miomoptera	2			2
Mecoptera	1			1
Grylloblattidae	1			1
Trichoptera	1			1
Hymenoptera	1			1
Indeterminate	57			57
Total	510	8	8	526

4.1.3. Cretaceous. The poorly known Cretaceous insect fauna from Argentina is reflected by only 5 described species and only 8 collected specimens (Table 1). These records are based on two Early Cretaceous localities: (a) the Anfiteatro de Ticó Formation, Bajo Grande locality, from Santa Cruz Province, and (b) the La Cantera Formation, Gigante Group of San Luis Province.

The Anfiteatro de Ticó Formation of the Baqueró Group is composed by siliciclastic and volcaniclastic deposits [60] and the insect fauna consisting of the species *Blattulopsis popovi* (Blattida) [61] and *Argentinopetala archangelskyi* (Odonata) [62].

The La Cantera Formation of the Gigante Group exhibits greenish to gray limonite and arcilite with intercalated red sandstones and shales in the upper part [63]. The La Cantera assemblage is composed of a large, diverse association of ostracods, insects (hemipterans, orthopterans, coleopterans and caddisfly larval cases), palynomorphs, plants (leaves and sphenopsid reproductive organs), and fish fragments [64]. The paleontological content of this deposit suggests a lakeshore environment, attributable to fish taxonomic diversity and the poor state of terrestrial insect preservation [65]. Insect remains comprise of *Canteronecta irajae* (Naucoroidea) [66], *Rhomboidella popovi* (Corixidae) [67] and

Notonecta mazzoniae (Notonectidae) [65]. Also are recorded the poorly preserved orders Coleoptera, Orthoptera, and Trichoptera.

5. Mesozoic Coleopterans from Argentina

The analysis of Coleoptera used information obtained from previous publications [21, 26–33, 39, 44]. Information was also obtained from observation of new unpublished material collected in the 2010 expedition to the Potrerillos Formation of Mendoza Province (Figures 4(f)–4(r)), Los Rastros Formation of La Rioja Province, and Jurassic specimens from Cañadón Asfalto Formation of Chubut Province.

Coleoptera is the most abundant and exhibits the greatest speciosity among Mesozoic insect fauna in Argentina. Until now, 262 specimens have been collected, covering about 50% of the total abundance, and 29 species have been described, including the permosynids *Ademosyne umutu*, *A. llantenesensis*, *A. rosenfeldi*, *A. arcucciae* (Figure 4(c)), *A. punctuada* (Figure 4(d)), *A. elongatus*, *A. hexacostata*, *Ademosyne sp. 1*, *Ischichucasyne cladocosta*, *and Delpuentesyne menendezi*; the schizocoleids *Argentinosyne ischichucaensis*, *A. duraznoensis*, *A. bonapartei*, *A. frengüelli* (Figure 4(b)), *A. rugosa*, *A. gualoensis*, *A. gonaldiae*, *and A. losrastrosensis*, Gen. et sp. indet. 1 and 2; the elaterids *Babuskaya elaterata, Gemelina triangularis, Cardiosyne obesa, C. elegans*; the cupedids *Argentinocupes sara, A. pulcher* (Figure 4(a)), *and A. abdalai* (Figure 4(e)); and two specimens Gen. et sp. indet. 1 tentatively assigned to the Permosynidae family and other specimen (Gen. et sp. indet. 1) of uncertain position.

From an analyses of the information about Triassic beetles, the best represented groups are families Permosynidae (135 specimens) and Schizocoleidae (40 specimens); the genera *Ademosyne* (129 specimens) and *Argentinosyne* (39 specimens); and the species *Ademosyne arcucciae* (96 specimens, Figure 4(c)) and *Ademosyne hexacostata* (17 specimens). Ninety-eight percent of the specimens collected are from Triassic continental sedimentary rocks, from Los Rastros Formation (69% of total abundance), Potrerillos Formation (17%), Cacheuta Formation (9%), Ischichuca Formation (4%), and Llantenes and Cerro de las Cabras Formations (2%).

The beetles preserved in the various localities appear as complete impressions, body part impressions (abdomens, thoraces), and as elytra moulds that can be complete or fragmentary, articulated or disarticulated, smooth or striated, with or without ornamentation.

Two analyses were performed. The first considered the state of preservation, namely, if the specimen was complete/incomplete, and its degree of articulation/disarticulation. For these measurements, there were used 262 elytra distributed in the different formations as shown in Table 2.

The beetle fauna consists mainly in complete and disarticulated elytra (211 samples; Figures 4(a), 4(d), 4(f)–4(l), 4(n)–4(r)), followed by incomplete and disarticulated elytra (41 samples), complete and articulated elytra (8 samples; Figures 4(b), 4(c), 4(e), 4(m)), and last incomplete and articulated elytra (2 samples) (Figure 5).

In the second analysis, elytra ornamentation was observed, used in describing the following specimens: 187 specimens from Bermejo Basin and 8 from Malargüe and Cuyo Basins. Accordingly, the ornamented elytra may be smooth (46 specimens; Figures 4(b), 4(f), 4(g), 4(k), 4(l), 4(m), 4(o), 4(q) and 4(r)) or striated (149 specimens; Figures 4(a), 4(c)–4(e), 4(h), 4(i), 4(n), 4(p)). In the case of striated elytra, costae may be smooth (91%; Figure 4(c)), punctuate (6%; Figure 4(d)), or granular (3%). The number of costae can vary between 6 and 11. Elytra also can have ornamentation (173 specimens) or lack it (22 specimens). Lastly, the ornamentation can be granular (93%; Figure 4(c)), rough (5%), with rows of cells (1%, Figure 4(a)), or striated (1%).

5.1. Jurassic and Cretaceous Coleopterans. Knowledge of beetles and of insects in general from Jurassic and Cretaceous sediments in Argentina is poorly developed. This absence of data highlights the importance of exploring for new fossil insect localities. In general, beetles are only referenced in works that treat other insect groups. By contrast, information generated for the Jurassic and the Cretaceous of Argentina will allow its characterization and comparison with other continental faunas from the Southern Hemisphere (South America, Antarctica, and Australia), currently which are better known. Therefore, owing to the scarce information about the Jurassic and Cretaceous specimens, the present study has considered only examination of Triassic material.

6. Comments on Other Insect Orders from Argentina (Figure 6)

Hemiptera is the second most common order in the Mesozoic from Argentina with 114 specimens collected (22%) and 19 described species from various assemblages. The material is preserved as impressions of wings (fore or hind, complete or fragmentary, with or without a clavus) and occasionally complete bodies of insects, most attributable to the families Dysmorphoptilidae (9 specimens) and Scytinopteridae (7 specimens); the genus *Gallegomorphoptila* (9 specimens); and the specie *Gallegomorphoptila acostai* (5 specimens). For the rest of the Mesozoic, there are only three recorded Cretaceous species: *Canteronecta irajae* (Naucoroidea) [66], *Rhomboidella popovi* (Corixidae) [67], and *Notonecta mazzoniae* (Notonectidae) [65].

The Blattida (cockroaches) includes 40 specimens (7%) and 18 species, preserved as disarticulated tegmina (complete or fragmentary, with missing or not the clavus). The assemblage is dominated by the families Mancusoblattidae and Mesoblattinidae (10 specimens) and by the genus *Hermosablatta* (9 specimens). The species *Samaroblatta corrientesina*, *S. gualoensis*, *Hermosablatta crassatella*, *H. pectinata*, *Lariojablatta neiffi*, and *Condorblatta lutzae* are the most abundant taxa. The *Blattulopsis popovi* species occurs in the Lower Cretaceous of Santa Cruz Province [61].

The rest of the orders, Plecoptera and Orthoptera with 16 specimens, Odonata with 8 specimens, Glosselytrodea with

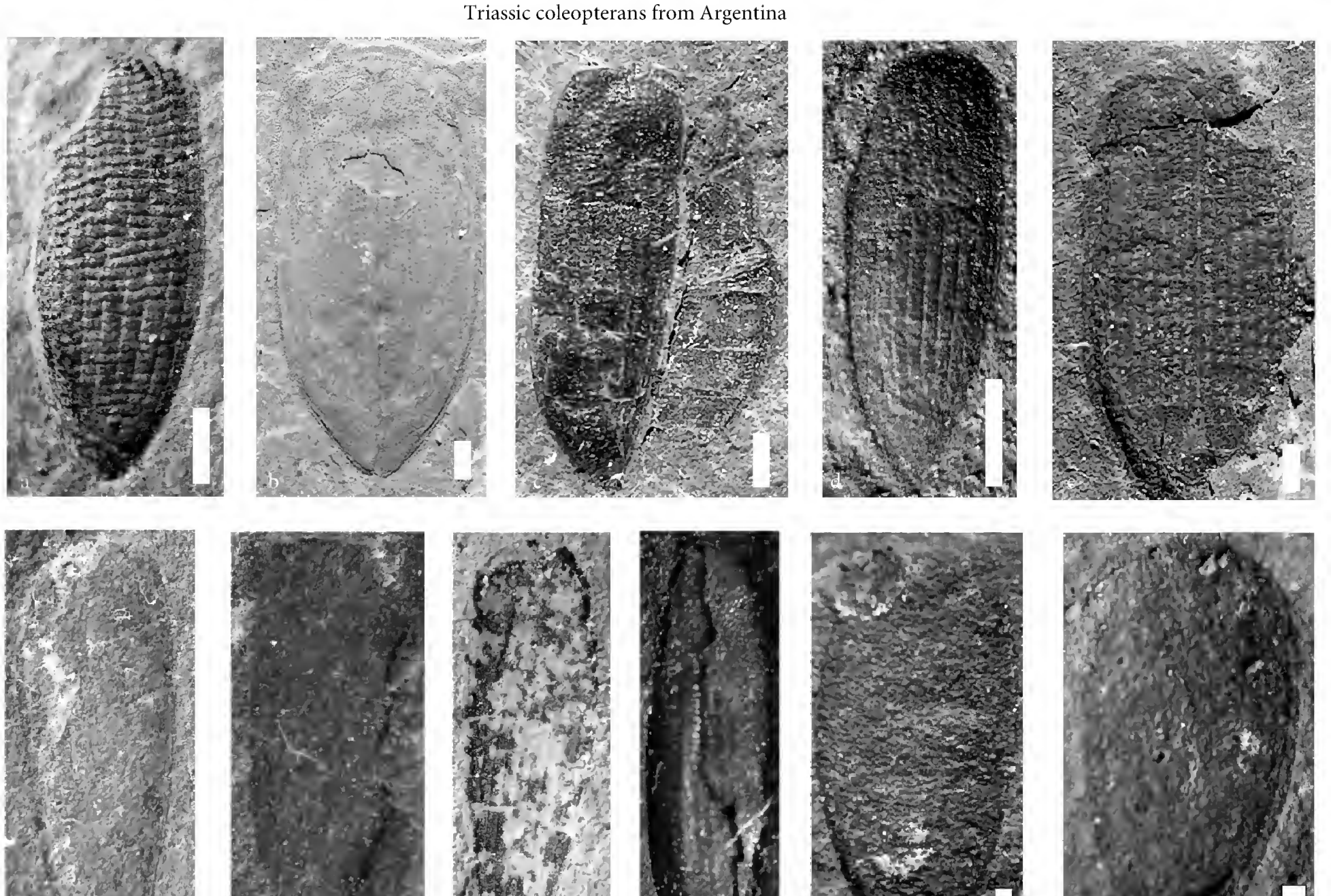

FIGURE 4: Examples of fossil insects collected from Triassic insects localities, Western Argentina, illustrating the stage of preservation seen in the localities. (a) *Argentinocupes pulcher*, complete desarticulated elytron. (b) *Argentinosyne frengüelli*, complete articulated elytron. (c) *Ademosyne arcucciae*, complete articulated elytron and thoracic and abdominal elements. (d) *Ademosyne punctuada*, complete desarticulated elytron. (e) *Argentinocupes abdalai*, complete articulated elytron (Martins-Neto et al., [27]); (f)–(r) Indet. material: complete articulated elytron (m) and complete desarticulated elytron ((f)–(l), (n)–(r)). Ornamentation of elytra: smooth ((b), (f), (g), (k), (l), (m), (o), (q), (r)) and striate ((a), (c)–(e), (h), (i), (n), (p)). Scale bar: 1mm.

Table 2: State of preservation of the Triassic beetles.

Elytron	Formation					
	Los Rastros	Ischichuca	Potrerillos	Cacheuta	L1antenes	Cerro de las Cabras
Articulated complete	5	0	3	0	0	0
Articulated incomplete	2	0	0	0	0	0
Disarticulated complete	140	5	41	24	1	0
Disarticulated incomplete	33	5	0	1	1	1
Total	180	10	44	25	2	1

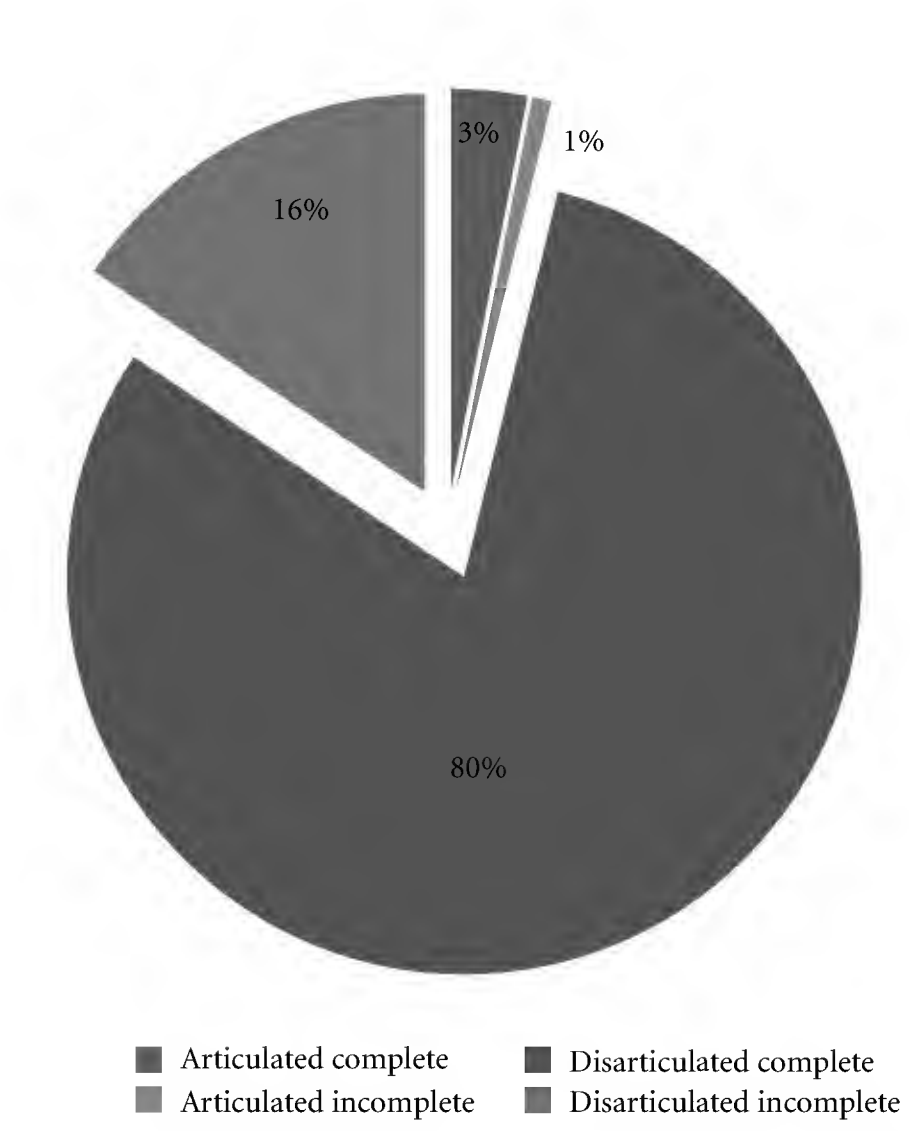

Figure 5: Pie-chart showing the relative percentage of specimens from beetle locality collection, grouped by element type.

3, Miomoptera with 2 specimens, and Mecoptera, Grylloblattida, Trichoptera, and Hymenoptera, all with one specimen each, constitute 9% of the total abundance of the material collected. Each one is represented by a handful of impressions of fore wings and complete or fragmentary bodies of nymphs such as head and abdominal sclerites.

The label "indeterminate" record (57 specimens, 11%) includes the complete, incomplete or poorly preserved elements, in which we were unable to be confidently assigned to any particular order or alternatively it is under study. They are represented by impressions of wings (complete or fragmentary), some elements associated with abdominal sclerites and partial bodies.

7. Paleoecology and Taphonomy

Given the aforementioned information on the Mesozoic insect fauna of Argentina, the material recovered includes disarticulated and fragmentary specimens (evidence of postmortem transportation) approximately 97.5% of which comprises mostly impressions of wings of hemipterans and blattids, elytra of beetles, and other isolated body parts (thoraces, abdomens, appendages) of the Orthoptera, Mecoptera, Grylloblattida, Plecoptera, Trichoptera, Miomoptera, Odonatoptera, Hymenoptera, and Glosselytrodea, in addition to head capsules and dwelling tubes of chironomid and caddisfly cases.

From an ecologic perspective, the analysis of this fauna shows that most groups have a subaerial or terrestrial habit as adults, and some are represented by immature (often aquatic forms), such as the Plecoptera, Grylloblattida, Hemiptera, and Odonata. This implies that the fossil record is biased by the absence of other autochthonous aquatic forms, the causes of which required future inquiry.

8. Final Considerations

Fossil beetles are one of the most interesting objects in paleontological and stratigraphical research, but they remain poorly understood, as they are difficult to study [68]. The great diversity and abundance that occurs in the fossil record is probably correlated with the composition of the elytra and its shape preservation, appearing as elytra moulds that can be articulated or disarticulated.

In South America, the previous literature reviews and the analyses of unpublished material indicate the relevance of Triassic and Tertiary beetle records. Nevertheless, the group is poorly studied and the intensity of collections is low. Therefore, it requires much more skilled work.

The record (Table 3) is restricted to continental sediments from Brazil, Argentina, Chile, and Peru. In Brazil, 17 species were discovered and come from Early to Middle Permian ages of Irati Formation [69, 70] and from Middle to Late Triassic ages of the Santa María Formation [71] of the Paraná Basin, from the Early Cretaceous age of Santana Formation in Araripe Basin [72–75], from the Oligocene age of Tremembé Formation in Taubate Basin and Fonseca Formation in Fonseca Basin [76–78]. In Argentina the order is restricted to Late Triassic age with twenty nine species described [25–31, 33] from Los Rastros and Ischichuca Formations in La Rioja Province, the Cortaderita Formation in San Juan Province, the Potrerillos, Cacheuta, Cerro de las Cabras, and Llantenes Formation in Mendoza Province; Middle to Late Jurassic ages of the La Matilde Formation in Santa Cruz Province and the Cañadón Asfalto Formation in Chubut Province [21, 24, 32]; Middle to Late Eocene

Triassic insects from Argentina

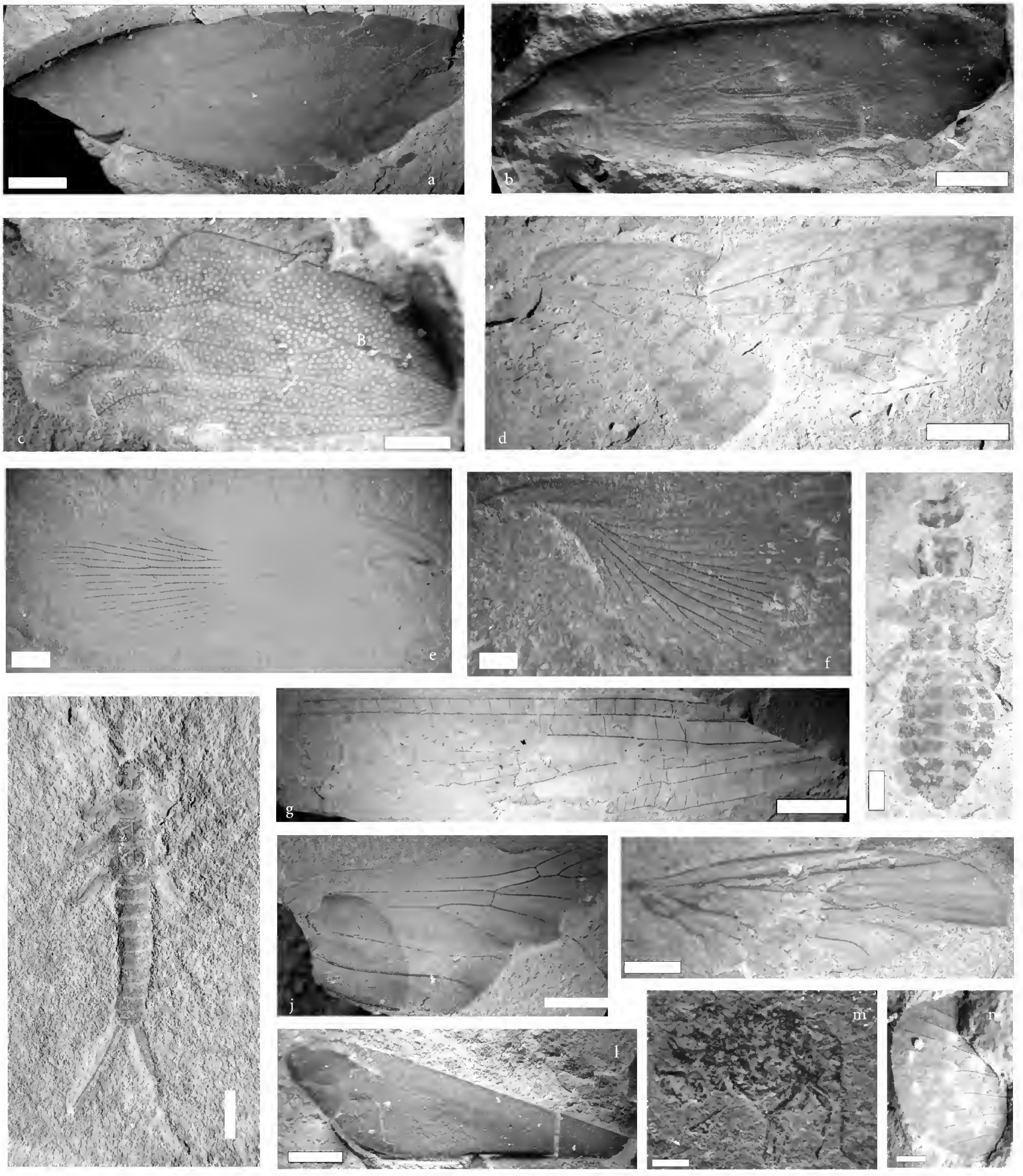

FIGURE 6: Examples of fossil insects collected from Triassic insects localities, Western Argentina, illustrating its diversity and the stage of preservation (impression) seen in the localities. (a), band (c): Hemiptera. (d): Orthoptera. (e): *Mancusoblatta pulchellaand* (f): *Hermosablatta crassatella* (Blattida), Martins-Neto et al., [45]. (g) and (h): Odonata adult and nymph, respectively. (i): *Platyperla marquati* (nymph), Gallego et al., [54]. (j), (k), (m), and (n): indet. material. l: Hemiptera clavus. Scale bar: 1 mm (except (a), (b), (c), and (i): 2 mm).

Table 3: Main stratigraphic units and beetle localities in South America.

Erathern Era	System Period	Series Epod	Argentina	Brazil	Chile	Perú
Cenozoic	Quaternary	Holocene				
		Pleistocene				Talara
	Neogene	Pliocene				
		Miocene	Palo Pintado Formation			
	Paleogene	Oligocene	Ventana Formation	Tremembé and Fonseca Formation		
		Eocene	Lumbrera Formation			
			Maíz Gordo Formation			
		Paleocene				
Mesozoic	Cretaceous	Upper			Dorothea Formation	
		Lower		Santana Formation		
	Jurassic	Upper	Cañadón Asfalto Formation			
		Middle	La Matilde Formation			
		Lower				
	Triassic	Upper	Llantenes Formation		Santa Juana Formation	
			Cacheuta Formation			
			Potrerillos Formation	Cortaderita Formation		
			Los Rastros Formation	Santa María Formation		
		Middle	Cerro de las Cabras Formation			
			Ischichuca Formation			
		Lower				
Paleozoic	Permian	Upper				
		Middle		Iratí Formation		
		Lower				

ages from the Lumbrera Formation (Grupo Salta) in Salta Province [79]; Late Paleocene to Early Eocene age from the Maíz Gordo Formation in Jujuy Province with thirty one species [80–84]; Eocene to Early Oligocene age from the Ventana Formation in Neuquen Province [85]; and Late Miocene age from the Palo Pintado Formation in Salta Province [86]. In Chile the material just comes from Santa Juana Formation of Late Triassic age with one species [87] and Dorothea Formation of Upper Cretaceous [88]. In Peru the specimens come from the Pleistocene tar-seeps of Talara in Piura Province [89].

The Mesozoic localities in Argentina, typically from the Triassic Period, have proven to be rich in fossil insect material; recent findings of insects in the strata of the Cuyo Basin have increased the number of taxa represented to currently 510 specimens. This makes Argentina one of the most important paleoentomologic regions not only in South America but also generally in the Southern Hemisphere, such as the Triassic sequences already known from Australia and South Africa.

All the knowledge provides invaluable information about the composition of the Mesozoic biota, essential for understanding biological processes that different groups of organisms experienced after the great Permian extinction event about 252 million years ago. In addition, time series of taxa within lineages could provide data for understanding beetles evolution.

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Research Article

Foraging Behavior of the Blue Morpho and Other Tropical Butterflies: The Chemical and Electrophysiological Basis of Olfactory Preferences and the Role of Color

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Inside a live butterfly exhibit, we conducted bioassays to determine whether the presence of color would facilitate the location of attractants by the butterflies. It was found that color facilitated odor attraction in some species that feed on flowers (*Parthenos silvia, Heraclides thoas, Dryas julia*, and *Idea leuconoe*), but not in the exclusively fruit-feeding species, such as *Morpho helenor*, hence demonstrating that species with different natural diets use different foraging cues. Green, ripe, and fermented bananas were evaluated for their attractiveness to butterflies together with honey and mangoes. The fermented bananas were determined to be the most attractive bait, and the electrophysiological responses to their volatiles were studied in *Morpho helenor* and *Caligo telamonius*. During GC-EAD evaluation, fifteen different aliphatic esters, such as isobutyl isobutyrate, butyl acetate, ethyl butanoate, and butyl butanoate (both fermentation products and fruit semiochemicals) were shown to be detected by the butterflies' sensory apparatus located in the forelegs, midlegs, proboscis, labial palpi, and antennae. Legs, proboscis, and antennae of *Morpho helenor* and *Caligo telamonius* showed similar sensitivity, reacting to 11 chemicals, while labial palpi had a lower signal-to-noise ratio and responded to seven chemicals, only three of which produced responses in other organs.

1. Introduction

Although the mechanisms involved in foraging for food have been studied in several model species of butterflies, there is still much unknown for this ecologically and physiologically diverse group of ca. 20,000 species. It is known that butterflies possess trichromatic vision, [1] which has a rather complex mechanism and evolutionary history [2]. It has been shown to play a role in the selection of potential mates [3], as well as in the location of adult food sources [4]. Butterflies possess the ability to discriminate even between fine variations of color, as it has been shown in *Heliconius charithonia* L. [5]. Many species have the capacity to learn to associate colors with a food source [6]. *Papilio xuthus* L., for example, was successfully trained to feed on a sucrose solution placed on a disk of a particular color and after a few such training sessions, the butterfly was able to select the color from an array of multi colored disks [7]. Butterflies can overcome their natural preferences, as in the case of newly emerged *Heliconius*, for which color stimulus can overtake scent in its importance after conditioning [8]. The same was found in monarchs, *Danaus plexipus* L., which show strong innate color preferences but can rapidly learn to associate colors with sugar rewards, doing so for noninnately preferred colors as quickly and proficiently as they do for innately-preferred colors [9].

Although butterfly foraging is normally associated with feeding on nectar or pollen, many butterfly species, especially in the tropics, do not feed on flowers but instead are attracted to nitrogen-rich substances, such as feces and carrion, or feed on fermented fruit, tree sap, and other less colorful, but odorous substrates [10]. These food sources contain

different volatile attractants than nectar, and species feeding on them are expected to use a different set of foraging cues than those used by purely flower-feeding species [11]. These rotting foods are characterized by low sugar concentrations and the presence of fermentation products (ethanol and acetic acid) [12]. However, the chemical composition of such rotting foods and the effects of these constituents on butterfly feeding behavior have rarely been investigated. As with other flower-visiting insects, the importance of visual and olfactory cues most likely varies with species, with each species having a unique favored combination of color cues and chemical compounds [13, 14].

Scent plays an important role in foraging, sometimes acting synergistically with color. In *Vanessa indica* Herbst, it was found that either scent or visual stimulus (artificial flowers used in experiments) acts as the important cue, depending on the particular color [4]. In another study, hawkmoths were attracted to flower models by either olfactory or visual cues, but only simultaneous exposure to both stimuli elicited feeding [15]. This reliance on perceiving both cues simultaneously is supported by neurological examinations of Lepidoptera [16], which revealed that activity in the mushroom bodies of the hawkmoth, *Manduca sexta* L., is influenced by both olfaction and vision during foraging. While testing color preferences in butterflies can be a relatively straight-forward task, identifying the specific compounds that they find attractive is often more difficult. Andersson and Dobson [17, 18] identified specific volatile compounds present in flowers commonly visited by butterflies and showed that there were antennal responses to most of these compounds. It was suggested that the presence of these compounds may be a result of adaptive pressure on flowers and host plants to specifically attract butterflies.

Not only do olfactory stimuli play an important role in locating food sources and potential mates, but they are crucial in locating the right host plants for oviposition. Plant odors, which are complex blends of dozens, if not hundreds of chemicals, are thought to have specific compounds, unique for different plants that are attractive to insects (e.g., [13]). It has been shown that electroantennographic responses can be elicited from butterflies with volatiles collected from the leaves of their corresponding host plants (e.g., [19–21]). Butterflies can be stimulated to lay eggs on certain plants by specific volatile compounds, as well as deterred from doing so by others (e.g., [22–27]). For instance, it was found that the oviposition behavior of female *Papilio xuthus* can be induced by methanol extracts of fresh leaves of *Citrus* plants [22], while hydroxybenzoic acid derivatives in a nonhost rutaceous plant deter both oviposition and larval feeding [28].

Considering the above studies, an extremely complex picture of butterfly sensory systems and of their foraging mechanisms emerges, it is clear that not only controlled experiments, but also field behavior and ecological studies involving a variety of species and scenarios, in combination with electro-antennographic analyses, are necessary, before we can fully understand the foraging strategies employed by Lepidoptera.

In the present study, by offering various combinations of color and scent, we examine how a diverse assemblage of tropical butterflies in a live butterfly exhibit responds to foraging cues. Red and yellow colors were chosen for the trials, as these colors are common in flowers, and they were most easily associated with a food source in a study of the swallowtail butterfly, *Papilio xuthus* [7]. The unique opportunity to examine foraging cues in a semicontrolled environment for multiple species of tropical butterflies with different ecologies and multiple individuals of different ages, sexes, and levels of foraging experience was presented by the Butterfly Rainforest facility at the University of Florida. Conducting experiments in these settings allowed the experiment to partially simulate a natural environment, while greatly increasing the chance of response due to a high density of butterflies and a variety of species present.

Following the initial bioassays, the most frequently observed species, *Morpho helenor* Cramer, was chosen for further electrophysiological examination. In this particular fruit-feeding forest species, color was expected to play little or no role in locating food, because rotten fruit on the forest floor is hardly discernible from the leaf litter. *M. helenor* must, therefore, rely on scent to find their food, and as a result, this species was expected to be particularly sensitive to olfactory stimuli. GC-EAD analyses were used to examine the antennal responses of *M. helenor* to the volatile compounds present in ripe banana, and another fruit feeding species, *Caligo telamonius* Felder, was used for comparison.

Previous studies showed that gustatory organs in insects often exhibit olfactory abilities [13] (and references within) and that olfactory receptors are present not only on antennae, but also on other appendages [29]. Hence, we decided to electrophysiologically examine not only the antennae, which are traditionally thought of as having an olfactory function, but also the proboscis, the foreleg, the midleg, and the labial palpi.

2. Materials and Methods

2.1. The Rainforest Bioassays. Bioassays were carried out in October-November 2009 and November-December 2010 in the Butterfly Rainforest exhibit facility located at the McGuire Center for Lepidoptera and Biodiversity, Florida Museum of Natural History, Gainesville, FL. The Butterfly Rainforest is a live butterfly exhibit consisting of a 600 m^2 by 30 m high-screened enclosure with ambient conditions and hundreds of species of tropical plants providing shade and nectar. The ca. 2000 individual butterflies of ca. 50 species that fly in the Butterfly Rainforest include swallowtails (Papilionidae), brush-footed butterflies (Nymphalidae), and sulphurs (Pieridae) (see http://www.flmnh.ufl.edu/butterflies/guide/ for details). The butterflies mostly feed on nectar from flowers, such as *Pentas lanceolata* (Rubiaceae) and *Symphyotrichum dumosum* (Asteraceaea). Some butterflies, however, such as *Morpho* and *Caligo* species, feed exclusively on fermenting fruit (mangoes and bananas) which are provided regularly on trays.

2.1.1. Bioassay I. Five separate replications were performed in the Butterfly Rainforest between October 30 and November 13, 2009. The trials were conducted for four hours between 13.00 h and 17.00 h on each occasion (RH: 68–85%, temp.: 21–30°C). Five cardboard landing pads covered with red (650 nm wave length, 49% reflectivity), yellow (570 nm wave length, 35% reflectivity), or black (7% reflectivity) paper were placed simultaneously on the railings ca. 1 m above the enclosure floor. Three of the pads (red, yellow and black) were covered with 10% honey solution, while two of the landing pads (red and yellow) were used as controls and were left unbaited. The landing pads were placed so that each pad received approximately the same amount of sunlight. The positions of the landing pads were rotated at random every hour to prevent butterflies from memorizing a specific location of bait. Honey solution was reapplied with a sponge every 30 min. Butterfly landings (physical contact with a circle, either by direct landing or by landing near the pad and then crawling onto it) were recorded and photographed.

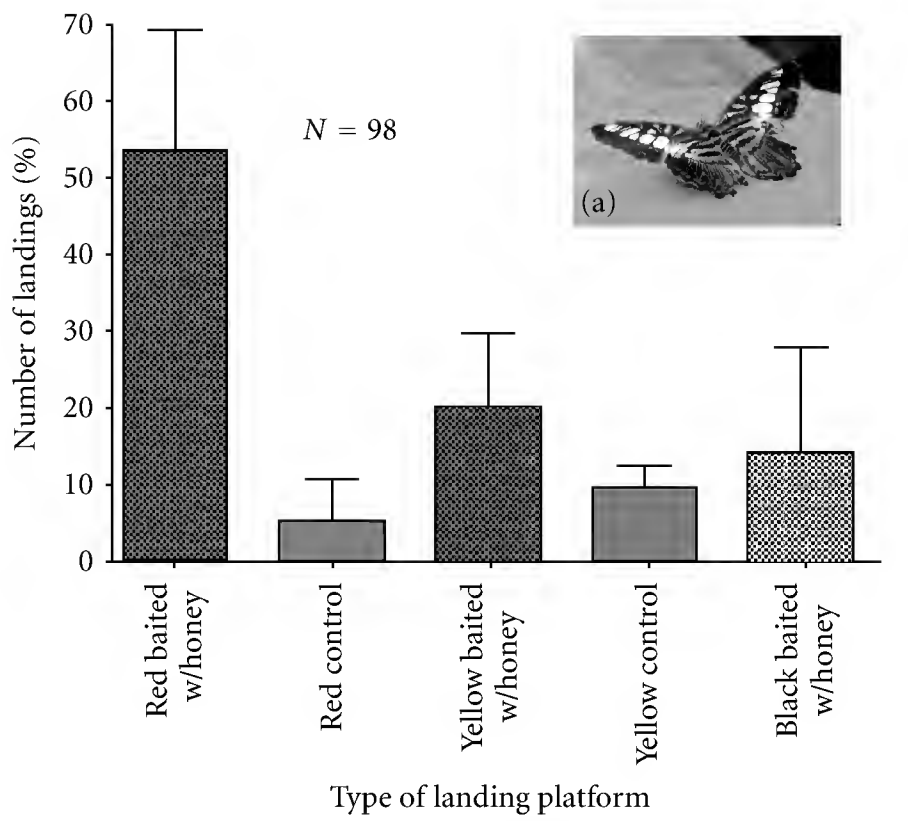

FIGURE 1: Bioassay I. The Clipper butterflies, *Parthenos sylvia* (a), showed a strong preference for the red baited landing platforms when offered an array of other choices.

2.1.2. Bioassay II. In 2010, a second bioassay was conducted to determine which bait (mango, banana (green, ripe, and fermented), or honey) was the most attractive to *M. helenor*. Four separate replications were performed between October 25 and November 29, 2010. The trials were conducted between 16.00 h and 17.00 h on each occasion (RH: 62–79%, temp.: 20–29°C).

In this bioassay, the butterflies were simultaneously offered a choice between eight "scent stations" which emitted scents of mango, banana, or honey. The control station contained no bait. Scent stations were comprised of a glass jar (7 cm tall, 4 cm in diameter) that contained the bait placed under an upended plastic cup (12 cm tall, 8 cm in diameter) with holes punctured in the bottom to allow scent to escape the cup (Figures 3(a)–3(c)). Each type of bait included one red (650 nm wave length, 49% reflectivity) and one clear cup to test for possible color preference. The distance from the holes to the cups with the attractants was greater than the proboscis length of a butterfly, preventing them from feeding. The bioassay was designed so that butterflies were unable to make physical contact with the attractants/bait and were only able to land individually on each "scent station." Thus, in Bioassay II, we were able to eliminate possible factors of gregarious behavior and conditioning from our experimental design (perceived shortcomings associated with Bioassay I). The scent stations were placed on the same railing as in Bioassay I and their positions were randomly rotated every 30 min. Butterfly landings (physical contact with a cup) were recorded by species for each scent station. Separately, green banana bait was compared with fermented banana. Landing data in Bioassay 1 were compared with a Mann-Whitney statistical test using PAST software. ANOVA was also used when analyzing the results of Bioassay 2.

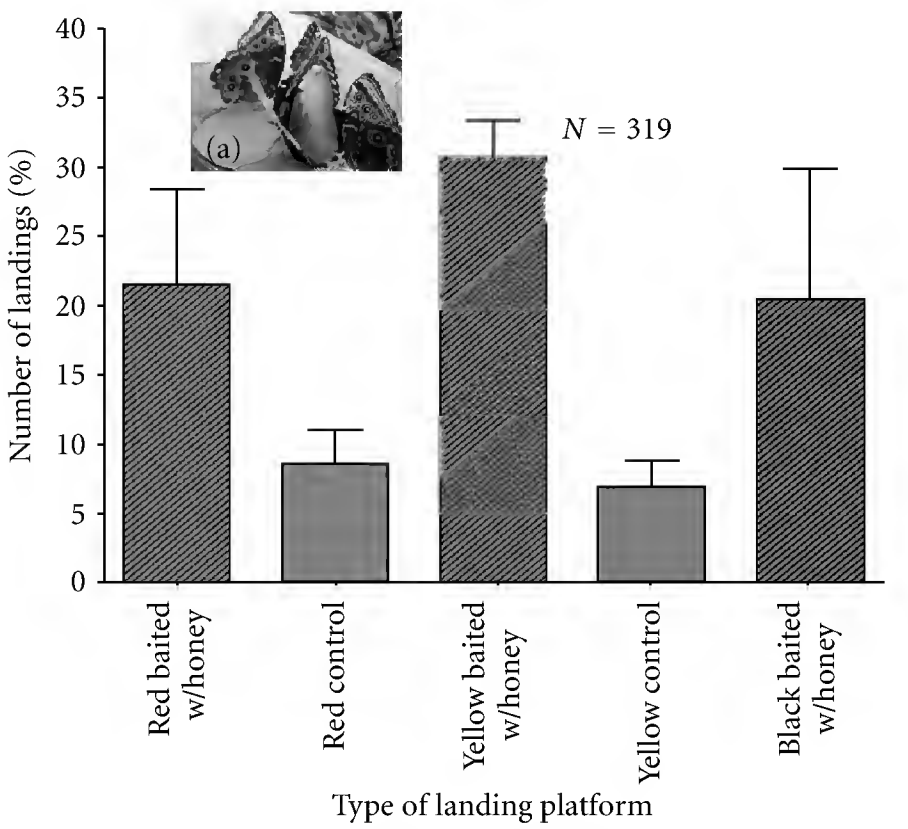

FIGURE 2: Bioassay I. Blue Morpho butterflies, *Morpho helenor* (a), showed a strong preference for baited landing platforms compared to nonbaited platforms.

2.2. Banana Volatile Collection and Analysis. Procedures described in Sections 2.2 and 2.3 were conducted at the Chemistry Research Unit of the Center for Medical, Agricultural and Veterinary Entomology (USDA-ARS, Gainesville, FL).

2.2.1. Volatile Collection. To obtain fermented banana, the unpeeled ripe bananas were cut into thin pieces and placed into a resealable plastic bag, which was stored outside for 6 days (RH: 80%; temp: 31°C), thus inducing the fermentation process. Banana volatile collections were conducted using dynamic headspace adsorption method. Bananas were sliced and placed on a sheet of aluminum foil, which was put into a 1.7L glass volatile collection chamber (Agricultural

Table 1: Butterfly species that visited landing platforms during Bioassay I.

Scientific name	Common name	Diet	Origin	Number of Landings
Morpho achilles	Banded Morpho	fruit	Central America	1
Hamadryas amphinome	Blue Cracker	fruit	Central America	3
Morpho helenor	Blue Morpho	fruit	Central America	319
Parthenos sylvia	Clipper	flower/fruit	Philippines	98
Neptis hylas	Common Sailor	fruit	Southeast Asia	14
Hypolimnas bolina	Great Eggfly	flower	Southeast Asia	10
Catonephele mexicana	Grecian Shoemaker	fruit	Central America	4
Eueides vibilia	Vibilia Longwing	flower	Central America	3
Kallima paralekta	Indian Leaf	fruit	Southeast Asia	1
Dryas julia	Julia	flower	Florida	14
Cethosia cyane	Leopard Lacewing	flower/fruit	Southeast Asia	2
Parides iphidamas	Pink Cattleheart	flower	Central America	1
Heraclides thoas	Thoas Swallowtail	flower	Central America	13
Papilio pilumnus	Three-tailed Swallowtail	flower	Central America	1
Idea leuconoe	Tree Nymph	flower	Southeast Asia	17
Myscelia cyaniris	Tropical Blue Wave	flower	Central America	4
Morpho polyphemus	White Morpho	fruit	Central America	1
Heliconius charithonia	Zebra Longwing	flower	Florida	1

(Photos of these and other species found in the Butterfly Rainforest can be viewed at http://www.flmnh.ufl.edu/butterflies/guide/.)

Research Systems, Gainesville FL). Clean air passed into the chamber through Teflon tubing (Cole Parmer, Vernon Hills, IL); air flow was regulated with a flow meter (Alborg, Orangeburg, NY) set to 110 mL/min. The air passed over the fruit before being drawn out with a vacuum (also regulated with a flow meter). As the air was pulled from the chamber, it passed through a filter containing 50 mg Super Q (Alltech, Nicholasville, KY) which captured volatiles. Volatile collections were conducted for 2 hrs. To elute volatiles trapped in the filter, 200 μL of methylene chloride (Sigma-Aldrich, St. Louis, MO) were added to filter and pushed through with nitrogen gas into a 1.5 mL glass vial (Sun-Sri, Rockwood, TN) with a 0.25 mL conical insert (Sun-Sri, Rockwood, TN). The volatiles collected for GC-EAD did not have an internal standard added. Those for identification and quantification had 5 μL of 80 ng/μL nonyl acetate (Sigma-Aldrich, St. Louis, MO) solution in methylene chloride added before the filter was eluted. Samples were stored at -80°C until they were used for assays.

2.2.2. Volatile Bioassay. Volatiles were tested in the Butterfly Rainforest by deploying 10 μL with a syringe onto 5.5 -cm qualitative filter paper and observing the number of butterfly landings for 1 h. Volatiles were reapplied every 30 min. It was determined that they maintained the same level of attraction for the butterflies as fermented banana.

2.2.3. GC-MS Analyses. Chemical analyses and quantifications were conducted using both gas chromatography-mass spectroscopy (GC-MS) and gas chromatography-flame ionization detection (GC-FID). Volatile chemical identities determined by GC-MS analyses (Figure 4) were confirmed by analysis of synthetic standards, by comparing retention times and MS fragmentation patterns for 2-methylbutyl acetate, butyl acetate, ethyl butyrate, hexyl acetate, butyl butyrate, and propyl acetate. Other chemicals were identified from their MS fragmentation patterns using ChemStation software (Agilent Technologies, Santa Clara, CA) and the NIST mass spectral library (NIST, Gaithersburg, MD): all chemicals matched at least 90%. For GC-FID, samples in extracts were injected (1 μL) in the splitless mode, injector purge at 1 min. Helium was used as a carrier gas at a linear flow velocity of 20 cm/sec. The oven was held at 35°C for 5 min and then increased at 5°C/min to 75°C and then 10°C/min to a final a temperature of 230°C. EI GC-MS analyses of extracts from Super Q filters were conducted using a Hewlett Packard HP6890 GC interfaced to an HP5973 MS and equipped with a 30 m $\times$ 0.25 mm ID HP1 Column. The ion source temperature was 220°C and the transfer line was held at 240°C. The GC oven was operated under the same conditions as for the GC-FID analyses.

2.3. Gas Chromatography Electroantennographic Detection. The coupled GC-EAD was conducted using a GC-FID (Hewlett Packard HP 6890 equipped with an Alltech Econo-Cap, EC-1 30 m $\times$ 0.25 mm Column), a heated transfer line and humidified cool air to introduce the separated chemicals to the insect organ. Conditions of chromatographs were the same as those used for the previously described GC-FID analyses. The GC was connected to both a flame ionization detector (FID) and an electroantennographic detector (EAD) (Syntech, The Netherlands), with a split ratio of 1 : 1. The EAD column discharged into a glass tube, and a humid air stream chilled and transported the volatile compounds to the organ situated on the electrode.

FIGURE 3: Bioassay II. (a-c) Scent stations attracting butterflies in the Butterfly Rainforest. (d) Graph showing the frequency of landings by *Morpho helenor* on bait stations emitting smells of mango (M), banana (B), or honey (H). C: clear cups, R: red cups. CC and CR are control (no bait) stations.

Labial palpi, antenna, legs, proboscis, and forelegs (SEMs of these organs are shown in Figure 6) were procured from two live female *Morpho helenor*. In total, four midlegs, four forelegs, four antennae, and two proboscises were examined. The antenna, proboscis, legs, and forelegs were cut in half. Electrode Gel (Spectra 360, Parker Labs, Fairfield, NJ) was spread evenly on both sides of a PRG-2 probe (Syntech, Netherlands). Under a microscope, both halves of the organ were placed 2 mm apart on the electrode. The ends of both halves of the organ were covered with the electrode gel, so that only the middle remained uncovered and suspended between the two sides of the electrode. When preparing the labial palpi for the GC-EAD analysis, they were not cut in half and were placed onto a 4 mm wide probe. Similar techniques were applied to a single female individual of an Owl butterfly, *Caligo telamonius* Felder. Once this was set up, the probe was attached to an Intelligent Data Acquisition Controller, IDAC-232 (Syntech, Netherlands) that interfaced with a personal computer running EAD2000, Version 2.6 (Syntech, The Netherlands), to record both the output from the FID and the antenna. This enabled the pairing of insect antennal responses and the corresponding FID signals.

2.4. Scanning Electron Microscopy. Morphology of the electrophysiologically examined organs was illustrated in *M. helenor* using a Scanning Electron Microscope (model JOEL JSM-5510-LV) at the Florida State Collection of Arthropods.

3. Results

3.1. Bioassay I. A total of 507 landings by 18 species were recorded (Table 1). Clipper, *Parthenos sylvia*, which landed a total of 98 times (19% of all landings) showed a strong preference for the red baited circle ($P < 0.02$) (Figure 1). Hence, in *P. sylvia*, the combination of the red color and the scent of honey produced the strongest response. Similarly, *Heraclides thoas* (L) (Papilionidae), *Dryas julia* (Fabricius), and *Idea leuconoe* Erichson (Nymphalidae), which, in combination, accounted for 9% of the landings, also showed a statistically significant preference for the red baited pad. For *H. thoas*, 85% of the landings ($N = 13$) were on the red baited pads; *D. julia* landed on red baited pads 100% ($N = 14$); *I. leuconoe*—71% ($N = 17$).

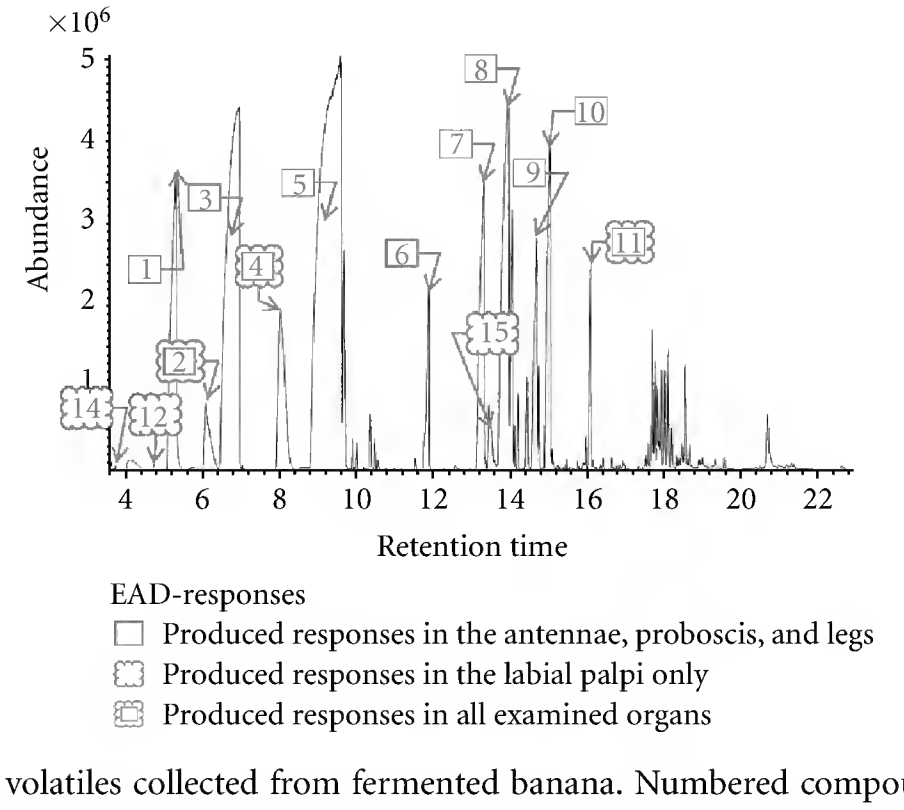

Figure 4: GC-MS chromatograms of volatiles collected from fermented banana. Numbered compounds produced EAD-responses in the antennae, proboscis, forelegs, midlegs, and labial palpi of *Morpho helenor* and *Caligo telamonius* (compound no. 13, propyl acetate, was determined using GS-FID). For compound names, see Table 2.

Morpho helenor was the dominant species in our study, responsible for 63% of all landings ($N = 319$). For this species, the three baited pads were significantly more attractive than the nonbaited pads ($P < 0.02$), but no significant color preference was found (Figure 2).

3.2. Bioassay II. In the second bioassay, which was primarily aimed at determining the preferred food source of *Morpho helenor*, banana was found to be more attractive than mango, honey, and the control. To test the significance of color in the foraging of this species, red and clear cups were offered for each type of bait. No significant difference ($P > 0.05$) was found in the color preferences of *Morpho helenor*: it landed as frequently on red cups as it did on the clear cups (Figure 3(d)), supporting the results of Bioassay I. Green, ripe, and fermented bananas were tested for their attractiveness, and the latter were found to be significantly more attractive.

3.3. GC-EAD Analyses. *Morpho helenor* showed electrophysiological responses to a total of fifteen compounds (all esters) from the fermented banana volatiles (Figure 5, Table 2). Four of the five organs analyzed (proboscis, foreleg, midleg, and antenna) reacted with similar intensity to the same 11 chemicals (Figure 5). The labial palpi reacted to three of these chemicals as well as four other chemicals, which did not elicit response from proboscis, legs, or antenna, and the responses were weaker. A single Owl butterfly, a female of *Caligo telamonius*, was analyzed in the same manner in which we studied *Morpho helenor*, and the EAD-responses of its organs were also similar. In Figure 4, we provide a chromatogram from fermented bananas to show the presence and abundance of attractive compounds. The chromatograms obtained initially from green, ripe (ready-to-eat stage), and fermented bananas indicate that number and abundance of volatile compounds increased as the fruit aged and the fermentation is responsible for a number of the compounds, absent in green fruit. The electrophysiological responses to these volatiles in *Morpho helenor* and *Caligo telamonius* (Figures 5(a) and 5(b)) indicate that different aliphatic esters, such as isobutyl isobutyrate, butyl acetate, ethyl butanoate, and butyl butanoate, which are both fermentation products and fruit semiochemicals, produce responses in the olfactory organs of fruit-feeding butterflies. Some of these compounds, such as 1-Methylbutyl acetate (aka isoamyl acetate) are responsible for banana odor in banana oil, while others, such as isobutyl isobutyrate or ethyl 2-methylpropanoate, are the products of fermentation.

3.4. Morphology. The examination of legs, proboscis, labial palpi, and antennae using SEM showed the presence of sensilla on all of these organs (Figure 6). Organs other than proboscis possess long sensilla that have been described as olfactory [29]. We also illustrate gustatory sensilla located on proboscis on the midproximal part of galeae (Figure 6(k)). These are possibly responsible for both gustatory and olfactory functions as is the case with sensilla styloconica found on proboscis of cabbage armyworm [30].

4. Discussion

Bioassay I showed that different species are likely to exhibit different foraging behaviors and that generalized conclusions should not be drawn from experiments conducted on one or two species. The fact that flower-feeding butterflies *Parthenos sylvia*, *Heraclides thoas*, *Dryas julia*, and *Idea leuconoe* showed a preference for a specific color (red) during these bioassays confirmed that color cues are important for butterflies during foraging. Many flowers in the Butterfly Rainforest are red and the nectar-feeding butterflies could have learned to associate this color with the nectar reward. Repeated landing on the red baited circles and obtaining the honey solution reward could have further conditioned these butterflies.

Table 2: Chemical volatile compounds collected from fermented banana and the presence (+) of EAD-responses to these compounds in various appendages of Blue Morpho butterfly (*Morpho helenor*).

Number in Figure 4	Volatile compound	Antenna	Mid-leg	Proboscis	Labial palp	Foreleg
1	2-Methylpropyl acetate	+	+	+		+
2	Ethyl butanoate	+	+	+	+	+
3	Butyl acetate	+	+	+		+
4	1-Methylbutyl acetate	+	+	+	+	+
5	3-methylbutyl acetate	+	+	+		+
6	Isobutyl isobutyrate	+	+	+		+
7	Butyl butanoate	+	+	+		+
8	Hexyl acetate	+	+	+		+
9	1-Methylhexyl acetate	+	+	+		+
10	Butyl 3-methylbutanoate	+	+	+		+
11	Pentyl pentanoate	+	+	+	+	+
12	Ethyl 2-methylpropanoate				+	
13	Propyl acetate				+	
14	Isopentyl formate				+	
15	Hexenyl acetate				+	

The outcome of this assay supports previous studies (e.g., [4, 8, 31]), which have found that a combination of stimuli are necessary to elicit feeding response and that some butterflies have the ability to discriminate between colors.

While *Parthenos sylvia*, *Heraclides thoas, Dryas julia*, and *Idea leuconoe* greatly preferred the red baited circles, the fruit-feeding *Morpho helenor* did not discriminate between colors and equally visited red, yellow, and black baited circles, while avoiding the unbaited controls (regardless of color). In the Butterfly Rainforest, where *M. helenor* are fed yellow mangoes and bananas, we expected a preference for yellow baited circles, yet this species exhibited no such preference. In the darkness of the forest floor, where *M. helenor* forage naturally for rotting/fermenting fruit that have fallen from the canopy, visual cues are reduced. Hence, the ecology of this species corresponds with our findings: *M. helenor* relies more upon volatile cues than color when foraging.

EAD-analyses performed on various organs of *Morpho helenor* and *Caligo telamonius* suggest that rotting fruit volatiles can be perceived not only by sensilla located on antennae (as has been traditionally thought), but also by those located on the proboscis, labial palpi, and legs. It is possible that the olfactory receptors covering the legs, antennae, proboscis, and labial palpi are simultaneously sending messages to the butterfly's brain, hence increasing the magnitude of the signal. This supports previous studies which indicated the possible olfactory role of the proboscis ([30] (and references within)). Previously proposed functions of the labial palpi include the detection of sexual pheromones, attunement to adult food sources, stimulants for migration, a shield for the proboscis, and wipers for cleaning the eyes of the butterfly [29]. The present study indicates that labial palpi are equipped to detect some of the volatile chemicals present in adult food sources. However, the array of chemicals perceived by the palpi is different from the array perceived by antennae, legs, or proboscis, with an overlap of only three compounds (out of 15 total, Table 2). Such a distribution of different functions between organs might contribute to the efficiency of olfactory processing in butterflies.

When butterflies are foraging for fermenting fruit, they use cues from the fruits themselves and from the fermentation products produced by the rotting fruit [11]. This notion has been confirmed by the present study: *Morpho helenor* reacted to several compounds that are products of fermentation (e.g., isobutyl isobutyrate; ethyl 2-methylpropanoate), as well as several compounds that are commonly found in fruit (e.g., butyl acetate; ethyl butanoate; butyl butanoate) and are responsible for fruity odor. When we initially compared the volatiles released by unripe, ripe, and fermented banana using GC-MS, it was generally observed that the abundance of the volatile compounds increased as the fruit fermented. It was also observed that the GC-MS of fermented banana volatiles contained compounds that are not present at all in the chromatograms of green and ripe bananas. This explains why fruit-feeding butterflies are not attracted to unripe fruit: to locate their food source, they require volatile compounds associated with both fruit and fermentation. Fermentation cues must also play a role when butterflies are locating other food sources, such as rotting fish, dung, and carrion.

The specific chemistry of banana volatiles and the responses these chemical compounds elicit in *Morpho helenor* and *Caligo telamonius* are the most analytical and controlled parts of our study, and yet they may also be the most ambiguous when it comes to drawing conclusions. For instance, although we determined that the most abundant compound in the fermented banana volatiles that *M. helenor* and *C. telamonius* reacted to was 3-methylbutyl acetate, this does not necessarily mean that it is the banana semiochemical that attracts butterflies, as we do not know what role the abundance of a chemical plays. It is known that semiochemicals, such as pheromones, do not need to be present in great volume to trigger a response [32].

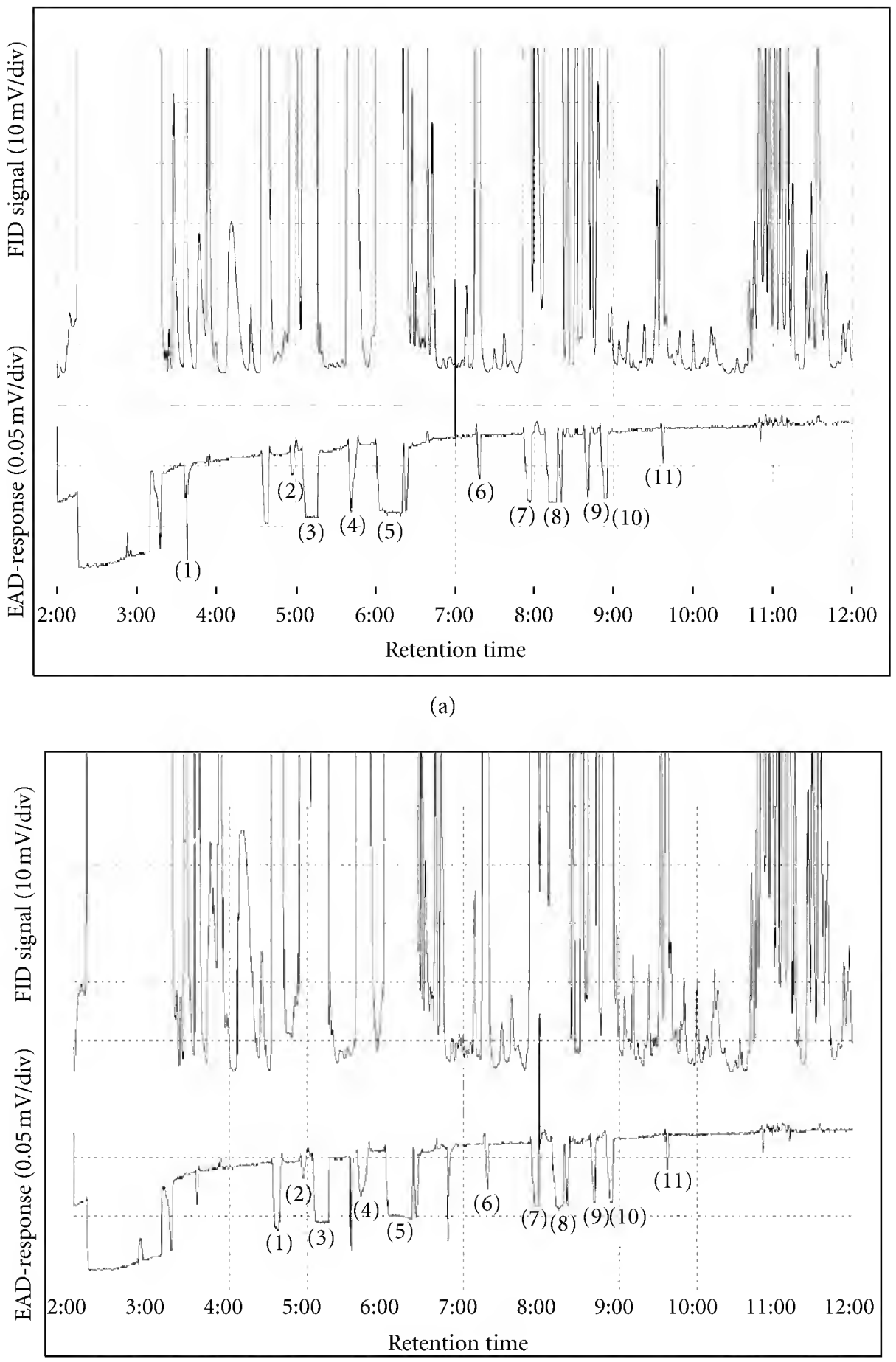

FIGURE 5: Eleven chemicals in fermented banana volatiles identified here, produced EAD-responses in olfactory organs of *Morpho helenor*. (a) Proboscis. (b) Foreleg, antenna, and midleg of *Morpho helenor* and the same organs of *Caligo telamonius* produced similar EAD-responses. (1) 2-Methylpropyl acetate. (2) Ethyl butanoate. (3) Butyl acetate. (4) 1-Methylbutyl acetate. (5) 3-Methylbutyl acetate. (6) Isobutyl isobutyrate. (7) Butyl butanoate. (8) Hexyl acetate. (9) 1-Methylhexyl acetate. (10) Butyl 3-methylbutanoate. (11) Pentyl pentanoate.

The response-triggering chemical could be any of the ones that are present in the volatiles or a combination of two or more chemicals. In fact, a detected chemical could be attractive, repellent, or completely ignored by a butterfly. Hence, the next step to advance the understanding of this system will be securing (through synthesis or commercial acquisition) the individual banana semiochemicals that were identified by the present study, and conducting bioassays with these compounds. This will allow us to determine which compound(s) are responsible for butterfly attraction.

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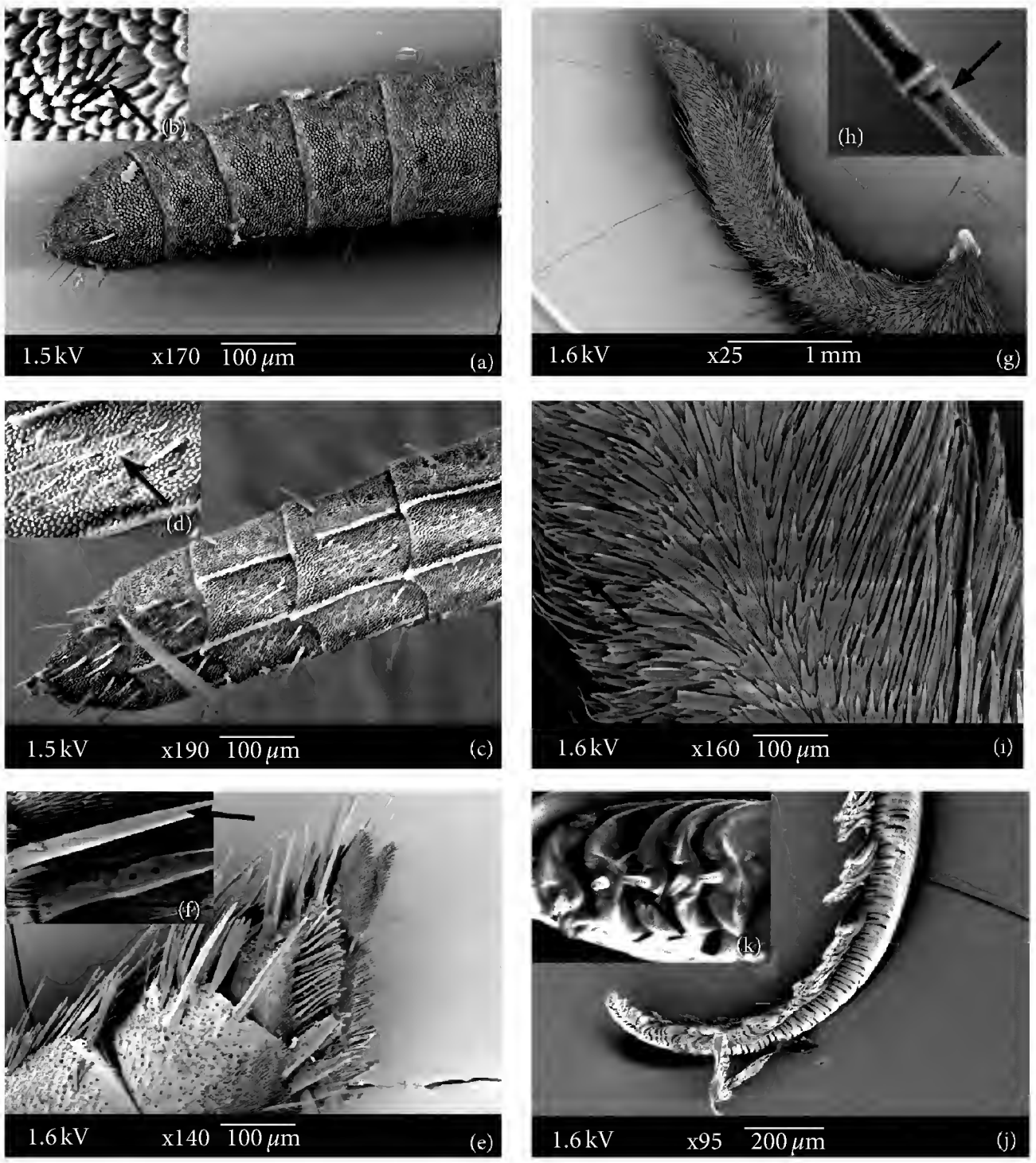

FIGURE 6: Scanning Electron Micrographs (SEMs) of *Morpho helenor* organs used for EAD analyses. (a-b) Antenna, dorsal surface. (c-d) Antenna, ventral surface. (e-f) Foreleg tip. (g–i) Labial palpus. (j-k) Galeae of proboscis. Closeup inserts show sensory organs potentially responsible for the observed EAD responses.

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Research Article

Splendid Hybrids: The Effects of a Tiger Beetle Hybrid Zone on Apparent Species Diversity

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Nonexpert citizen groups are being used to monitor species to track ecosystem changes; however, challenges remain for proper identification, especially among diverse groups such as beetles. Tiger beetles, *Cicindela* spp., have been used for biological diversity monitoring because of their diversity and the ease of recognition. The finding of an apparent hybrid zone among *Cicindela denverensis* Casey, *Cicindela limbalis* Klug, and *Cicindela splendida* Hentz in central Nebraska prompted a detailed study of the biogeography of this species group within Nebraska, a test of characteristics that could be used by citizen scientists, and limited breeding experiments. This study suggests that while *C. denverensis* appears to hybridize with both *C. limbalis* and *C. splendida* within the hybrid zone, all three species maintain their integrity across most of their ranges, largely occupy unique geographic regions, and at least *C. denverensis* and *C. splendida* cooccur in many areas with no evidence of hybridization. Evidence of hybridization between *C. limbalis* and *C. splendida* was found at only two sites. Furthermore, breeding experiments with virgin *C. splendida* and *C. denverensis* showed that they are capable of producing hybrid larvae in the laboratory. The presence of morphological intergrades serves as a cautionary note when using biological indicator species.

1. Introduction

Hybridization of distinct lineages has been recognized as an important area of evolutionary research since the time of Charles Darwin. Although much of the past research has been on plant hybridization, attention to animal species has been increasing and has become the subject of focused research by evolutionary biologists [1–4]. Unfortunately for the field of conservation biology, hybridization can be extremely problematic. Moreover, the challenge of hybridization to the conservation of unique species has increased as anthropomorphic changes to environment and globalization and introduction of exotic species have combined to increase interactions among species [5].

As global changes take place and loss of biodiversity is a growing concern, many research organizations have sought to increase biological monitoring by citizen groups. A growing number of examples exist for monitoring of aquatic ecosystems for pollution [6]. More recently, citizen scientist groups have successfully detected both invasive species [7] and rare native species such as the nine-spotted lady beetle, *Coccinella novemnotata* [8]. Citizen science programs have also been used to collect data over broad scales such as the case for determining monarch butterfly, *Danaus plexippus*, migration routes [9].

Despite these and many other benefits in the use of citizen scientists for ecosystem monitoring, many challenges remain, including training citizen scientists, coordinating monitoring programs, and ensuring the accuracy of identification (e.g., [6, 7]). Relatively, few citizen monitoring programs exist for terrestrial invertebrate diversity, likely as a result of the enormous diversity of terrestrial insects. Among groups that have been monitored, dragonflies, butterflies, and ladybird beetles have received the most attention. Another candidate group is the tiger beetles, Coleoptera: Carabidae: Cicindelinae.

The tiger beetles of North America have been studied thoroughly and are well known even to the subspecies level,

Table 1: Identification characters used to differentiate between species in the *Cicindela splendida* group.

Character	*C. denverensis*	*C. limbalis*	*C. splendida*
Dorsal head	Green to blue-green	Purple, red, or dull red	Green to blue
Margins of head	Green to blue-green	Green to blue	Green to blue
Dorsal pronotum	Green to blue-green	Purple, red, or dull red	Green to blue
Margins of pronotum	Green to blue-green	Green to blue	Green to blue
Elytra	Green to blue-green	Purple, red, or dull red	Red, purple, or (rarely) green
Margins of elytra	Green to blue-green	Green to blue	Green to blue
Proepisternum	Green to blue	Red to orange	Green to blue

Table 2: Locality information for specimens of *C. denverensis* examined.

Species	County	County total	Location	Location total
C. denverensis	Banner	2	Bull Canyon	2
	Buffalo	54	Amherst	8
			Cherry Creek	3
			Kearney	43
	Custer	13	Ansley	13
	Dawes	4	Chadron	2
	Dawson	103	Crawford	2
			Gothenburg	41
			Sumner	62
	Garfield	9	Burwell	9
	Kimball	1	Pine Bluffs	1
	Red Willow	1	McCook	1
	Scotts Bluff	14	Scottsbluff	14
	Sherman	90	E Loup City	3
			Hazard	77
			W Loup City	10
	Sioux	58	Crawford	6
			Harrison	52
	Valley	11	Arcadia	3
			Elyria	4
			Ord	4
	Total counties = 12		Total sites = 20	Total = 360

Table 3: Locality information for specimens of *C. limbalis* examined.

Species	County	County total	Location	Location total
C. limbalis	Buffalo	4	Cherry Creek	2
			Kearney	2
	Burt	1	Decatur	1
	Butler	10	Bellwood	10
	Cass	1	Murdock	1
	Douglas	8	Omaha	8
	Greeley	4	Scotia	2
			Wolbach	2
	Howard	28	St. Paul	28
	Lancaster	4	Lincoln	4
	Merrick	4	Palmer	4
	Nance	5	Fullerton	3
			Palmer	2
	Polk	1	Osceola	1
	Sarpy	4	Ashland	1
			Gretna	3
	Saunders	3	Otoe Creek	3
	Sherman	26	E Loup City	18
			Hazard	8
	Valley	2	Arcadia	1
			Davis Creek	1
	Washington	3	County Line Road	3
	Total counties = 15		Total sites = 22	Total = 108

although variation is considerable and the validity of many is still debated [11]. Because many tiger beetles are diurnally active predators, regionally diverse, and identified by color markings, they can potentially be useful for citizen groups as a biological indicator group [12]. Indeed, worldwide, tiger beetles have been used to predict species richness patterns in other taxa and have shown strong correlation with butterfly species richness [13–15]. In the United States, tiger beetle diversity varies by region, with the highest diversity found in the southwest and generally lower diversity found in the north [11]. The state of Nebraska has recorded 32 species of tiger beetles [16, 17]. Among the 93 Nebraska counties, as few as 0 and as many as 22 tiger beetle species have been recorded with the highest numbers recorded in areas with the most intensive sampling [16]. No pattern in number of species has been detected by latitude, ecoregion, or county size [16].

Among the tiger beetles occurring in Nebraska, one group, the *Cicindela splendida* group, remains controversial. The group consists of three named species, *Cicindela denverensis* Casey, *Cicindela limbalis* Klug, and *Cicindela splendida* Hentz, which are morphologically very similar and may only be readily separated by color. Schincariol and Freitag [18] determined that each of these three forms represented valid species and that they could be distinguished on the basis of elytral pattern, percent maculation, elytral color, and nonsensory setae number. These authors noted that the genitalia were very similar in all three of these forms. Interspecific copulation between species in this group has

Table 4: Locality information for specimens of *C. splendida* examined.

Species	County	County total	Location	Location total
C. splendida	Buffalo	70	Amherst	3
			Cherry Creek	7
			Kearney	57
			Pleasanton	3
	Butler	11	Bellwood	11
	Cass	4	Murdock	2
			Nebraska City	2
	Custer	6	Ansley	5
			Merna	1
	Dawson	37	Gothenburg	26
			Sumner	11
	Douglas	7	Omaha	7
	Franklin	28	Bloomington	27
			Naponee	1
	Gage	1	Virginia	1
	Garfield	2	Burwell	2
	Gosper	4	Elwood	4
	Greeley	6	Scotia	4
			Wolbach	2
	Harlan	9	Harlan Reservoir	1
			Oxford	5
			Ragan	3
	Howard	14	Ashton	4
			St. Paul	10
	Lancaster	22	Lincoln	19
			Spring Creek	3
	Merrick	3	Palmer	3
	Nance	1	Fullerton	1
	Phelps	14	S Holdrege	14
	Red Willow	4	McCook	4
	Saline	1	Crete	1
	Sarpy	2	Gretna	2
	Saunders	1	Otoe Creek	1
	Scotts Bluff	2	Scottsbluff	2
	Sherman	133	E Loup City	48
			Hazard	70
			W Loup City	15
	Sioux	8	Harrison	8
	Valley	7	Arcadia	3
			Davis Creek	1
			Ord	3
	Total counties = 25		Total sites = 41	Total = 397

been reported in the literature [19], and, in Nebraska is frequently observed. However, these observations do not verify that these matings result in offspring or if offspring are viable. Moreover, Schincariol and Frietag [18] suggested that spermatophore ejection by the female allows these species to maintain their integrity.

Table 5: Colors used in character analyses (from [10]).

Code	Color definition
1	Very deep purplish red (257)
2	Deep red (13)
3	Grayish reddish orange (39)
4	Dark greenish yellow (103)
5	Deep yellowish green (132)
6	Deep bluish green (161)
7	Deep blue, royal blue (179)
8	Deep violet (208)

During extensive sampling by the senior author, a number of apparent hybrids between *Cicindela denverensis* and *C. limbalis* were collected in central Nebraska from a zone extending north to south and about 30 km wide [17]. Apparent hybrids between two other species, *C. denverensis* and *C. splendida*, occur regularly across a zone in central Nebraska extending north to south and approximately 80 km wide.

In this study we conducted a morphological study of members of the *Cicindela splendida* group throughout Nebraska and did selective interspecific breeding experiments. In order to determine the occurrence of hybrids within this group, we tested the following hypotheses: (1) hybrids are most frequent in specific geographic areas, (2) the geographic areas in which hybrids are most frequent are related to the range and relative abundance of the species present, and (3) hybrid offspring would be produced in the laboratory using virgin males and females with interspecific pairings.

2. Materials and Methods

2.1. Character Analyses and Geographic Location. A total of 865 Nebraska specimens from this group were examined from the personal collections of Mathew Brust, Steve Spomer, and Paul Nabity (Tables 2, 3, 4). Individuals were identified to species based on the characters presented in Tables 1 and 6. Based on these characters, any specimen with a distinct blue to dark green margin on the head and pronotum was classified as *C. limbalis*, while any lacking this character but having the color of the head and pronotum differing from the color of the elytra or having distinct dark green to blue margins on the elytra was considered *C. splendida*. These designations were made to allow analyses with the null assumption of no hybridization.

Character analysis generally followed those of Schincariol and Frietag [18] and Schincariol [20]. One additional grade for color based on Kelly and Judd [10] was added to account for an unusual morph that was found at several locations. Thus, elytral color (1–8) and pronotal color (1–8) were scored for each specimen (Table 5) and analyzed in order to determine hybridization.

Maculation characters were also analyzed to test whether maculation could be used to differentiate these species, as suggested by Schincariol and Frietag [18]. The following

Table 6: Variations in color found in apparent hybrids between species in the *Cicindela splendida* group.

Character	*C. denverensis* × *limbalis*	*C. denverensis* × *splendida*	*C. limbalis* × *splendida*
Dorsal head	Orange-green, yellow-green, or green	Green to blue	Orange-green to red
Margins of head	Green-blue to blue	Green to blue	Orange-green to red, limited greenish
Dorsal pronotum	Orange-green, yellow-green, or green	Green to blue	Orange-green to red
Margins of pronotum	Green-blue to blue	Green to blue	Orange-green to red, limited greenish
Elytra	Orange-green, yellow-green, or green	Bronze, orange-green, yellow-green or green	Purple, red, or dull red
Margins of elytra	Green-blue to blue	Green to blue	Orange-green to red, limited greenish
Proepisternum	At least partially red or orange	Green to blue	At least partially red or orange

characters of maculation were graded: development of the humeral lunule (A–E), development of the middle band (A–E), development of the apical lunule (A–E), and overall development of maculation (A–E).

All characters were then analyzed across latitude and longitude using the PROC GLM procedure [21] with each one degree increment represented as a categorical variable (latitude = 1–3, longitude = 1–9). The results were checked for latitude by longitude interactions as well. When significant differences ($P < 0.05$) were found for a character by latitude or longitude, a post-hoc Tukey test was performed.

2.2. Hybridization in the Laboratory. Adults of *C. denverensis* and *C. splendida* emerge briefly in Fall after pupation, but do not mate until spring [11, 22]. Specimens for hybridization experiments were collected in October from the vicinity of Kearney, Nebraska. Three conspecific pairs consisting of a male *C. denverensis* and a female *C. splendida* were placed in individuals plastic aquaria (3.8 liter) with loess soil (approximately 70 cm deep) from collection sites. The aquaria were maintained at room temperature for about one week and were then placed into a refrigerator (approximately 6°C) for 8 weeks because a cool period is needed to trigger diapause and that this diapause is required for sexual maturity [22]. Aquaria were then placed at room temperature and adults were allowed to mate and oviposit. Resulting eggs and larvae were counted once the female in each aquarium had died. Adults and resulting larvae were fed apterous *Drosophila melanogaster* Meigen.

3. Results

3.1. Character Analyses and Geographic Location. Examination of more than 860 specimens belonging to the *C. splendida* group revealed considerable variation in elytral and pronotal color among the group (Figure 1, Table 6). For *C. denverensis*, no significant differences in elytral color were detected, while the elytral coloration for both *C. splendida* and *C. limbalis* differed significantly by longitude (Figures 4, 6, and 8). Elytral color converged for all three species between approximately 98° and 100° west longitude (Figures 10, 11, and 13).

Pronotal colors also varied by region with significant differences found for *C.denverensis*, *C. limbalis*, and *C. splendida* (Figures 5, 7, and 9). Among species, no latitude by longitude interactions were found. In the region between 98° and 100° west longitude, the pronotal color of *C. limbalis* became significantly more like that of *C. denverensis* (Figure 7).

Character analyses based on Schincariol and Freitag [18] revealed no consistent differences in elytral maculation that would allow the three species to be consistently distinguished. Significant differences in markings occurred for all species across their distribution for at least some of the elytral markings. For *C. denverensis*, differences in total maculation, humeral lunule, middle band, and apical lunule varied by longitude (Figure 1). For both *C. limbalis* and *C. splendida* significant differences were detected for the middle band and apical lunules but not for total maculation (Figures 2 and 3).

3.2. Hybridization in the Laboratory. The three females used in the hybridization experiments produced 66, 23, and 4 eggs respectively. Of these eggs, 39, 12, and 0 hatched, respectively. Attempts were made to rear the larvae to adulthood but all died before maturity as a result of mold infection.

4. Discussion

Pearson and Cassola [12] suggest that tiger beetles represent a well-characterized fauna that is suitable for use by non-experts as a biological indicator group. In Nebraska, the three species examined in this study display an apparent hybrid zone based on color and markings across the central region of the state. Field observations of interspecific pairings along with the small-scale laboratory breeding experiments reveal that hybridization is possible, although these results should be cautiously interpreted. To determine the extent of hybridization, molecular studies over a large region should be conducted as was accomplished for *Cicindela dorsalis* Say [23] and *C. splendida* and *C. limbalis* [24] Further studies of interbreeding and rearing conditions should also be

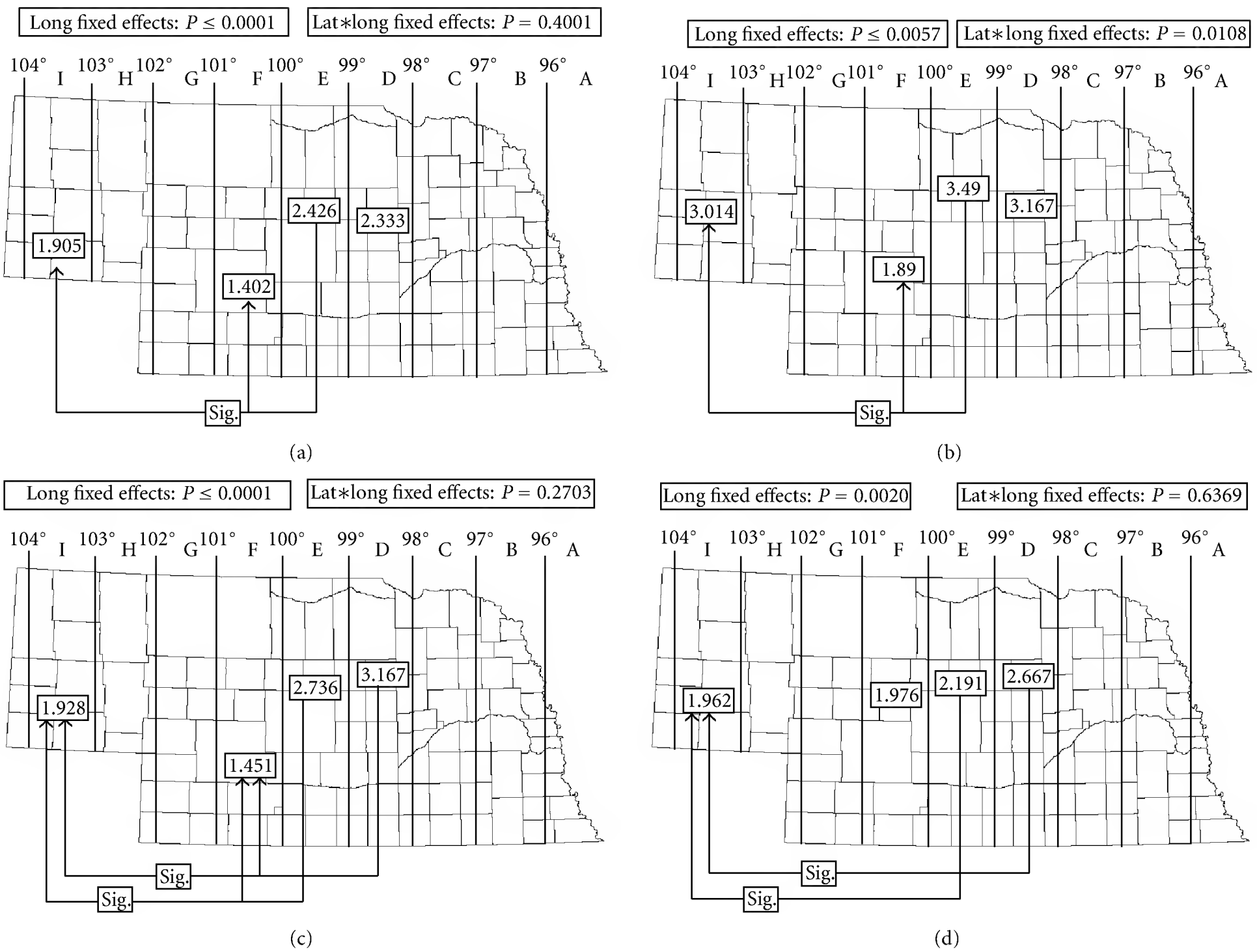

FIGURE 1: Elytral maculation character states for *C. denverensis* (adapted from [18]). (a) Total maculation, (b) humeral lunule, (c) middle band, and (d) apical lunule. Significant differences between longitudes shown by arrows and mean value presented in each longitudinal grouping. Fixed effects and effects of latitude by longitude interactions presented at top of each map.

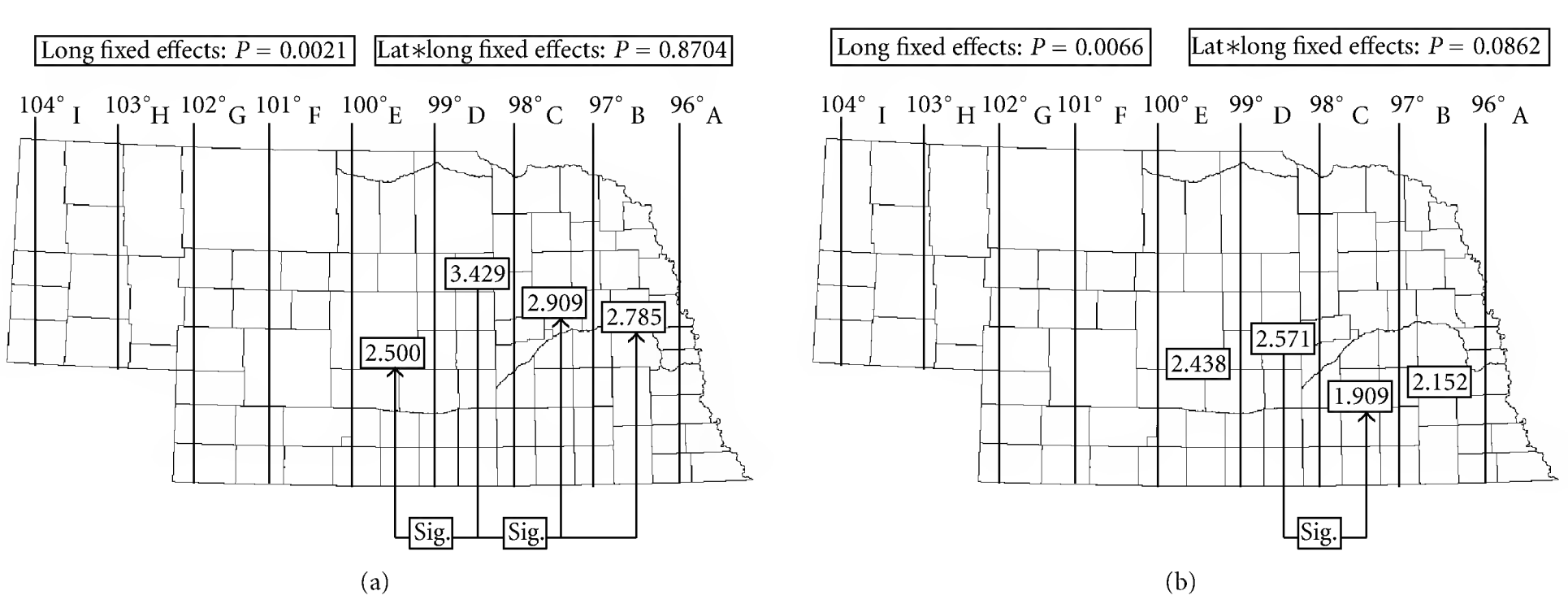

FIGURE 2: Elytral maculation character states for *C. limbalis* (adapted from [18]). (a) Middle band and (b) apical lunule. Total maculation and humeral lunule not presented as no significant differences found. Significant differences between longitudes shown by arrows and mean value presented in each longitudinal grouping. Fixed effects and effects of latitude by longitude interactions presented at top of each map.

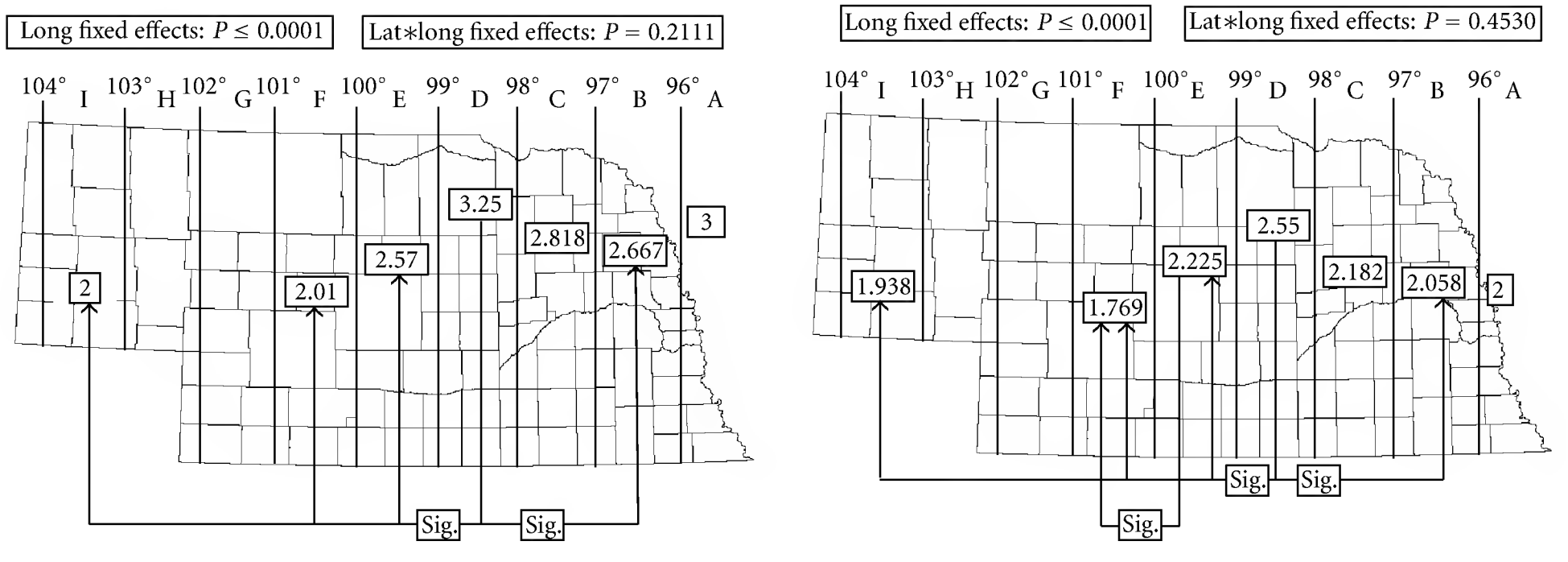

FIGURE 3: Elytral maculation character states for *C. splendida* (adapted from [18]). (a) Middle band and (b) apical lunule. Total maculation and humeral lunule not presented as no significant differences found. Significant differences between longitudes shown by arrows and mean value presented in each longitudinal grouping. Fixed effects and effects of latitude by longitude interactions presented at top of each map.

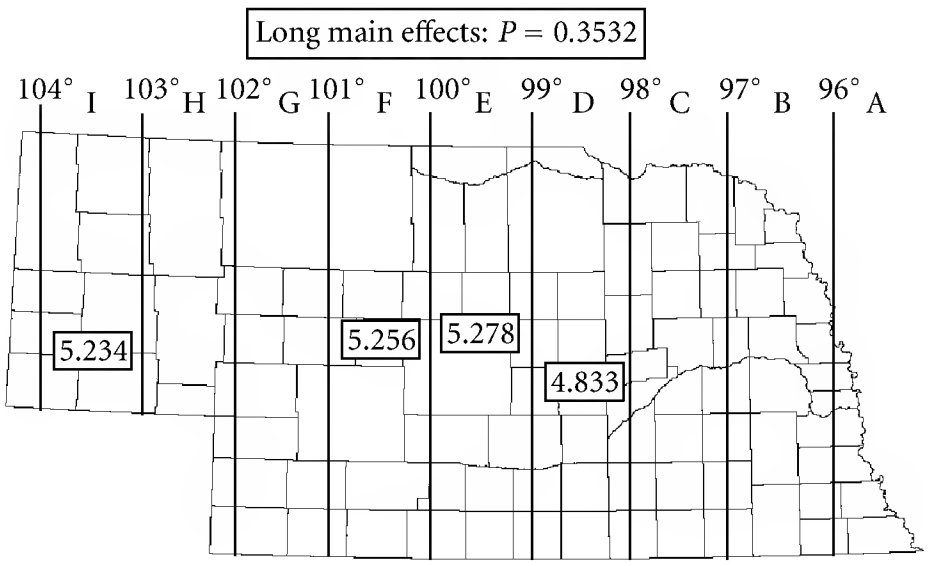

FIGURE 4: Mean elytral color by code for *Cicindela denverensis* by longitudinal region. Significant differences between longitudes shown by arrows and mean value presented in each longitudinal grouping. Fixed effects presented at top. No latitude by longitude interactions found.

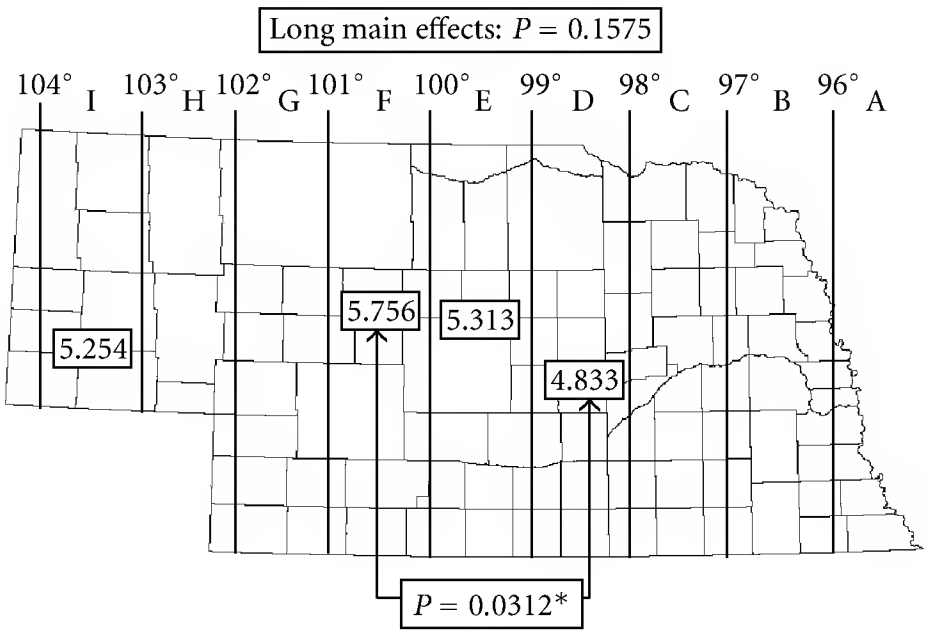

FIGURE 5: Mean pronotal color by code for *Cicindela denverensis* by longitudinal region. Significant differences between longitudes shown by arrows and mean value presented in each longitudinal grouping. Fixed effects presented at top. No latitude by longitude interactions found.

conducted because environmental conditions can influence adult coloration patterns [25]. Because Nebraska's tiger beetle fauna consists of 32 species and most counties have 8 or less [16], the inability to properly identify species or the presence of hybrids will affect estimates of biological diversity.

In Nebraska, the apparent hybrid zone affects parts of at least seven counties and approximately 20% of the state (Figure 12). The geographic and morphological analyses indicate a hybrid zone extending from central Custer and Dawson Counties east to the eastern third of Valley and Hall Counties (Figure 12). The termination of this hybrid zone to the north and south coincides with a general lack of suitable habitat as the Rainwater Basin occurs south of this area, and the Sand Hills occur to the north.

In the eastern half of the hybrid zone, all three species cooccur west at least to Kearney. Nearly all of the *C. limbalis* collected in this area exhibit at least a moderate greenish hue, suggesting hybridization with *C. denverensis*. Greenish *C. limbalis* have been recorded in other areas as well [26, 27], mostly where *C. denverensis* and *C. limbalis* cooccur. Across the entire zone, the majority (especially toward the east) exhibit coppery bronze to greenish elytra, which in some cases might suggest the "*ludoviciana*" [28] phenotype. However, the majority of these specimens have a green to bluish green pronotum, while in "*ludoviciana*" the pronotum is deep blue. Some specimens have variable amounts of coppery bronze on the anterior parts of the elytra, diffusing into green elsewhere. For the analyses, these were classified as coppery green, but, importantly, this phenotype has not been previously documented in *C. splendida* elsewhere in its range. Interestingly, specimens with features suggesting hybridization between *C. limbalis* and *C. splendida* were found only on the eastern edge of the hybrid zone.

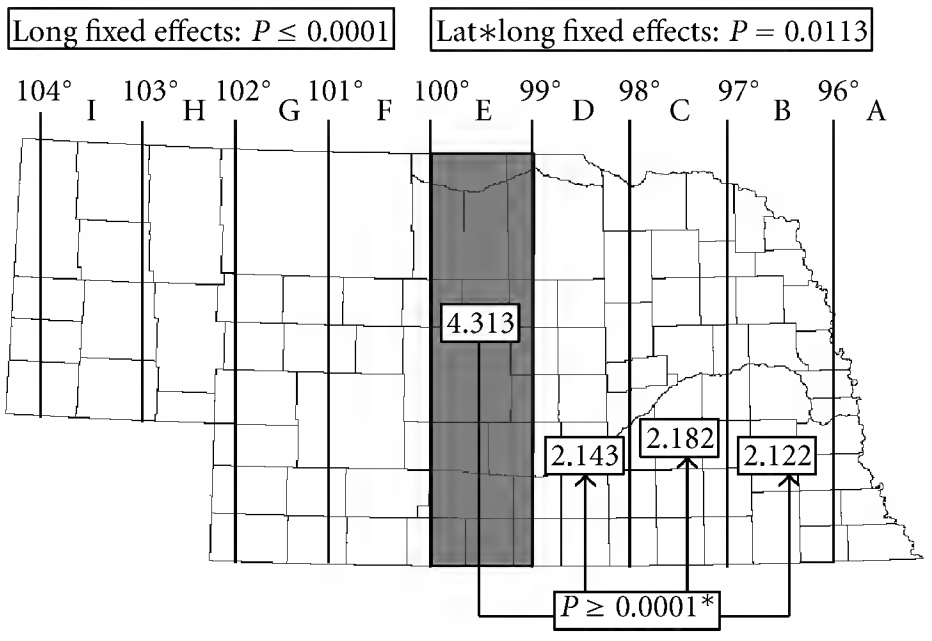

Figure 6: Mean elytral color by code for *Cicindela limbalis* by longitudinal region. Significant differences between longitudes shown by arrows and mean value presented in each longitudinal grouping. Fixed effects presented at top. No latitude by longitude interactions found. Shaded area depicts longitudinal regions differing from all others.

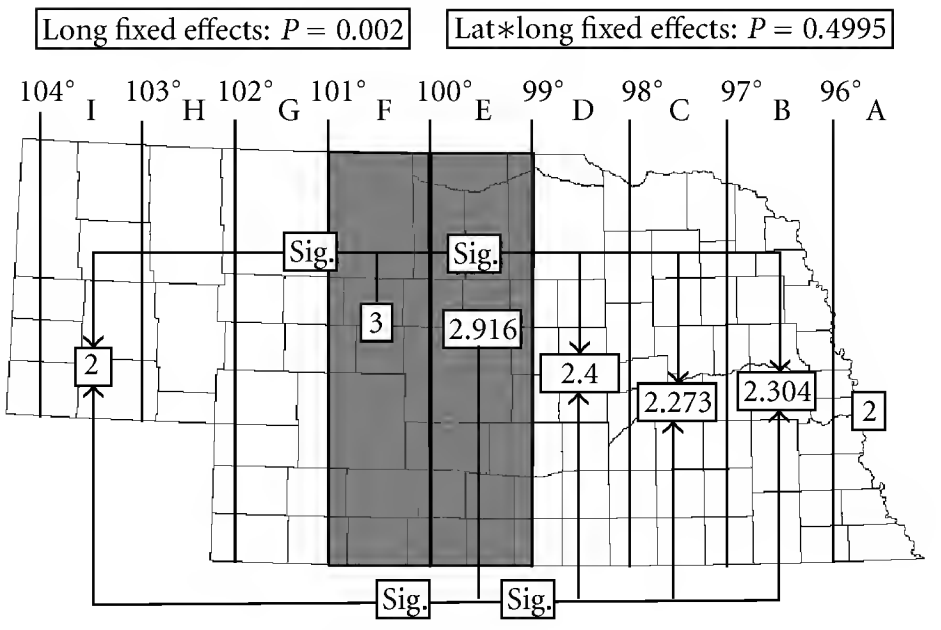

Figure 8: Mean elytral color by code for *Cicindela splendida* by longitudinal region. Significant differences between longitudes shown by arrows and mean value presented in each longitudinal grouping. Fixed effects presented at top. No latitude by longitude interactions found. Shaded area depicts longitudinal regions differing from others.

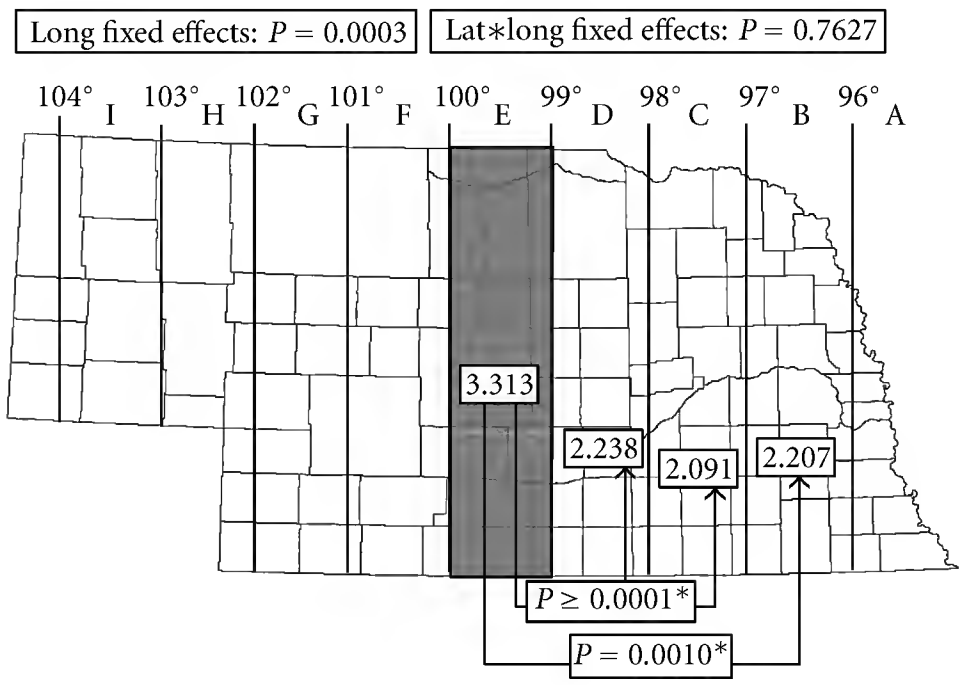

Figure 7: Mean pronotal color by code for *Cicindela limbalis* by longitudinal region. Significant differences between longitudes shown by arrows and mean value presented in each longitudinal grouping. Fixed effects presented at top. No latitude by longitude interactions found. Shaded area depicts longitudinal regions differing from all others.

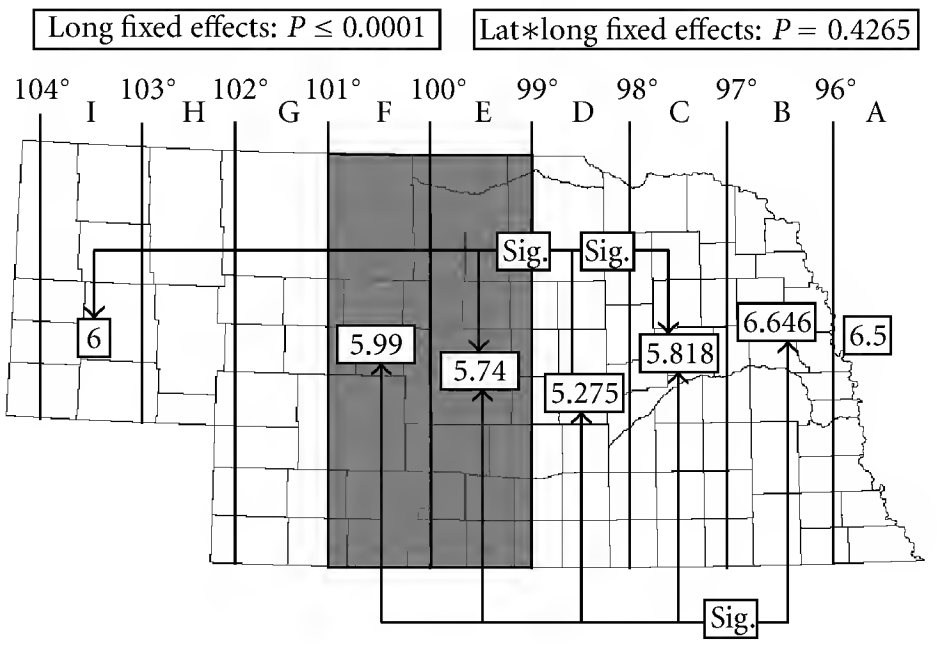

Figure 9: Mean pronotal color by code for *Cicindela splendida* by longitudinal region. Significant differences between longitudes shown by arrows and mean value presented in each longitudinal grouping. Fixed effects presented at top. No latitude by longitude interactions found. Shaded area depicts longitudinal regions differing from all others except each other.

Observed hybridization among members of the butterfly genus *Liminitis* in North America appears correlated to one species occurring at extremely low densities alongside a sister species that is more numerous. Under such conditions, a male of the rare species may choose to mate with a female of the more common species if he is unable to find a mate of his own species [29]. However, Wirtz [30] concluded in a review of the literature that females are the choosier sex and that in most cases females of rare species will mate with males of more common species as a last resort. Although genetic analysis is needed to determine the direction of crossing in these species in Nebraska, rarity of individuals of a species appears to contribute to interbreeding at least for *C. limbalis*.

Rarity of individuals does not appear to explain intergrades between *C. denverensis* and *C. splendida* which often and widely co-occur in Nebraska and elsewhere with little evidence of interbreeding. It does seem possible that hybridization between *C. denverensis* and *C. limbalis* could lead to a cascade of hybridization events perhaps causing hybrid offspring to interbreed with any of the three species, resulting in offspring of a broad range of phenotypes. Elsewhere, *C. denverensis* and *C. limbalis* may hybridize where they cooccur, but they are mostly geographically separated, potentially as a result of differing moisture preferences. In Colorado, Kippenhan [31] reported few locations where both species had been collected. It appears that *C. limbalis* dominates sites with a long and stable history. For example, although the steep loess bluffs in Fremont County Iowa just across the Missouri River from Nebraska City present habitat suitable for *C. limbalis* and *C. splendida* and are within the range

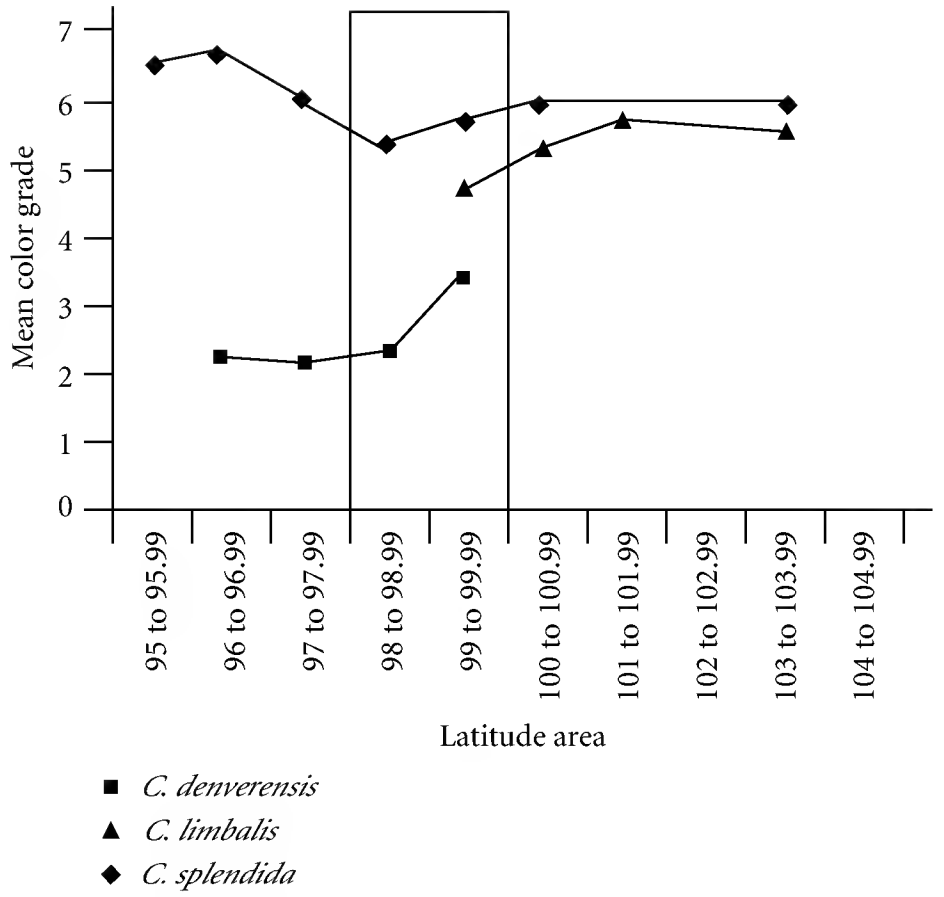

FIGURE 10: Graph of mean pronotal color by code for *C. denverensis*, *C. limbalis*, and *C. splendida* by longitude. Region of character convergence for *C. denverensis* and *C. splendida* depicted by rectangle.

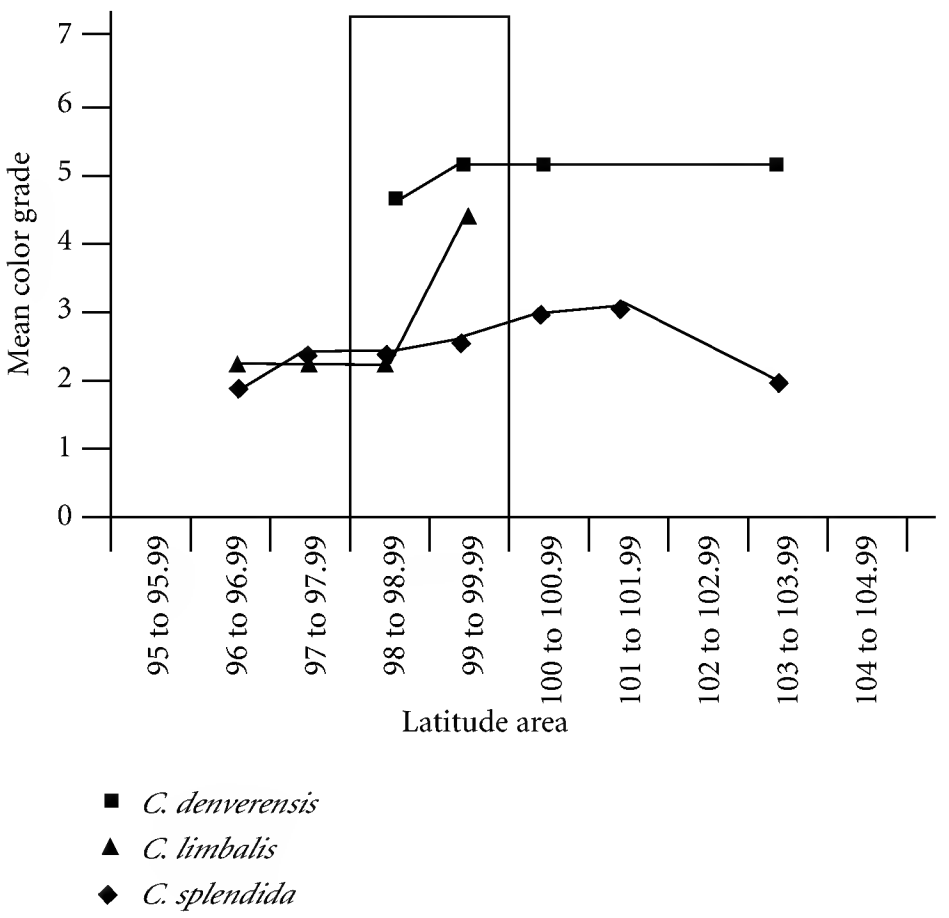

FIGURE 11: Graph of mean elytral color by code for *C. denverensis*, *C. limbalis*, and *C. splendida* by longitude. Region of character convergence for *C. denverensis* and *C. splendida* depicted by rectangle.

of both species, *C. limbalis* is common while *C. splendida* is rare there. Thus, disturbance, either from natural causes or anthropomorphic changes, may also influence the hybrid zone.

It is also unknown if the location of the hybrid zone is stable over time. Dasmahapatra et al. [32] found that a hybrid zone in the lepidopteran genus *Anartia* had moved significantly in Central America over a twenty-year period. Future collection in the hybrid zone in Nebraska should reveal if the

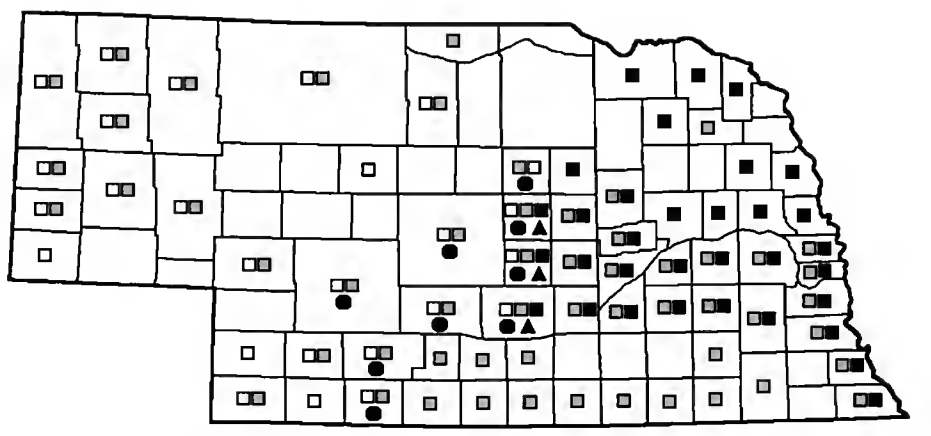

FIGURE 12: Approximate delineation of hybrid zones in the *Cicindela splendida* group in Nebraska. The occurrence of *C. denverensis* is depicted by an empty square, *C. splendida* by a gray square, and *C. limbalis* by a black square. A black circle indicates presence of *C. denverensis* × *splendida* hybrids, and a black triangle indicates the presence of *C. limbalis* × *denverensis* hybrids.

FIGURE 13: Series of *Cicindela splendida* showing variation in maculation and color. Top row: Largely pure *C. splendida*, bottom row: Hybrid *C. denverensis* × *splendida*.

zone is spatially stable. It is also unknown if the hybrids suffer from reduced fitness as has been found in some other studies [33–35].

Finally, it is unknown whether assortative mating [36, 37], female choice, or male choice are the major factors influencing the hybrid zone. Male tiger beetles will attempt to mate with nearly any other tiger beetle of similar size [38]. Thus, it may be that females make the final choice in determining if the spermatophore is suitable. It is also possible that the dispersal ability of each of these three species may also play a role in cooccurrence [39]. Carter [40] suggested that *C. limbalis* did not colonize new sites as rapidly as *C. splendida*, and this matches our own observations.

Mitochondrial studies used to distinguish between closely related species are sometimes of limited value. For example, Schmidt and Sperling [3] suggested that rare hybridization between tiger moth species in the genus *Grammia* might explain why their mtDNA tree appeared to follow geographic distribution rather than previously supported phylogeny. The authors also suggested that while mtDNA analyses can

be misleading for distinguishing closely related species, these tools are an excellent tool for detecting hybridization [3]. Of particular interest in such cases is why such mitochondrial lineages are passed on and proliferate. Perhaps such phenomena support the hybrid vigor hypothesis.

Mitochondrial DNA evidence suggests that all three species in the *C. splendida* group may represent a single variable species [22, 24]. However, ecological preferences and the complex phenotypic interrelationships between these forms suggests otherwise. Even the concept of subspecies does not apply as this would suggest that across much of the United States, two subspecies occur sympatrically without interbreeding. The remaining explanations are (1) unique phenotypes within a single species which affect coloration, mating preference, and habitat associations, perhaps as a result of differing selection pressures, (2) ecological species, or (3) a ring species phenomenon.

If the first explanation is correct, it would suggest a group in the process of speciation. Indeed, if phenotype affects mating preference, this would largely keep each of these forms distinct. It is apparent that while males will attempt to mate with females of any of these three forms, many observed matings between forms resulted in rejection of the spermatophore [20]. Both the second and third explanations suggest overlaps in habitat preference, but differences in optimal habitat. If these tiger beetles qualify as a ring species, the geographic pattern of phenotypes suggests that *C. denverensis* would form the middle of the ring, and *C. limbalis* and *C. splendida* the two ends. If this phylogenetic relationship is true, it would differ from the hypothesis presented by Schinckariol and Freitag [18], who suggested that *C. limbalis* is most representative of the ancestral form, that *C. denverensis* represents an early split, and that *C. splendida* represents a later split from a *C. limbalis* type ancestor.

While the biological species concept suggests that the occurrence of any hybrids represents incomplete speciation [41, 42], the fact that these three tiger beetle species maintain their integrity over most of their range suggests that they "function" as individual species in most areas. For now, based on morphological and mating studies, it appears that *C. denverensis* is phylogenetically closer to both *C. limbalis* and *C. splendida* than these two species are to each other. Perhaps more sensitive genetic studies may reveal the true phylogenetic relationships among these three species. This study is an example of the difficulty in applying species concepts for closely related species that differ in a small number of characters and hybridize in at least limited areas.

This study shows the complexity of species definitions, especially based on color morphologies. Across much of their ranges, these forms function as distinct species; however, the observed hybrid zone in central Nebraska causes the validity of this conclusion to be questioned. Our findings have important implications for conservation and for monitoring biological diversity. Based on the frequency of hybridization in this group of species in Nebraska all three species could be lumped into a single species. Alternatively, if only morphology is used, hybrids could be viewed as different species, leading to the possibility of four or five species being present. Doing rapid biodiversity assessment in central Nebraska using tiger beetles could result in either underestimating or overestimating tiger beetle diversity or both if citizen scientists were used for these surveys [6].

In Nebraska, the Salt Creek tiger beetle, *Cicindela nevadica lincolniana* Casey, is a federally endangered subspecies of the much more widely distributed *C. nevadica* [43]. Thus, the designations of subspecies based on phenotypes can have important consequences for conservation as well. Our findings of morphological variation and hybridization among multiple species suggests that tiger beetle taxonomy based on morphological characters alone should be cautiously interpreted and that additional research using molecular and behavioral techniques is warranted. Because tiger beetles are among the most charismatic and well-studied beetle groups, it is likely that similar or even greater problems will be encountered for other beetle groups that are potential indicators of ecosystem changes.

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Research Article

A Survey of Bee Species Found Pollinating Watermelons in the Lower Rio Grande Valley of Texas

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Using a combination of flower traps and visual observations, we surveyed three watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) fields in the Lower Rio Grande Valley to determine what bees inhabit this crop in this region. No managed honey bee (*Apis mellifera* L.) hives were in any of the fields; however, two contained managed hives of the common eastern bumble bee, *Bombus impatiens* (Cresson). A total of 15 species were collected or observed from all three fields combined. Of these species, only four were found to be very abundant: *Agapostemon angelicus* Cockerell/*texanus* Cresson, *A. mellifera*, *Lasioglossum coactum* (Cresson), and *Melissodes thelypodii* Cockerell. *Apis mellifera* comprised 46% of all bees collected from all three fields combined and was highly abundant in two of the three fields. In the third field, however, *A. mellifera* and *Agapostemon angelicus/texanus* were equally abundant. Surprisingly, *B. impatiens* comprised only 1% of the total bees surveyed in all three fields combined, despite two of the fields having several managed hives each. As *B. impatiens* is not native to this region, it was not surprising that none were collected or observed in the field with no managed hives.

1. Introduction

Watermelon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai; Cucurbitaceae] is a crop that has been well documented for its dependence on insect pollinators for fruit and seed set due to its monoecious flowering condition of separate staminate (male) and pistillate (female) flowers [1, 2]. In fact, numerous studies have even shown that watermelon plants in exclusion cages will not set fruit [1–3]. Each female watermelon flower also requires approximately 500 to 1000 or more viable pollen grains for complete fertilization of ovules [1, 4]. Therefore, each female watermelon flower has been found to require at least 6–8 honey bee (*Apis mellifera* L.; Hymenoptera: Apidae) visits for successful pollination [1, 5].

Historically, *A. mellifera* has been generally recognized as the most important pollinator for commercial crop production [4, 5], including watermelon. Due to their manageability and large perennial colonies, *A. mellifera* is easily transported to different fields as needed [4–6]. Recently, however, many *A. mellifera* colonies have been significantly weakened or lost due to exotic parasites, diseases, loss of bee-keeping subsidies, colony collapse disorder, Africanization, and pesticide exposure [3–8]. In fact, the supply of *A. mellifera* colonies has been reduced more than 50% since the 1950s despite a growing demand for *Apis* pollination services [4].

In response to declining *A. mellifera* populations, watermelon growers are now pollinating their fields using commercial bumble bees (*Bombus* spp.; Hymenoptera: Apidae). Although *Bombus* spp. have small annual colonies made up of fewer workers than *A. mellifera* hives have and they are also labor-intensive to produce, *Bombus* colonies require far less maintenance in the field. Moreover, their workers are active at lower temperatures and fly in higher winds than *A. mellifera* do. On a per-bee basis, *Bombus* spp. are also more efficient watermelon pollinators than *A. mellifera* are [6]. In USA, the primary commercial *Bombus* species is the common eastern bumble bee, *B. impatiens* (Cresson).

Researchers and growers have recently turned their focus to evaluating wild bee species as pollinators in crop production, especially in crops that are heavily dependent on insect pollinators, such as watermelon, for example [4, 7, 8]. In certain crops, some wild bee species are more effective pollinators than are *A. mellifera* workers [4]. Also, wild bees provide their pollination services free of charge [7]. Kremen et al. [7] found that organic farms in California located near native habitat (defined as having ≥30% native habitat within a 1 km radius of the farm) could receive adequate pollination from wild bees alone. However, as agricultural intensification increases, pollination services decrease by 3- to 6-fold [7].

Watermelon is one of many important crops grown in the Lower Rio Grande Valley of Texas. In an attempt to better understand watermelon pollination in the Lower Rio Grande Valley, bee species, both wild and managed, were surveyed at flowering watermelon in this region.

2. Materials and Methods

Observations and collections were undertaken in three fields located in Hidalgo County, TX, during Spring 2011. Two fields, designated as Mile 13 Field (26.38881° N, 98.23451° W) and Mile 14 Field (26.40230° N, 98.23282° W), located northwest of Edinburg, TX, were the larger of the three fields at 3.237 ha and 7.284 ha, respectively. Both fields are owned by a local watermelon grower and were planted in mid-Feb. 2011 with both seeded and seedless varieties interplanted within each row. While neither of these fields had managed *A. mellifera* hives placed in them, they both did have multiple managed hives of *B. impatiens*. Native vegetation consisting predominantly of common sunflower (*Helianthus annuus* L.; Asteraceae) was allowed to grow along the field edges as a refuge for beneficial insects. Both of these fields had adjacent citrus orchards and grassy fields with remnant citrus trees and mesquite.

The third field (ARS Field) was a small solid planting (0.352 ha) of a seeded variety (Legacy) located on the USDA-ARS property in Weslaco, TX (26.15850° N, 97.96364° W). This field was established a month later on 8 Mar. 2011. Native vegetation along the field edges was kept mowed, except for a 30 m strip of golden crownbeard (*Verbesina encelioides* (Cav.) Benth. & Hook. F. ex A. Gray; Asteraceae) growing along a fence ~30 m from the field's western edge. ARS Field was also bordered on the south side by a corn field. No managed *A. mellifera* or *B. impatiens* hives were placed in this field.

To target just the bees visiting watermelon flowers, flower traps were used primarily [9]. Traps consisted of clear 4.5 oz. Falcon specimen cups filled with approximately 80 mL of soapy water solution (3 mL of liquid dishwashing soap/3.785 L of water). A single male watermelon flower was submerged in each trap during trap placement. A total of 10 points approximately 6 m apart were marked in each field along three 60 m transects for a total of 30 points per field. Transects began approximately 6 m from the field edge following the row. During peak watermelon flowering, traps were placed at each point between 0900 HR and 1000 HR and removed between 1400 HR and 1430 HR. Contrary to findings in North Carolina where watermelon flowers opened around 0700 HR [10], watermelon flowers in our study were just opening at the time of trap placement and were just about to close at the time of trap removal. All samples were brought back to the lab for processing and identification. Due to the short flowering period, Mile 13 Field and Mile 14 Field were sampled weekly a total of three times (31 Mar., 7 Apr., and 14 Apr.), and ARS Field was sampled weekly a total of five times (19 Apr., 26 Apr., 3 May, 10 May, and 17 May). A single trap was placed beside a single hive quad (Koppert Biological Systems, Inc.; Michigan, USA) at Mile 13 Field and Mile 14 Field (2 traps total) to see if traps would collect worker *B. impatiens*.

Trap samples were supplemented with visual observations and hand collections. During trap placement and removal, the surrounding flowers were scanned for the presence of bees. If a bee could be identified by sight, its identity and the location it was observed were recorded. If a bee could not be identified by sight, it was collected using a Dirt Devil Detailer (Model CV 2000) and brought back to the lab for processing and identification. To eliminate counting nonvisiting bees, only bees observed in watermelon flowers were recorded or collected. Approximately 1.5 h after trap placement on 14 Apr., sample points of Mile 13 Field were each visually surveyed for an additional period; however, additional visual observations were not made at either of the remaining fields.

All identifications were made by one of us (CSH) primarily using the identification keys provided on the Discover Life website (http://www.discoverlife.org/mp/20q?search=Apoidea). In cases where the Discover Life key to species for a specific genus did not cover the Lower Rio Grande Valley, an appropriate published key was used. Identification of *Lasioglossum coactum* (Cresson) (Hymenoptera: Halictidae) was aided with correspondence with Jason Gibbs, who also confirmed this identification after viewing representative specimens.

Due to morphological similarities between *Agapostemon angelicus* Cockerell and *A. texanus* Cresson (Hymenoptera: Halictidae), a definitive identification cannot be made without molecular testing [11]. Based on species collection records for both species mapped on the Discover Life website, it is likely that the correct identification is *A. texanus*. However, no males of either species, which are distinctly different, were collected to support this assumption.

3. Results

A total of 15 species of bees were collected from our watermelon fields in the Lower Rio Grande Valley (Table 1). ARS Field was found to be the most diverse field with 11 species. Eight and nine species of bees were collected from Mile 13 Field and Mile 14 Field, respectively. ARS Field had a higher overall abundance of bees than the other two fields, possibly a result of this field being sampled two more times than Mile 13 Field and Mile 14 Field.

Only four species were found to be abundant: *A. angelicus/texanus*, *A. mellifera*, *L. coactum*, and *Melissodes thelypodii* Cockerell (Hymenoptera: Apidae). *Apis mellifera* was

Table 1: Bee species collected from three Lower Rio Grande Valley watermelon fields during 2011.

Species	ARS		Mile 13		Mile 14	
	Traps	Obs.	Traps	Obs.	Traps	Obs.
*Agapostemon angelicus/texanus**	1	—	12	1	38	—
Apis mellifera	2	67	10	44	11	28
Augochlorella aurata	—	—	—	—	1	—
Augochlorella bracteata	—	1	—	—	—	—
Bombus impatiens	—	—	—	3	1	1
Exomalopsis snowi	1	—	—	—	—	—
Florilegus condignus	1	—	—	—	—	—
Halictus ligatus	3	2	—	—	1	1
Lasioglossum coactum	38	—	5	—	6	1
Lasioglossum viridatum	—	—	—	—	3	—
Lasioglossum sp. 1	—	—	1	—	—	—
Lasioglossum sp. 2	3	—	8	—	3	—
Lasioglossum spp.	—	2	—	—	—	—
Melissodes thelypodii	19	1	18	1	8	1
Nomada crucis	—	1	—	—	—	—
Triepeolus helianthi	3	1	1	—	—	—
Unknown Halictid	—	1	—	—	—	—
Total:	**71**	**76**	**55**	**49**	**72**	**32**
Overall Total:	**147**		**104**		**104**	

*Females of *A. angelicus* and *A. texanus* are morphologically identical and cannot be separated where the distribution of the two species overlap [11].

the most abundant bee, comprising 46% of the overall total number of bees collected and observed from all three fields combined. *Agapostemon angelicus/texanus*, *L. coactum*, and *M. thelypodii* each comprised 13% to 15% of the overall total number of bees surveyed. The remaining 11 species collectively comprised the remaining 11%. Despite Mile 13 Field and Mile 14 Field having approximately 3-4 managed hives of *B. impatiens* each, only 5 workers from these colonies were collected or observed from both fields. *Bombus impatiens* workers comprised only 1% of the overall total of bees surveyed from the three fields.

Apis mellifera was the most abundant pollinator in ARS Field and Mile 13 Field, while *A. angelicus/texanus* was equally abundant to *A. mellifera* in Mile 14 Field. Only one individual of *A. angelicus/texanus* was collected at ARS Field. However, *A. angelicus/texanus* numbers began to decline as the season progressed (Figure 1). Conversely, ARS Field had 3x the abundance of *L. coactum* than either of the two other fields combined, with a large peak in abundance on the last sample date (17 May) (Figure 1).

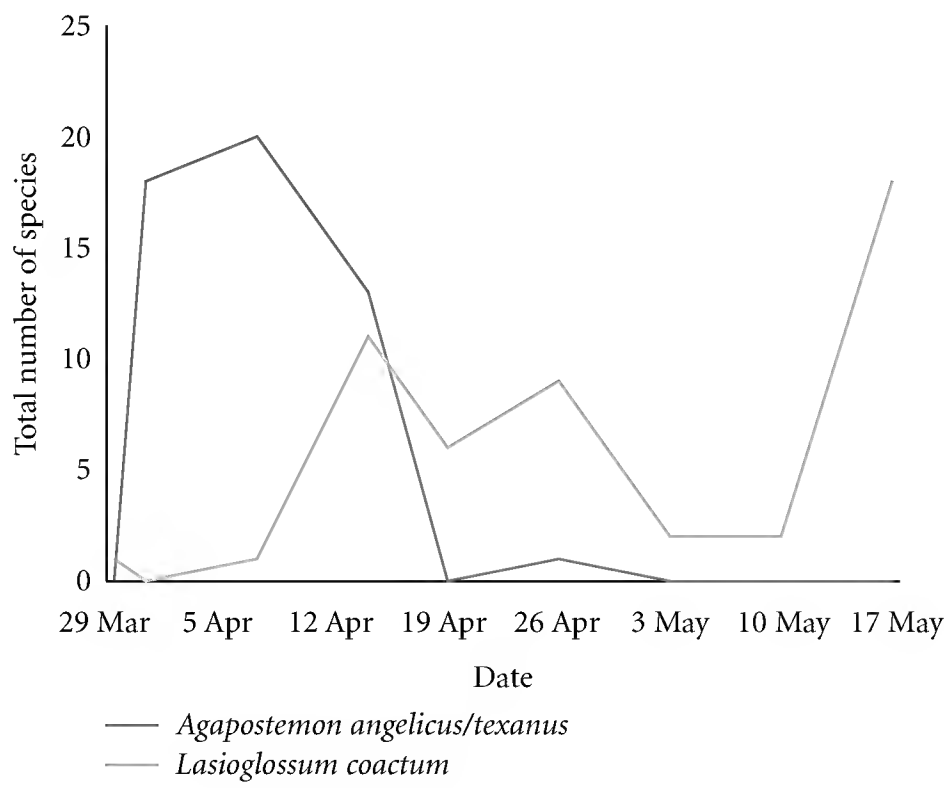

Figure 1: Seasonality of *Agapostemon angelicus/texanus* and *Lasioglossum coactum* collected from three Lower Rio Grande Valley watermelon fields during 2011.

4. Discussion

Our results were similar to those found in a small survey near Leesburg, FL [12]: the most abundant species was *A. mellifera*, followed by three fairly abundant species and other less common species. In total, Goff [12] collected eight species of bees, which is approximately the number of species that were found in our study fields. However, with the exception of *A. mellifera*, the bees Goff [12] collected were solely from the family Halictidae. Our study found roughly equal number of species from Halictidae and Apidae, including a few cleptoparasites presumably oudrinking nectar.

As no managed hives of *A. mellifera* were placed in any of these fields, the high number of this species collected and observed likely derives from feral colonies living nearby.

Grassy habitats located in close proximity to our fields were observed to contain suitable nesting sites for feral *A. mellifera*. It is equally likely that some of these bees may have originated from managed hives that were observed in a grassy field approximately 1 to 2 km east of Mile 13 Field and Mile 14 Field. Previous studies have indicated that this distance is well within the typical foraging range of *A. mellifera,* for example [13, 14]. In fact, Visscher and Seeley [13] found the radius surrounding 95% of their observation colony's foraging sites to be 6 km. Beekman and Ratnieks [14] found that 95% of the bees from their observation colony foraged within an even greater distance of 10 km.

Porter [15] noted that *A. texanus* was frequently found in fields and open places in scrub and woodland, such as abandoned citrus groves in the Lower Rio Grande Valley. Populations of *A. texanus* most likely occur year-round, but its population appeared to peak in December and January before becoming scarce by March and May [15]. This seasonality may explain the relative lack of *A. angelicus/texanus* in ARS Field compared to Mile 13 Field and Mile 14 Field as ARS Field was sampled a month later than the other two fields due to a later planting date. Porter [15] also noted that the main nectar and pollen source for *A. texanus* was common sunflower, which may, again, contribute to the low abundance of *A. angelicus/texanus* in ARS Field. While Mile 13 Field and Mile 14 Field both had common sunflower growing abundantly along the edges, ARS Field did not.

Life history information is lacking for *L. coactum*, which belongs to the predominantly primitively eusocial subgenus *Dialictus* [16, 17]. *Dialictus* contains numerous, commonly collected, "morphologically monotonous" species [17, 18]. In fact, a recent molecular study revealed that the easily identified species *Lasioglossum tegulare* (Robertson) (Hymenoptera: Halictidae) was instead a species complex containing several cryptic species, including *L. coactum* [17]. Gibbs [17] notes that the range of *L. tegulare* is more restricted to the northeast than previously reported and that records from Texas are probably *L. coactum*. As *Dialictus tegularis*, Mitchell [19] records a flight season of March or April through October for *L. coactum*.

The apparent higher abundance of *L. coactum* at ARS Field than Mile 13 Field and Mile 14 Field is likely a reflection of the later sampling at ARS Field than Mile 13 Field and Mile 14 Field. Primitively eusocial species tend to start with a single, solitary female completing all necessary nesting tasks. Upon emergence of her offspring, division of labor between queen and workers arises [20]. The lower number of *L. coactum* at both Mile 13 Field and Mile 14 Field are likely females just emerging from winter diapause at the beginning of the flight season. The increase at ARS Field likely reflects the natural increase in population as the season progresses. The drop in *L. coactum* abundance seen during early May could be attributed to residues from insecticides targeting whiteflies in late April. As we did not test for this, nor was this pesticide used on either of the two other fields, this relationship could also be coincidental.

Such a low abundance of *B. impatiens* both in the traps and during visual observations was surprising when considering the presence of multiple colonies at Mile 13 Field and Mile 14 Field. This was contrary to a study in North Carolina [10] looking at watermelon and cucumbers, which compared the diurnal activity, floral visitation rate, and pollen deposition rate of *B. impatiens* to *A. mellifera* to determine the most efficient pollinator of the two. Because *B. impatiens* was found to be out foraging earlier and longer, visited more flowers/minute and deposited more pollen grains, it was found to be the most efficient pollinator. However, no distinction was made in this study between managed and feral *B. impatiens* [10].

Bombus impatiens has a published distribution of Ontario and Maine, south to Florida, and west to Michigan, Illinois, Kansas, and Louisiana [21, 22]. The low abundance of *B. impatiens* in our study compared to the North Carolina study may be due to the fact that *B. impatiens* is not native to Texas or the Lower Rio Grande Valley as it is to North Carolina. Therefore, the managed colonies brought in from Michigan may not have been adequately adapted for the Lower Rio Grande Valley climate. In North Carolina, *B. impatiens* was found to be out foraging in watermelon flowers approximately 30 min. earlier than *A. mellifera* and, in some cases, even attempting to forcibly enter unopened watermelon flowers. Both species were observed foraging until the watermelon flowers closed for the day [10]. While our traps were placed in the field later in the morning than those in North Carolina, we do not feel we missed early morning *B. impatiens* foraging in watermelon as our traps were placed at the time of flower opening. In fact, on at least one occasion, trap placement was delayed until the male flowers were open enough to be used in the traps.

Colored pan traps are a passive collection method with the advantage of limiting potential sampling biases associated with the sampler's observational and netting skills. However, they have been known to have several biases, one of which is that they catch fewer individuals of *Bombus* spp. than expected [23]. In an attempt to avoid these biases as well as target our catches to bees attracted to watermelon, we employed flower traps, which shift the attractant from bowl color to the target flower. While this study did not specifically test the traps for any biases, we do not believe that trap biases, if any, were a contributing factor in the low *B. impatiens* abundance at both Mile 13 Field and Mile 14 Field. Visual observation data as well as unrecorded observations made in these fields during nonsampling times also indicated low *B. impatiens* abundance.

This study was conducted solely with the intent to establish baseline knowledge of the bees present in Lower Rio Grande Valley watermelon fields. As no major surveys of the Lower Rio Grande Valley bee fauna had been undertaken previously, our prior expectations were limited to *A. mellifera*, based on surrounding vegetation types as well as *Peponapis pruinosa* (Say) (Hymenoptera: Apidae), due to its dependence on the flowers of other cucurbits (i.e., squash). Therefore, the relatively high abundance of *A. mellifera* was not very surprising. Neither was the relatively high abundance of *L. coactum* as the genus *Lasioglossum* is globally occurring, commonly collected, and well known to dominate faunas with its abundance [18]. The most surprising finding of this study was the low abundance of

B. impatiens at Mile 13 Field and Mile 14 Field despite having managed hives within these fields.

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Research Article

Pseudacteon Parasitoids of *Azteca instabilis* Ants in Southern Mexico (Diptera: Phoridae; Hymenoptera: Formicidae)

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Three new species of the genus *Pseudacteon* are described, all from Chiapas, Mexico, and all of which are parasitoids of the ant *Azteca instabilis*. Sternite 6 of *Pseudacteon dorymyrmecis* Borgmeier is illustrated for the first time, and *P. confusus* Disney is synonymized with this species. The natural history of the *Azteca-Pseudacteon* interaction is described.

1. Introduction

The species of the phorid fly genus *Pseudacteon* Coquillett have been under intense scrutiny lately because of their potential to control invasive species of fire ants (*Solenopsis invicta* and *S. saevissima* in North America; *S. geminata* elsewhere) [1–3]. Other lesser-studied species of *Pseudacteon*, many of them undescribed, attack different hosts, including species of *Crematogaster*, *Lasius*, *Liometopum*, *Nylanderia*, *Pseudolasius*, and other small ants.

In a series of papers [4–7], a new host record, with *Azteca instabilis* Fr. Smith, has been documented from southern Mexico. Below, the so-far-known species of *Pseudacteon* associated with these ants are described, their identification clarified, and natural history summarized.

2. Materials and Methods

Specimens were collected into 70% alcohol and dried using hexamethyldisilazane [8]. They were deposited in the following collections:

CEET: El Colegio de la Frontera Sur, Colección de Insectos Asociados a Plantas Cultivadas en la Frontera Sur, Tapachula, Chiapas, Mexico,

LACM: Natural History Museum of Los Angeles County, California, USA,

MCZC: Museum of Comparative Zoology, Harvard University, Massachusetts, USA,

MUCR: Universidade de Costa Rica, San Jose, Costa Rica,

USNM: Smithsonian Institution, Washington, DC, USA.

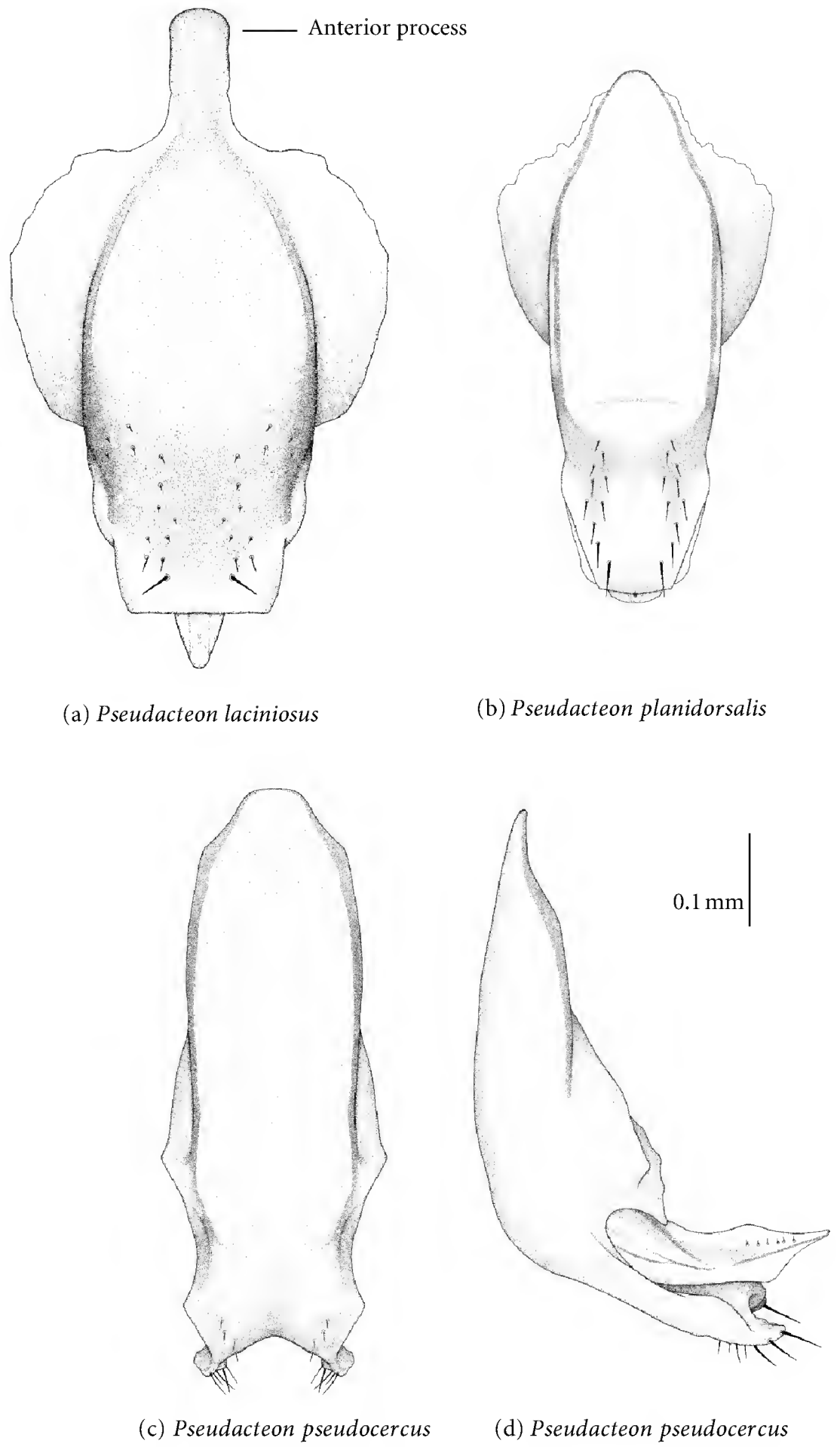
3. Systematics

Pseudacteon Coquillett [9]; full synonymy in Borgmeier, 1968 [10]; type species. *Pseudacteon crawfordi* Coquillett, original designation.

Note on gender: the word *Actaeon* is a Greek name for a (male) hunter; thus, the name *Pseudacteon* means "false Actaeon" and is masculine in gender.

Pseudacteon laciniosus new species (see Figures 1(a) and 2(a)).

Diagnosis. The last general key to adults of *Pseudacteon* is that of Borgmeier [11]. This new species keys to couplet 10, where a user is given the alternatives of "ovipositor lanceolate" versus "ovipositor subcylindrical, tapering at apex." Since the protruding stylet could fool users into accepting the first alternative, these flies could key out to either *P. dorymyrmecis* Borgmeier in the first lead or *P. onyx*



(a) *Pseudacteon laciniosus* (b) *Pseudacteon planidorsalis*

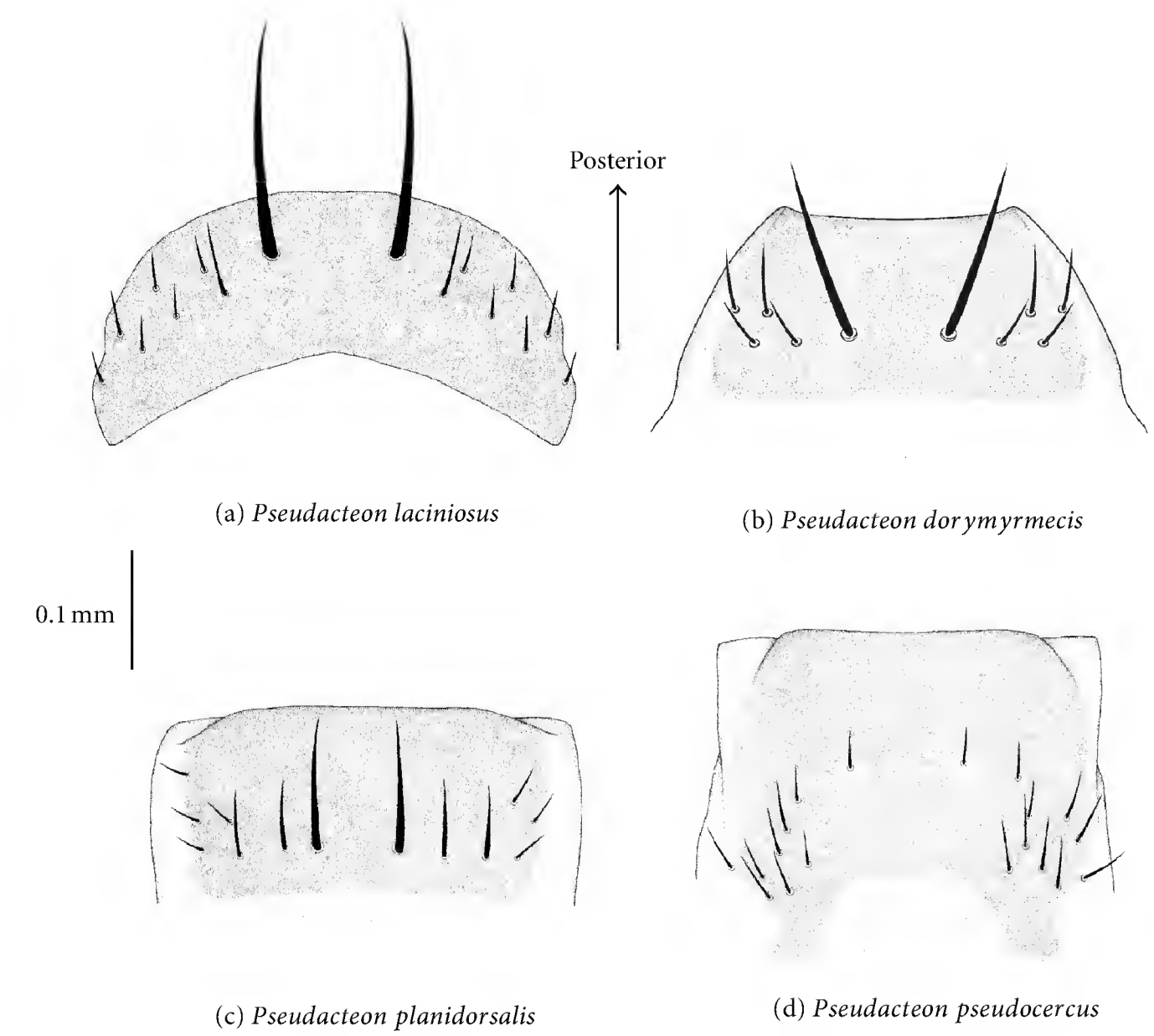
(c) *Pseudacteon pseudocercus* (d) *Pseudacteon pseudocercus*

FIGURE 1: *Pseudacteon* species, female oviscapes, dorsal and right lateral (Figure 1(d)).

Steyskal in the second. Both of these species are markedly smaller than *P. laciniosus*, and both have narrower oviscapes. Further, based on examination of the holotype specimen, sternite 6 of *P. dorymyrmecis* has a long pair of medial setae originating basally on the segment (see Figure 2(b)). The oviscape of *P. onyx* was illustrated by Borgmeier [12] and is much more ventrally curved than that of *P. laciniosus*. Finally, unlike both of the other species, *P. laciniosus* has a dark brown body that strongly contrasts with its yellowish legs.

Disney (in [13]) described a new species, *Pseudacteon confusus*, that also keys to *P. dorymyrmex*. We examined a paratype female of *P. confusus*, comparing it to the holotype female of *P. dorymyrmex*, and conclude the two are the same species. Therefore, *P. confusus* is a junior subjective synonym of *P. dorymyrmex* (new synonymy).

Description. *(Female)* Body length 1.2–1.5 mm (mean = 1.3). Frons dark brown, with 2-4-4-4 setae and one pair of proclinate supraantennal setae. Flagellomere 1 dark brown, rounded, flat; length of arista about two times that of flagellomere 1. Palpus light brown, setulae thick. Thorax dark brown. Scutellum with two pairs of large setae, anterior



(a) *Pseudacteon laciniosus*

(b) *Pseudacteon dorymyrmecis*

(c) *Pseudacteon planidorsalis*

(d) *Pseudacteon pseudocercus*

FIGURE 2: *Pseudacteon* species, venter of segment six.

pair 0.8 length of posterior pair. Legs yellowish brown, except forecoxa lighter, mid- and hind coxae darker brown. Wing with mean costal length 0.36 wing length, range 0.35–0.37. Halter yellow. Abdominal tergites dark brown, ventral abdominal membrane dark gray. Tergite 6 with large posterior emargination and lateral pair of setae. Sternite 6 with one large (0.18 mm) pair of slightly divergent setae; more lateral pair much smaller (0.06 mm), but larger to subequal in size to median pair on one or both sides in some specimens; with scattered setulae (see Figure 2(a)). Oviscape (Figure 1(a)) with narrow anterior process, convex dorsally, with large lateral flanges anteriorly, terminating bluntly, although preserved specimens often with stylet protruding, making oviscapes appear pointed. Minute setae on dorsal surface of oviscape arranged in laterally concave lines, with larger, divergent, apical pair.

Holotype. ♀, Mexico: Chiapas: Finca Irlanda, 15°11′ N, 92°20′ W, July 2010, S. Philpott, over *Azteca instabilis* (barcode LACM ENT 139561) (CEET).

Paratypes. 16♀, same data as holotype (CEET, LACM, MCZC, USNM).

Etymology. The specific epithet is from a Latin word for "fringed," referring to the enlarged lateral margin of the oviscape.

Pseudacteon planidorsalis new species (see Figures 1(b) and 2(c))

Diagnosis. The species is similar to *Pseudacteon dorymyrmecis,* but differs in the presence of longer setae flanking the relatively shorter medial pair on sternite six.

Description. (Female) Body length 1.1–1.4 mm (mean = 1.3). Frons dark brown, with 2-4-4-4 setae and one pair of proclinate supra-antennal setae. Flagellomere 1 dark brown, rounded, flat; length of arista about two times that of flagellomere 1. Palpus yellow, setulae thick. Thorax brown. Scutellum with two pairs of large setae, anterior pair 0.47–0.60 length of posterior pair. Legs yellowish brown, except forecoxa lighter. Wing with mean costal length 0.38 wing length, range 0.37–0.39. Halter yellow. Abdominal tergites dark brown, ventral abdominal membrane dark gray. Tergite 6 with large posterior emargination and lateral pair of setae. Sternite 6 with one large (0.09 mm) pair of large setae and smaller lateral setae in basal transverse row; smaller lateral setae scattered more posteriorly (see Figure 2(c)). Oviscape (Figure 1(b)) lacking narrow anterior process, flat dorsally, with large lateral flanges anteriorly, terminating in rounded point (which is difficult to see in Figure 1(b) because the oviscape is downturned apically). Minute setae on dorsal surface of oviscape arranged in laterally convex lines, with slightly larger apical pair.

Holotype. ♀, Mexico: Chiapas: Finca Belen, 15°15′ N, 92°23′ W, 8.ii.2003, S. Philpott, over *Azteca instabilis* (barcode LACM ENT 294148) (CEET).

Paratypes. 3♀, same data as holotype (LACM).

Etymology. The specific epithet is from a Latin word for "flat backed," referring to the surface of the oviscape.

Pseudacteon pseudocercus new species (see Figures 1(c), 1(d) and 2(d))

Diagnosis. This species can be recognized by the strongly downturned, lightly sclerotized oviscape with the pair of cercuslike apical processes. Other species of *Pseudacteon* with a bilobed oviscape have the structure strongly sclerotized and dark brown in color.

Description. (Female) Body length 1.2 mm. Frons dark brown, with 2-4-4-4 setae and one pair of proclinate supra-antennal setae. Flagellomere 1 dark brown, rounded, flat; length of arista about two times that of flagellomere 1. Palpus light brown, setulae small, thin. Thorax brown. Scutellum with two pairs of large setae, anterior pair 0.47–0.60 length of posterior pair. Legs yellowish brown, except forecoxa lighter. Wing with costa 0.40 wing length. Halter yellow. Abdominal tergites brown, ventral abdominal membrane gray. Tergite 6 with large posterior emargination, pair of lateral setae near midline, and longer, thicker seta more laterally. Sternite 6 anteriorly emarginate, with scattered small setae (see Figure 2(d)). Oviscape (see Figures 1(c) and 1(d)) without narrow anterior process, convex dorsally, apically downturned with pair of cercuslike lobes.

Holotype. ♀, MEXICO: Chiapas: Finca Belen, 15°15′ N, 92°23′ W, 8.ii.2003, S. Philpott, over *Azteca instabilis* (barcode LACM ENT 294147) (CEET). No other specimens preserved.

Etymology. The specific epithet is from Latin words for "false circus," referring to the apex of the oviscape.

4. Natural History

Little is known about the life cycle of *P. laciniosus, P. planidorsalis,* and *P. pseudocercus*, as the three species are only known from adults. Further, most natural history information available treats the flies as a genus, rather than as individual species, so more work will be necessary to distinguish between them. Adults have been observed in a range of shaded coffee plantations varying in canopy cover from ~25 to 100% in the Soconusco region of Chiapas, Mexico. Specifically, *P. laciniosus, P. planidorsalis,* and *P. pseudocercus* have been observed in Finca Irlanda, Tapachula municipality (15°11′ N, 92°20′ W), between 800–1100 m elevation, Finca Hamburgo, Tapachula municipality, between 800 to 1100 m elevation (15°10′ N, 92°19′ W), and in Finca Belen, Huixtla municipality, between 800 to 1200 m elevation (15°15′ N, 92°23′ W). The shade coffee habitats from which the phorids have been seen range from shaded monocultures with relatively low levels of canopy cover, tree diversity, and density to rustic coffee plantations with a high diversity and density of shade trees and nearly 100% canopy cover [14]. Preliminary work indicates that the relative abundance of the three species in a range of coffee agroecosystems is similar, even as canopy conditions change [15].

Within the shade coffee habitats, females of *P. laciniosus, P. planidorsalis,* and *P. pseudocercus* have only been observed when hovering over or ovipositing in the host ant species, *A. instabilis*, or flying out of leaf litter collected from the ground or on tree trunks and branches near to *A. instabilis* nests. Males of the three species have not been collected or identified. As a group, the flies are attracted to the host ant by an alarm pheromone (1-acetyl-2-methylcyclopentane) released from the dorsal section of the ant gaster, but they do not attempt to oviposit without ant movement [16]. Work is underway to determine whether visual and similar chemical cues are used by each species in host location and host selection processes. Once a female fly locates host individuals, it will remain in the area for up to several minutes, closely hovering over and following moving ant individuals. Individual flies have been observed to attempt to oviposit at least a dozen times before disappearing from view; actual oviposition has not yet been quantified. Several individuals of the three species (up to 8–10) have been observed simultaneously around the same *A. instabilis* nest.

Phorids strongly modify the behavior of the *A. instabilis* ants and thereby indirectly affect other insects in coffee agroecosystems. *Azteca instabilis* is an aggressive, canopy-dominant ant that has important impacts on many members of the coffee insect food web [17]. In the presence of *Pseudacteon* flies, *A. instabilis* ant foraging is reduced (by about 50%) for up to 90 min. after the first appearance of the phorid [4]. Once the *Pseudacteon* arrives near an *A. instabilis* nest, the ants will either (1) run back to their nest, or to hiding places under tree bark or (2) remain motionless with their heads tilted back [17]. This reduction in ant activity allows other species of ants to gain access to food resources [3, 5] and reduces the predatory effects of ants on lepidopteran larvae [4] and the coffee berry borer (*Hypothenemus hampei* Ferrari) [18]. Furthermore, *A. instabilis* normally prevent adults of the coccinellid beetle *Azya orbigera* Mulsant from feeding on scale insects (*Coccus viridis* Green), a keystone mutualist of the ant. When the *A. instabilis* are under attack by the phorids, *A. orbigera* greatly increase their feeding rates [19] and oviposition rates (Hsieh and Perfecto, *unpublished data*). The host ant, *A. instabilis,* is patchily distributed within coffee agroecosystems, and one force maintaining this distribution and relative abundance of colonies within sample areas may be attacks from the phorid flies [7]. Thus the *Pseudacteon* flies, through their influence on the activity and distribution of this keystone species, likely have widespread impacts on the coffee insect food web. This result contrasts with conclusions of studies with *Pseudacteon tricuspis* Borgmeier and *P. curvatus* Borgmeier and fire ants (*S. invicta*). At least some studies have concluded that phorids attacking *S. invicta* do not have long-term impacts on the ants or associated arthropods (e.g., [20]).

Phorids that attack *A. instabilis* do not lower population sizes, similar to findings with *P. tricuspis* and *P. curvatus*, but likely impact coffee food webs due to their role in maintaining a similar number of colonies and reducing ant behavior.

Field evidence and observations suggest that *P. laciniosus*, *P. planidorsalis*, and *P. pseudocercus* adults probably live near their host, in leaf litter on the ground. Data suggest that the fly population is likely distributed in a density dependent manner, as number of attacks on *A. instabilis* individuals are more frequent and more numerous where *A. instabilis* densities are greater [6, 7]. Likewise, field evidence indicates that *Pseudacteon* adults are usually located within the leaf litter on the ground because time to first oviposition attempt on *A. instabilis* adults placed on the ground is much less than for ants placed at 1.5 m above ground [6]. However, more rapid arrival on the ground could mean that the phorids primarily search for hosts at ground level.

5. Key to Females of *Pseudacteon* Attacking *Azteca* in Southern Mexico

This key is intended for ant ecologists who need to identify phorid parasitoids from known ant hosts. A new general key to New World *Pseudacteon* is needed, as researchers currently must use a combination of Borgmeier [11], Porter and Pesquero [21], Plowes et al. [1], the key below, and reference to species not covered in the previously listed keys [22–26].

(1) Apex of oviscape with lobelike processes (Figures 1(d) and 2(a)); venter of segment 6 with short setae only (Figure 2(d)).....*P. pseudocercus* new species
- Apex of oviscape without lobe-like processes (Figures 1(a) and 1(c); venter of segment 6 with some long setae.....2

(2) Oviscape anteriorly with narrow process, dorsally domelike, with small setulae scattered posteriorly, but longer pair near apex; apex of oviscape truncate (Figure 1(a)); enlarged ventral setae of segment 6 placed posterior to midpoint of sternite, much longer than other ventral setae (see Figure 2(b)).....*P. laciniosus* new species
- Oviscape anteriorly with broad apex; dorsally flattened on apical third (except for downturned tip); small setulae in laterally convex rows; apex of oviscape pointed; enlarged ventral setae of segment 6 placed anterior to midpoint of sternite, only slightly longer than those directly lateral (see Figure 2(d)).....
.....*P. planidorsalis* new species

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Research Article

Oriental *Hydrocyphon* (Coleoptera: Scirtidae: Scirtinae): Seven New Species from Indonesia, Thailand, Malaysia, and India

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Application Specific Instruction-set Processors (ASIPs) expose to the designer a large number of degrees of freedom. Accurate and rapid simulation tools are needed to explore the design space. To this aim, FPGA-based emulators have recently been proposed as an alternative to pure software cycle-accurate simulator. However, the advantages of on-hardware emulation are reduced by the overhead of the RTL synthesis process that needs to be run for each configuration to be emulated. The work presented in this paper aims at mitigating this overhead, exploiting a form of software-driven platform runtime reconfiguration. We present a complete emulation toolchain that, given a set of candidate ASIP configurations, identifies and builds an overdimensioned architecture capable of being reconfigured via software at runtime, emulating all the design space points under evaluation. The approach has been validated against two different case studies, a filtering kernel and an M-JPEG encoding kernel. Moreover, the presented emulation toolchain couples FPGA emulation with activity-based physical modeling to extract area and power/energy consumption figures. We show how the adoption of the presented toolchain reduces significantly the design space exploration time, while introducing an overhead lower than 10% for the FPGA resources and lower than 0.5% in terms of operating frequency.

1. Introduction

The genus *Hydrocyphon* Redtenbacher is represented by 100 species divided into 13 species groups from the Palaearctic and the Oriental Regions (see, e.g., [1, 2] and Tables 1 and 2). The larvae of this genus inhabit running water, for example, small rivers and streams, and the adults are frequently collected by sweeping around the larval habitat. The genus is well defined by certain characteristics (e.g., small body, deeply notched anterior margin of the mesosternum, well-developed parameres and parameroids), and has been comparatively well studied taxonomically [1, 2]. In the present paper, I describe seven new species from Indonesia, Thailand, Malaysia, and India. In addition, new combination and additional specimens examined are presented.

This is the twelfth part of my comprehensive study of "Scirtidae of the Oriental Region" [2–12].

2. Materials and Mandhods

This study was conducted based on the dried specimens preserved in the following public collections.

Ehime University Museum, Matsuyama (EUMJ).

Systematic Entomological Laboratory, Hokkaido University (SEHU).

Staatliches Museum für Naturkunde Stuttgart (SMNS).

The methodology was as shown in a previous study [2]. The photographs in Figure 1 were taken under a Leica MZ95 and produced by automontage software Combine ZM.

The abbreviations used in the present paper are as follows: PL: length of pronotum; PW: width of pronotum; EL: length of elytra; EW: width of elytra; TL: total length (PL plus EL). The average value is given in parentheses after the range.

3. Description of the New Species

3.1. Hydrocyphon jogjaensis sp.n. (See Figures 1(a), 1(b), 2, and 11(a))

Type Material. Holotype male (EUMJ): "Ngaglik, Yogyakarta 7°42′28.34″S 110°24′45.34″E Java, INDONESIA 28. II. 2010 H. Yoshitomi leg."

Paratype female (EUMJ): same data as for the holotype.

Table 1: The list of the species, distribution, ZooBank LSID, and species group of the genus *Hydrocyphon*. An Excel file version is also available at the following URL: https://sites.google.com/site/waterbeandlesofjapan/home/support-files-on-articles/Appendix 1.xls.

No.	Species	Description	Distribution	Zoobank LSID	Species group
1	*alticola*	(Klausnitzer, 1976)	Bhutan: Gogona, India	urn:lsid:zoobank.org:act:F8C20A8C-A3AA-4D0D-9B03-57FCA48F24A4	*kambaiticus*
2	*amaurus*	(Klausnitzer, 1980)	India	urn:lsid:zoobank.org:act:4536CA5C-FFAB-49A6-BE87-05601C01612C	*nyholmi*
3	*aritai*	Yoshitomi, 2001	Taiwan	urn:lsid:zoobank.org:act:578D5320-29EF-4ED3-9063-C682CD9F14D5	*kambaiticus*
4	*auratus*	Ruta, 2004	Vietnam	urn:lsid:zoobank.org:act:F2DF47E7-3368-4940-AA4E-3FABFAF260E2	*pallidicollis*
5	*australis*	Linder, 1864	France, Spain, Algeria, Italy, Sicily,	urn:lsid:zoobank.org:act:DC94FAD9-F097-4480-A753-70AD4B3D536D	*australis*
6	*baliensis*	Yoshitomi and Satô, 2005	Indonesia	urn:lsid:zoobank.org:act:DA3B36F3-6003-4C5E-B95D-7540D9CF956C	*pallidicollis*
7	*bhutanensis*	Klausnitzer, 1976	Bhutan: Tongsa, Nepal	urn:lsid:zoobank.org:act:55404EFA-F485-4608-80E1-2208B26A6B1D	*australis*
8	*bicolor*	Yoshitomi and Satô 2003	Laos	urn:lsid:zoobank.org:act:65E8EFE3-F292-4E78-A1AD-F9B209AE5A0F	*bicolor*
9	*bicornis*	Yoshitomi and Klausnitzer, 2003	China	urn:lsid:zoobank.org:act:642BDF4D-0977-4496-A971-12E2A9CE2CDB	*bicornis*
10	*bifidus*	Yoshitomi and Satô, 2005	China	urn:lsid:zoobank.org:act:B468746C-1769-4061-A792-271FC252C675	*kambaiticus*
11	*boukali*	Yoshitomi and Satô, 2005	India	urn:lsid:zoobank.org:act:033F7904-B6A4-4CA8-A92D-534263880C19	*pallidicollis*
12	*celatus*	Klausnitzer, 1980	India	urn:lsid:zoobank.org:act:3D4EC532-1502-408A-9466-2DF296B8D9C9	*deflexicollis*
13	*championi*	Reitter, 1903	Spain	urn:lsid:zoobank.org:act:0656F99B-8CBB-4EB2-8D84-91FCB0B899E0	*deflexicollis*
14	*chiangmaiensis*	Yoshitomi and Satô, 2005	Thailand	urn:lsid:zoobank.org:act:19450260-7983-471F-8586-A143F268FF29	*pallidicollis*
15	*consolatorius*	Klausnitzer, 1990	Iran	urn:lsid:zoobank.org:act:392F24BC-1E1A-454F-894B-90792E23A22A	*australis*
16	*deflexicollis*	(Müller, 1821)	Europe	urn:lsid:zoobank.org:act:40EFD794-C074-4959-9964-30D7BE6FA9D9	*deflexicollis*
17	*deformis*	Yoshitomi, in present study	India	urn:lsid:zoobank.org:act:110BF6C5-79A4-4E12-9BA6-6A563EA40B38	*pallidicollis*
18	*dentatus*	Yoshitomi and Satô 2003	Laos	urn:lsid:zoobank.org:act:F4E68B0F-5FA9-4906-8818-32800FEE837B	*dentatus*
19	*dispar*	Yoshitomi and Satô, 2005	Thailand	urn:lsid:zoobank.org:act:CEC2D61D-EFB7-4BAF-8C9A-D5AF14DBD1D8	*pallidicollis*
20	*doiinthanonensis*	Yoshitomi, in present study	Thailand	urn:lsid:zoobank.org:act:E8C8ED04-BD9D-4AE1-AFBC-5C6001B6D29D	*deflexicollis*
21	*dubius*	(Klausnitzer, 1980)	India	urn:lsid:zoobank.org:act:A74EFB59-0D2D-47F7-AD69-66735D85C5DF	*renati*
22	*dudgeoni*	Yoshitomi and Klausnitzer, 2003	China	urn:lsid:zoobank.org:act:DB9A3BAA-B439-4405-8205-E583C6DAC007	*pallidicollis*
23	*elongatus*	Nyholm, 1981	Burma	urn:lsid:zoobank.org:act:28F2AC64-C3BA-436B-8B9C-985BFB8DC50E	*renati*
24	*finitimus*	Nyholm, 1977	Turkey	urn:lsid:zoobank.org:act:8670FE8B-5559-4B6A-8283-7BF9DFBA5D48	*australis*
25	*forficulatus*	Nyholm, 1981	Burma	urn:lsid:zoobank.org:act:009E27DE-0576-497B-8D6B-11081577B50E	*deflexicollis*
26	*fulvescens*	Nyholm, 1977	Spain	urn:lsid:zoobank.org:act:F54C50AC-4EEB-45E8-A204-98DBB374FD33	*deflexicollis*
27	*fuscatus*	Klausnitzer, 1970	Albania: Kruma	urn:lsid:zoobank.org:act:3733A42A-4A10-4769-A161-DDE68F73CFFC	*deflexicollis*
28	*gereckei*	Hernando, Aguilera, Ribera 2004	Morocco: Oued Zloul	urn:lsid:zoobank.org:act:194ED02C-FAFF-420A-925B-B67CBF4C4FB3	*pallidicollis*
29	*graseri*	Klausnitzer, 2006	Nepal	urn:lsid:zoobank.org:act:74A0D485-1433-4B3E-B2FF-731B28A6E166	*renati*
30	*guangxiensis*	Yoshitomi and Klausnitzer, 2003	China	urn:lsid:zoobank.org:act:BD810938-3FB8-4413-BE0B-F54F6D009F50	*pallidicollis*
31	*hainanensis*	Yoshitomi and Klausnitzer, 2003	China	urn:lsid:zoobank.org:act:D9D2302B-A302-48FA-BD23-BC3186C5A2AB	*pallidicollis*
32	*hamiota*	Nyholm, 1972	Spain	urn:lsid:zoobank.org:act:25875BEA-7E78-4C3D-A3EB-42ACC0F155C0	*pallidicollis*
33	*hydrocyphonoides*	(Tournier, 1868)	S. Italy, Sicily, Tunisia, Algeria	urn:lsid:zoobank.org:act:6A2A9ECF-4168-4241-A581-DE5A66B0266C	*pallidicollis*
34	*illiesi*	Klausnitzer, 1991	Algeria	urn:lsid:zoobank.org:act:CB511D28-1FFF-4860-A340-A31D6E3B20C4	*deflexicollis*
35	*indonesianus*	Yoshitomi and Satô, 2005	Indonesia	urn:lsid:zoobank.org:act:615A6523-2FD4-42DF-9ED1-B293E09D81C4	*Pallidicollis*

Table 1: Continued.

No.	Species	Description	Distribution	Zoobank LSID	Species group
36	*interrogationis*	Klausnitzer, 1980	Pakistan	urn:lsid:zoobank.org:act:5022A70A-8612-4DEC-B192-22F337EC6611	*deflexicollis*
37	*iriomotensis*	Yoshitomi, 2001	Japan	urn:lsid:zoobank.org:pub:53354434-8857-4500-8FE1-C3E009029BE0	*renati*
38	*jaechi*	Yoshitomi and Klausnitzer, 2003	China	urn:lsid:zoobank.org:act:EA5536A7-A98F-492F-ABB4-95D97CFE2C0B	*renati*
39	*javanicus*	Yoshitomi and Satô, 2005	Indonesia	urn:lsid:zoobank.org:act:7CF900C5-A969-40D6-9BF5-D18368B9E90A	*pallidicollis*
40	*jogjaensis*	Yoshitomi, in present study	Indonesia	urn:lsid:zoobank.org:act:CC3549AD-2680-4182-93F0-2E9E7509D070	*pallidicollis*
41	*kachinensis*	Yoshitomi and Satô, 2005	Myanmar	urn:lsid:zoobank.org:act:F8E9C354-6DD9-4033-B740-55F0821EF452	*deflexicollis*
42	*kambaiticus*	Nyholm, 1981	Burma	urn:lsid:zoobank.org:act:8F1AA735-F07C-499A-A171-17E4EE8DB7D1	*kambaiticus*
43	*kaszabi*	Klausnitzer, 1980	Vietnam	urn:lsid:zoobank.org:act:26FD1BD2-E452-41FA-BE50-08EAA71D0BDE	*deflexicollis*
44	*keralaensis*	Yoshitomi and Satô, 2005	India	urn:lsid:zoobank.org:act:60A84554-498B-4344-B778-CE47153FE7AC	*pallidicollis*
45	*kinabalensis*	Yoshitomi and Satô, 2005	Malaysia	urn:lsid:zoobank.org:act:101D411A-4D72-45BB-A1FB-FBAA75B8C4BC	*kinabalensis*
46	*klapperichi*	Yoshitomi, in present study	Indonesia	urn:lsid:zoobank.org:act:82B11C88-DDBF-4B12-9D94-19F74AAAEB7A	*pallidicollis*
47	*klausnitzeri*	Yoshitomi and Satô, 2005	Thailand	urn:lsid:zoobank.org:act:776698B5-109A-4323-814D-FFEA49622164	*pallidicollis*
48	*kodadai*	Yoshitomi and Satô, 2005	Philippines	urn:lsid:zoobank.org:act:F53BFE82-CB92-45A8-938D-C22374CC3AE3	*pallidicollis*
49	*komareki*	Yoshitomi, 2008	China	urn:lsid:zoobank.org:act:1D12096D-BDBD-440E-A3D9-E86811FEADB0	*kambaiticus*
50	*kopanddaghensis*	Ruta, 2007	USSR	urn:lsid:zoobank.org:act:E63FC1A7-8AFD-400E-B075-200977093FD8	*australis*
51	*laandicolor*	Nyholm, 1967	Spain	urn:lsid:zoobank.org:act:6AAA825E-E1CA-4E78-BC55-6E86A24E7C93	*pallidicollis*
52	*laosensis*	Yoshitomi and Satô 2003	Laos	urn:lsid:zoobank.org:act:D03DCA00-B60A-4232-8D3F-6475A3AAC74B	*pallidicollis*
53	*lii*	Yoshitomi and Klausnitzer, 2003	China	urn:lsid:zoobank.org:act:3992B473-B847-4672-AD45-E20CF2E75EAB	*pallidicollis*
54	*lusonensis*	Yoshitomi and Satô, 2005	Philippines	urn:lsid:zoobank.org:act:96F9212D-8494-4ED6-A927-2AB719A2E22B	*deflexicollis*
55	*malaysianus*	Yoshitomi and Satô, 2005	Malaysia	urn:lsid:zoobank.org:act:432974B2-E5F0-47BD-BE78-4A6D212F1B27	*pallidicollis*
56	*manfredi*	Yoshitomi and Satô, 2005	Indonesia	urn:lsid:zoobank.org:act:197987B9-91AF-4953-AA96-0DF26ECB2501	*pallidicollis*
57	*masatakai*	Yoshitomi, 2008	China	urn:lsid:zoobank.org:act:2A22EF58-D756-45C2-A94B-859B23CAA9F0	*deflexicollis*
58	*minous*	Nyholm, 1967	Crete	urn:lsid:zoobank.org:act:2C9A3DD3-5A36-4DF3-8004-9C6A78816611	*australis*
59	*mirabilis*	Yoshitomi and Satô, 2005	China	urn:lsid:zoobank.org:act:E40910F4-028A-4D9C-8B6A-C1AD5E13D769	*mirabilis*
60	*nakanei*	Yoshitomi, 2001	Japan	urn:lsid:zoobank.org:act:5AF6E7DB-3A8D-4DB8-BB32-11CA423E45A2	*kambaiticus*
61	*narraensis*	Yoshitomi and Satô, 2005	Philippines	urn:lsid:zoobank.org:act:11147154-F37B-4CC9-ADFE-A28E1A7FEBAD	*pallidicollis*
62	*nepalensis*	Yoshitomi and Satô, 2005	Nepal	urn:lsid:zoobank.org:act:F1F7DEB9-B007-49E6-BD56-F137E101E723	*renati*
63	*novaki*	Nyholm, 1967	Italy, Yugoslavia, Albania, Greece	urn:lsid:zoobank.org:act:6ACC76F1-7F7F-4D71-B191-3D3C63E7FA62	*deflexicollis*
64	*nuristanicus*	Klausnitzer, 2004	Afghanistan	urn:lsid:zoobank.org:act:849E2F10-EE37-44C4-9D17-724ED68D23AB	*pallidicollis*
65	*nyholmi*	Yoshitomi and Satô, 2005	Nepal	urn:lsid:zoobank.org:act:23F104FD-61CB-480A-9252-A114A46E6558	*nyholmi*
66	*oblongulus*	Nyholm, 1967	Cyprus	urn:lsid:zoobank.org:act:0ACA5B94-7CAB-4ADC-956D-C308859596C6	*australis*
67	*ovatus*	Nyholm, 1967	Italy	urn:lsid:zoobank.org:act:9D3B8572-8ADC-4EEA-9D64-181977729C1A	*deflexicollis*
68	*palawanensis*	Yoshitomi and Satô, 2005	Philippines	urn:lsid:zoobank.org:act:9289FAC1-3398-47E3-A114-892DFEB4D90C	*pallidicollis*
69	*pallidicollis*	Raffray, 1873	Corsica, Sardinia, Algeria, Morocco	urn:lsid:zoobank.org:act:BF53220B-9EDE-4E40-A427-90798C2291E3	*pallidicollis*
70	*palniensis*	Yoshitomi and Satô, 2005	India	urn:lsid:zoobank.org:act:2C983331-0F6B-4EDA-9C7C-9A76CF8CF5D2	*pallidicollis*
71	*panensis*	Yoshitomi and Satô 2003	Laos	urn:lsid:zoobank.org:act:E148DD4D-5CAB-4494-9796-DC517B8AAE1B	*pallidicollis*
72	*pernigrans*	Nyholm, 1967	Spain	urn:lsid:zoobank.org:act:098642CD-DC7D-4229-A722-E29D5D13F671	*deflexicollis*
73	*proximus*	Nyholm, 1967	Italy	urn:lsid:zoobank.org:act:C56E7ACA-6D6D-4220-A2DE-5C68D4EFA124	*deflexicollis*

Table 1: Continued.

No.	Species	Description	Distribution	Zoobank LSID	Species group
74	*pulchellus*	Klausnitzer, 1980	Nepal	urn:lsid:zoobank.org:act:C7793CC3-C759-4CD8-B843-FF36EE5987A7	*deflexicollis*
75	*rectangulus*	Klausnitzer, 1991	Algeria	urn:lsid:zoobank.org:act:BE470234-B4AB-4860-AE6A-EF17BA092ABF	*pallidicollis*
76	*renati*	Nyholm, 1981	Burma	urn:lsid:zoobank.org:act:40BCCB76-42A7-4094-B4C4-50F686FB3107	*renati*
77	*rivulorum*	Nyholm, 1977	Turkey	urn:lsid:zoobank.org:act:3DA3840B-6AD2-4BAA-883B-848DF04E3E15	*deflexicollis*
78	*rufithorax*	(Gemminger, 1869)	India/Sri Lanka	urn:lsid:zoobank.org:act:4DD9F8CA-E157-4039-A17B-C3F4D6396F9A	*pallidicollis*
79	*sagaingensis*	Yoshitomi and Satô, 2005	Myanmar	urn:lsid:zoobank.org:act:3BD8291D-9F48-4348-BA10-E0EF70715AEF	*pallidicollis*
80	*sagittiger*	Yoshitomi, in present study	Indonesia	urn:lsid:zoobank.org:act:0D309EFD-80D3-456D-821F-4C011C5A6598	*pallidicollis*
81	*sakaii*	Yoshitomi and Satô 2003	Laos	urn:lsid:zoobank.org:act:4A679CAE-4C32-417A-B49B-8F1BF14B2167	*deflexicollis*
82	*sarawakensis*	Yoshitomi and Satô, 2005	Malaysia	urn:lsid:zoobank.org:act:07DDD979-4676-484E-A7F0-FBBE4B516A16	*pallidicollis*
83	*satoi*	Yoshitomi, 2001	Japan, Taiwan, Korea	urn:lsid:zoobank.org:pub:53354434-8857-4500-8FE1-C3E009029BE0	*renati*
84	*schoenmanni*	Yoshitomi and Klausnitzer, 2003	China	urn:lsid:zoobank.org:act:9DABE069-D895-4ACD-97DD-3EDF94BFBACF	*pallidicollis*
85	*segrex*	Nyholm, 1972	Turkey, Anatolia, Iran, Caspian Sea	urn:lsid:zoobank.org:act:1D970446-0F80-4967-B9FD-CF69C16C5C32	*australis*
86	*serratibasialis*	Yoshitomi, in present study	India	urn:lsid:zoobank.org:act:1E9F878B-737F-4B31-99B7-FA7663EFFF3C	*deflexicollis*
87	*sieberi*	(Klausnitzer, 2010)	India	urn:lsid:zoobank.org:act:88BA77DB-C779-46C1-BE0C-069DFC296A53	*pallidicollis*
88	*similis*	Ruta, 2004	Vietnam	urn:lsid:zoobank.org:act:C88A15E4-2F83-4DBD-B515-AD418A16CB01	*pallidicollis*
89	*sinicus*	Pic, 1934	China	urn:lsid:zoobank.org:act:77951535-1B3B-494F-AC41-EBD6A45B53F6	*kambaiticus*
90	*spinosus*	Yoshitomi and Satô, 2005	India	urn:lsid:zoobank.org:act:2408AC80-003B-41E4-8834-CEFEEB5CA987	*pallidicollis*
91	*steueri*	Klausnitzer, 2006	Nepal	urn:lsid:zoobank.org:act:93909299-F337-490A-9E8A-BF4C6D008FC9	*kambaiticus*
92	*stupendus*	Nyholm, 1981	Burma	urn:lsid:zoobank.org:act:46B80B2B-8178-4EB0-B3F3-7F0D4D15C2E1	*kambaiticus*
93	*subcelatus*	Yoshitomi and Satô, 2005	India	urn:lsid:zoobank.org:act:1115FB1D-1947-4199-92FA-755C4AEBA401	*deflexicollis*
94	*submalaysianus*	Yoshitomi and Satô, 2005	Malaysia	urn:lsid:zoobank.org:act:95BAB60F-C4C7-42F7-B756-0757C10827FD	*pallidicollis*
95	*subrotundus*	Yoshitomi and Satô, 2005	Thailand	urn:lsid:zoobank.org:act:45CDFA7B-AEDE-47B8-AE79-C7A055FD9ADD	*deflexicollis*
96	*subtrilobus*	Yoshitomi and Satô, 2005	Indonesia	urn:lsid:zoobank.org:act:4EB51544-94BF-4305-ABF4-E979ACC971AA	*pallidicollis*
97	*sumatrensis*	Yoshitomi and Satô, 2005	Indonesia	urn:lsid:zoobank.org:act:5CD0CE1E-AED2-481B-9FD2-CF5087C1299E	*pallidicollis*
98	*taiwanus*	Yoshitomi, 2001	Taiwan	urn:lsid:zoobank.org:act:3A5B6F9A-E586-4BC8-9FAF-9F6B749AFEAB	*renati*
99	*takizawai*	Yoshitomi, in present study	Malaysia	urn:lsid:zoobank.org:act:9BB8C12B-3602-491D-81F9-58C3FDC8831F	*pallidicollis*
100	*tamilensis*	Yoshitomi and Satô, 2005	India	urn:lsid:zoobank.org:act:9C01DBD6-2538-408C-98BA-C53BACFB6F42	*tamilensis*
101	*thailandicus*	Yoshitomi and Satô, 2005	Thailand	urn:lsid:zoobank.org:act:E584D03E-D879-49C5-BF37-3F7F6BD5A20F	*Renati*
102	*triforius*	Yoshitomi and Satô, 2005	Malaysia, Thailand	urn:lsid:zoobank.org:act:6DBD3361-AD78-430E-8B0E-662012550497	*renati*
103	*trilobus*	Yoshitomi and Satô, 2005	Thailand	urn:lsid:zoobank.org:act:DBBB00C2-5B88-45C0-A3AD-8A72C1D424E6	*pallidicollis*
104	*uenoi*	Yoshitomi and Klausnitzer, 2003	China	urn:lsid:zoobank.org:act:A48EC5C2-38F1-4CCC-A832-CEF4B14C09F4	*kambaiticus*
105	*vicinans*	Nyholm, 1972	Turkey, Israel	urn:lsid:zoobank.org:act:DB707751-48AB-4693-A863-8671E0DCFDCC	*australis*
106	*wakaharai*	Yoshitomi and Satô 2003	Laos	urn:lsid:zoobank.org:act:8A75EBCF-66F9-4E52-B555-A6F4A6155E79	*renati*
107	*wangi*	Yoshitomi, 2008	China	urn:lsid:zoobank.org:act:C64DD841-7071-4993-8F36-61CE488BA954	*kambaiticus*
108	*yoshitomii*	Klausnitzer 2002	Nepal	urn:lsid:zoobank.org:act:C690CF7A-9E10-4D03-8FD7-558279A45203	*yoshitomii*

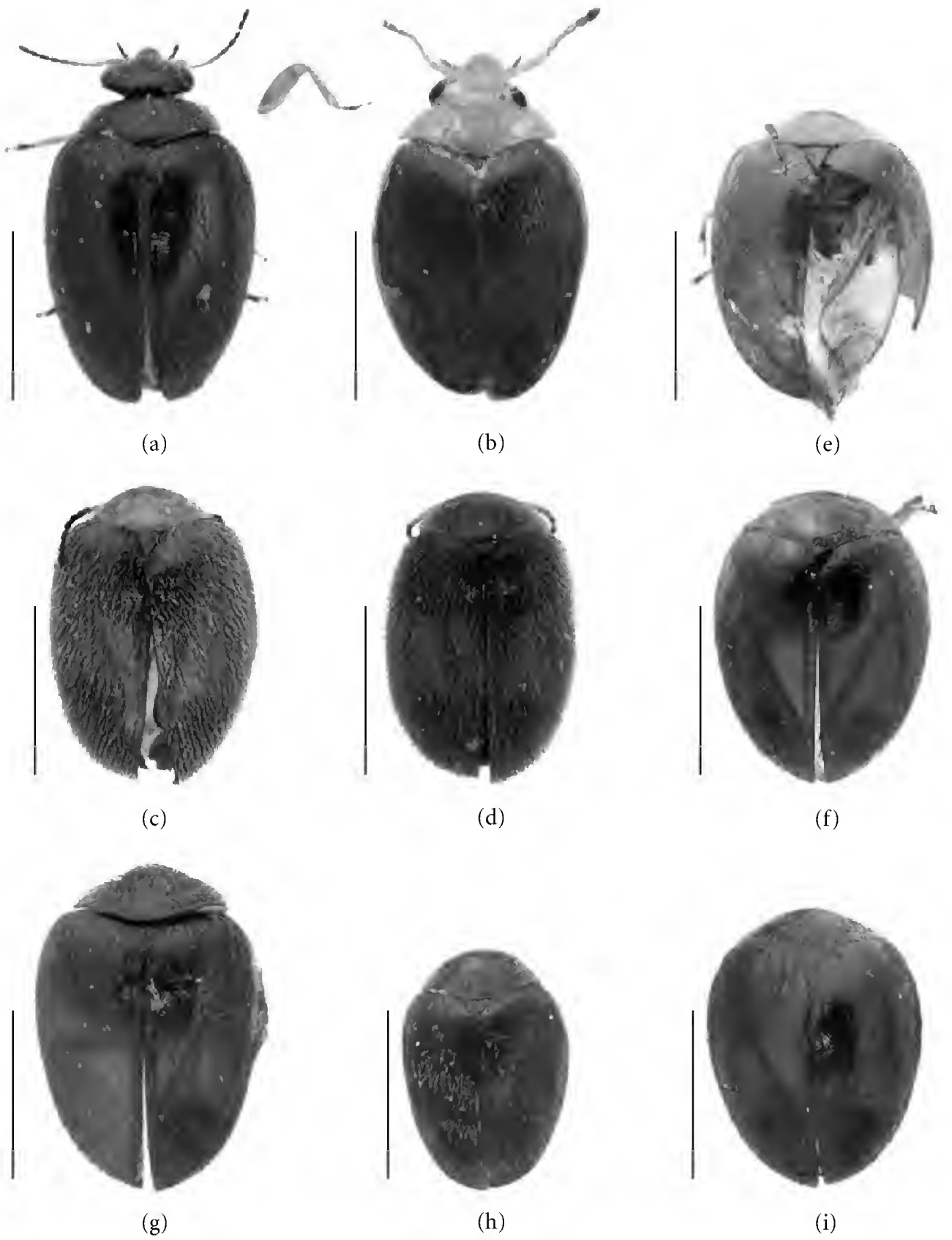

FIGURE 1: Habitus of *Hydrocyphon* spp., holotypes, male (a, c, e–i) and paratypes, female (b, d). (a, b) *H. jogjaensis* sp.n.; (c, d) *H. takizawai* sp.n.; (e) *H. sagittiger* sp.n.; (f) *H. serratibasialis* sp.n.; (g) *H. doiinthanonensis* sp.n.; (h) *H. klapperichi* sp.n.; (i) *H. deformis* sp.n. Scale = 1.0 mm.

TABLE 2: The list of the species excluding from the genus *Hydrocyphon*. An Excel file version is also available at the following URL: https://sites.google.com/site/waterbeandlesofjapan/home/support-files-on-articles/Appendix 2.xls.

No.	Species	Description	Distribution	Zoobank LSID	Transferred
1	*Hydrocyphon atratus*	Motschulsky, 1863	Ceylon	urn:lsid:zoobank.org:act:7CE1145A-0783-4196-A201-B05397235AE5	*Cyphon*

Male Description. Body oval, well-convex dorsally, shiny, closely covered with short yellowish-white setae. Coloration of body blackish-brown, but antennal segments I–V, lateral part of pronotum and legs yellowish-brown.

Head moderate in size, lightly convex dorsally, finely punctuate, with straight front margin of clypeus; the distance between eyes about 1.9 times as long as the maximum diameter of an eye. Eyes moderate in size, prominent. Antennae short, slender, reaching at basal part of elytra. Pronotum punctuate as in head, lightly convex dorsally, lightly depressed ventrally in lateral parts; front margin almost straight; anterolateral corners obtuse; posterolateral corners right-angle; lateral and posterior margins gently arcuate; PW/PL 2.69. Scutellum small, equilateral-triangular, punctuate as in head. Elytra oval, convex dorsally, broadest at basal 1/3, punctuate as in head; humeral parts indistinct; EL/EW 1.22; EL/PL 4.80; EW/PW 1.47; TL/EW 1.47. Legs relatively long, slender.

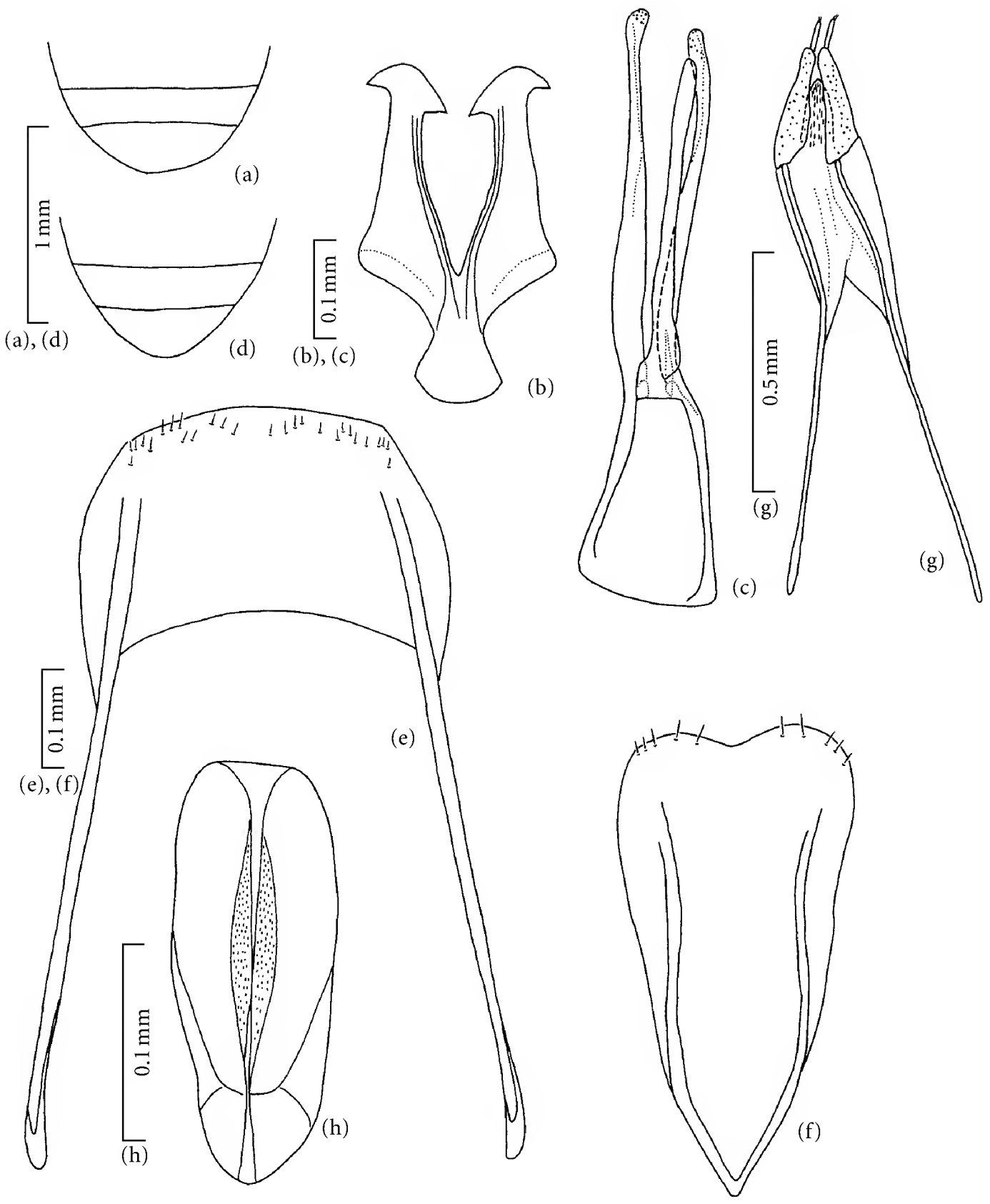

FIGURE 2: *Hydrocyphon jogjaensis* sp.n., holotype, male (a–c) and paratype, female (d–h). (a, d) Sternites V–VII; (b) tegmen; (c) penis; (e) tergite VIII; (f) sternite VIII; (g) ovipositor; (h) prehensor.

Caudal margin of sternite VII gently arcuate. Tergites VIII—IX moderately sclerotized, trapezoidal. Tegmen short, well sclerotized; proximal part short, fan-shaped, arcuate in basal margin; parameres stout, gently expanded laterally in basal parts, arrow-like shape in apical parts. Penis long, well sclerotized, asymmetrical, about 1.7 times as long as tegmen; pala subtrapezoidal, widest at base; parameroids long and slender, slightly asymmetrical, gently widened and punctuate in apical parts, obtuse at apices; trigonium with one long and slender projection, a little shorter than parameroids, obtuse at apex; median plate indistinct.

Female. Similar to male; pronotum yellow (probably teneral specimen); antennae relatively stout; PW/PL 2.55; EL/EW 1.23; EL/PL 4.25; EW/PW 1.35; TL/EW 1.52.

Caudal margin of sternite VII slightly pointed. Tergite VIII moderately sclerotized, trapezoidal, bearing short setae in caudal parts, with long apodemes; sternite VIII slightly sclerotized, oblong, bearing short setae along caudal margin. Ovipositor relatively short; relative length of stylus, coxite, and baculus ($n = 1$) as 1.0 : 4.0 : 15.3. Prehensor small, well sclerotized, oblong, bearing short spines in mesal part.

Measurements. Male ($n = 1$): TL 2.03 mm; PW 0.94 mm; PL 0.35 mm; EL 1.68 mm; EW 1.38 mm. Female ($n = 1$): TL 2.10 mm; PW 1.02 mm; PL 0.40 mm; EL 1.70 mm; EW 1.38 mm.

Remarks. The species belongs to the *pallidicollis* species group. It is similar to *H. trilobus* Yoshitomi and Satô and *H. subtrilobus* Yoshitomi and Satô with respect to the shape of

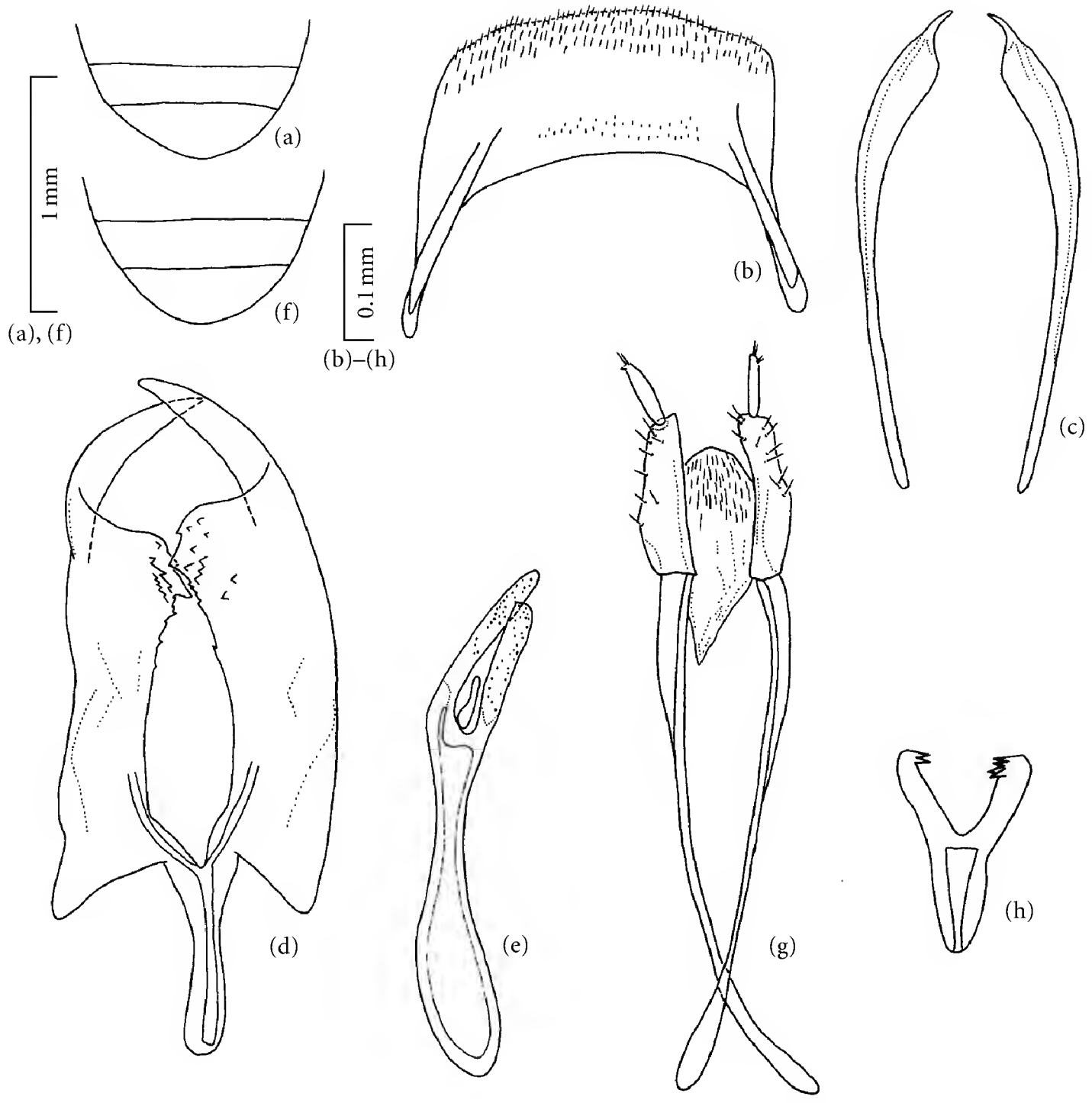

FIGURE 3: *Hydrocyphon takizawai* sp.n., holotype, male (a–e) and paratype, female (f–h). (a, f) Sternites V–VII; (b) tergite VIII; (c) sternite IX; (d) tegmen; (e) penis; (g) ovipositor; (h) prehensor.

the penis, but differs from them by the apices of the parameres which have an arrow-like shape.

Biological Notes. The type locality was a small river situated halfway up Mount Merapi (Figure 11(a)). The river was somewhat polluted by waste water flowing from cichlid fish farms.

Etymology. The species is named after the type locality.

3.2. Hydrocyphon takizawai sp.n. (See Figures 1(c), 1(d), 3, 4, 5, and 11(b))

Type Material. Holotype male (EUMJ): "Kinabalu Park, HQ Sabah, MALAYSIA 2–4. V. 2010 H. Yoshitomi leg."

Paratypes 2 females (EUMJ): same data as for the holotype.

Male Description. Body oval, well convex dorsally, shiny, closely covered with yellowish white short setae. Coloration of head, mouth parts, antennal segments I–IV, prothorax and legs yellowish-brown, but posterior part of head and tarsi infuscate; antennal segments V–XI, scutellum, elytra, meso- and metaventries, and abdominal segments brown.

Head moderate in size, slightly convex dorsally, finely punctuate; clypeus rather long, straight in front margin; the distance between eyes about 1.7 times as long as the maximum diameter of an eye. Eyes moderate in size, prominent. Antennae short, reaching about proximal 1/6 of elytra. Pronotum punctuate as in head, slightly convex dorsally, depressed ventrally in lateral parts; front margin straight; antero- and posterolateral corners obtuse; lateral and posterior margins gently arcuate; PW/PL 2.51. Scutellum small, equilateral-triangular. Elytra oval, convex dorsally, broadest at the middle; humeral parts gently projecting dorsally; EL/EW 1.41; EL/PL 4.83; EW/PW 1.36; TL/EW 1.70.

Caudal margin of sternite VII arcuate. Tergite VIII moderately sclerotized, transversal trapezoidal, bearing short spines in caudal part, with a pair of short apodemes. Sternite IX well sclerotized, consisting of a pair of hemisternites, with pointed at apices. Tegmen large, well sclerotized; proximal part short, subparallel-sided; parameres long, minutely serrate in apical 1/3 of inner parts, distinctly protruding

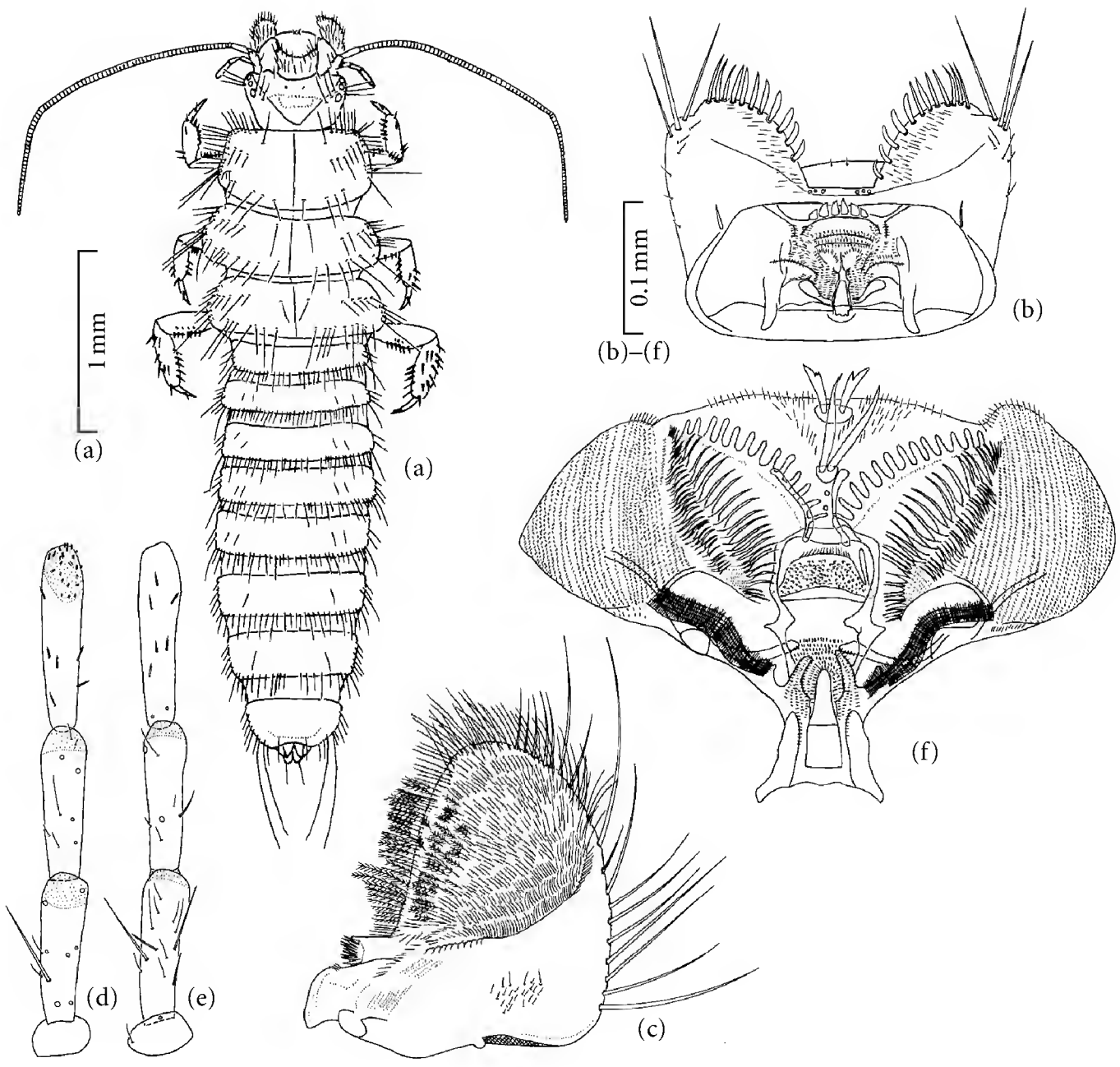

FIGURE 4: *Hydrocyphon takizawai* sp.n., larva. (a) Dorsal habitus; (b) labrum in ventral aspect; (c) mandible in ventral aspect; (d) maxillary palpus in ventral aspect; (e) ditto in dorsal aspect; (f) hypopharynx.

postero-interiorly in postero-lateral corners, projecting anteriorly in anterolateral corners. Penis asymmetrical, short, well sclerotized, about 0.8 times as long as tegmen; pala oblong, widest near base, tapered in proximal 2/3; parameroids short and almost straight, obtuse at apices, finely punctuate, left one long and slender, right one short and stout; trigonium consisting of a small lobe.

Female. Sexual dimorphism indistinct, but mesal part of pronotum infustate in paratype; PW/PL 2.24; EL/EW 1.42; EL/PL 4.59; EW/PW 1.45; TL/EW 1.73.

Caudal margin of sternite VII arcuate. Ovipositor relatively short; stylus with two pairs of apical setae; coxite bearing short spines; baculus without branch; relative length of stylus, coxite and baculus ($n = 1$) as 1.0 : 2.3 : 7.5. Prehensor small, slightly sclerotized, Y-shaped, bearing short spines in inner margins of apices.

Measurements. Male ($n = 1$): TL 2.04 mm; PW 0.88 mm; PL 0.35 mm; EL 1.69 mm; EW 1.20 mm. Female ($n = 1$): TL 2.07 mm; PW 0.83 mm; PL 0.37 mm; EL 1.70 mm; EW 1.20 mm.

Larvae. Body about 4.0 mm length in fully expanded specimens, subparallel-sided in thorax and abdomen which bearing short and long setae on lateral and posterior margins. Coloration of body right brown.

Head slightly protruding laterally, with three pairs of nonmelanized stemmata situated near anterolateral corners. Antennae relatively long, reaching at abdominal segment I; scape slightly curved posteriorly; flagellum 51–73 (64) segmented ($n = 4$). Labrum transverse, covered with long setae on dorsal surface; ventral lobes projecting anteriorly, with 12 pairs of stout and short setae on inner margins. Maxillary palpi long and slender; 1st segment covered sparsely with short and long setae on dorsal surface; 3rd rounded at apex, with widely apical sensory area; relative length of each segment ($n = 1$) as 1.0 : 1.0 : 1.3. Mandibles and hypopharynx typical for the genus. Thorax widest at posterior margin of mesothorax. Abdomen subparallel-sided, widest at segment V, then gently tapering posteriorly, bearing two (II–V) or one (VI–VII) pairs of short setae on lateral part. Tergite VIII trapezoidal, shallowly concave in posterior margin, with a pair of very long setae protruding from posterolateral corners. Sternite VIII semicircular, bearing long setae on lateral and posterior margins, two of those very long. Tergite IX semicircular, convex at apex, with a pair of long setae at apex, bearing pectinate short setae on lateral margin. Sternite IX transversal semicircular, with pectinate setae on posterior margin.

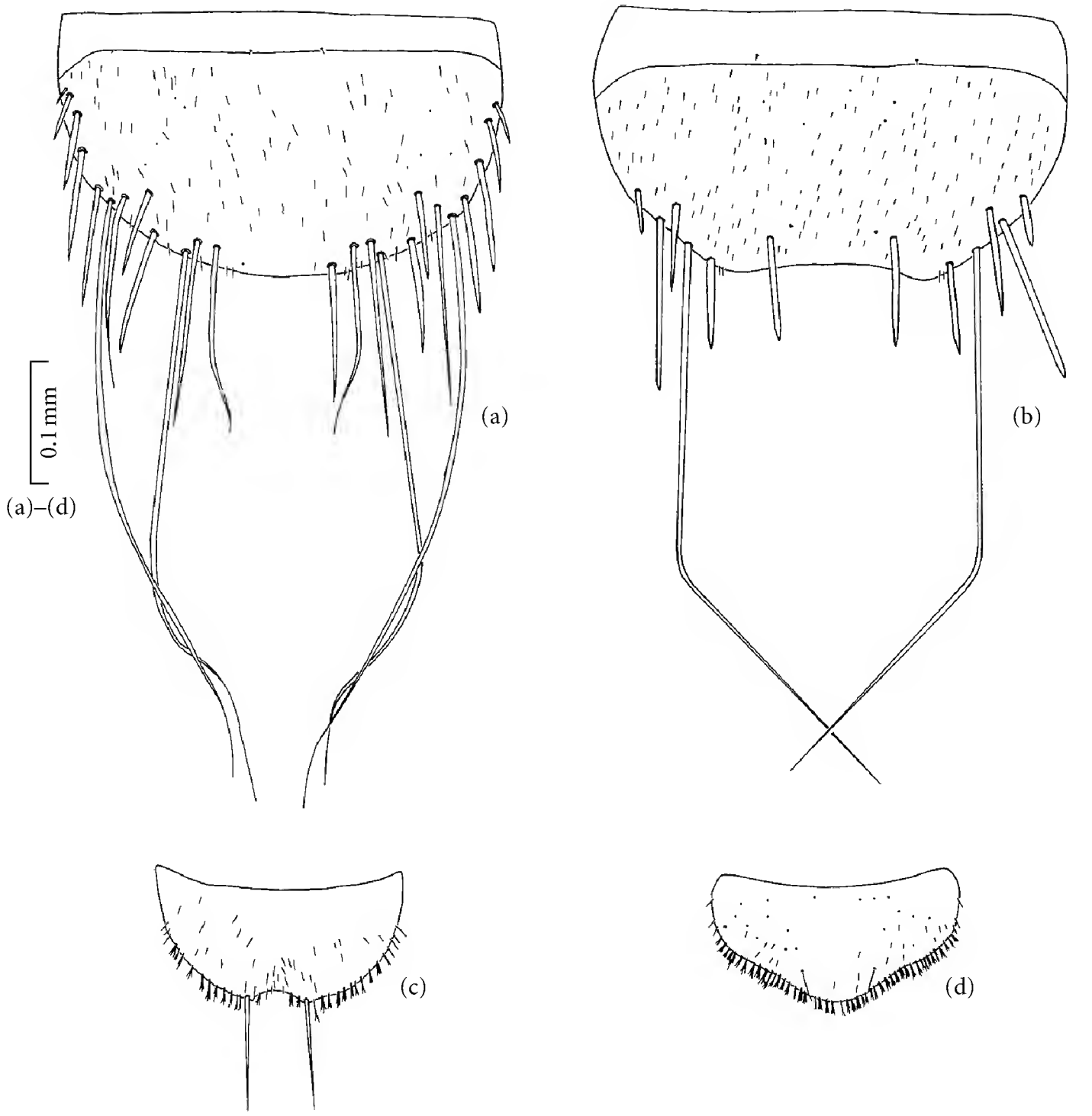

FIGURE 5: *Hydrocyphon takizawai* sp.n., larva. (a) Sternite VIII; (b) tergite VIII; (c) tergite IX; (d) sternite IX.

Measurements of Larvae (n = 3). TL 5.40–6.50 (5.80) mm; HW 0.80–0.90 (0.83) mm; PL 0.50–0.55 (0.52) mm; PW 1.05–1.20 (1.15) mm; TW 1.20–1.40 (1.33) mm.

Specimens Examined of Larvae. 29 exs. (mature larvae), Kinabalu Park, HQ Sabah, Malaysia, 2–4. V. 2010, H. Yoshitomi leg.; 5 exs. (mature larvae), Liwagu river, Kinabalu Park, HQ, Sabah, Malaysia, 28. II. 2009, H. Uno leg.

Remarks. The species belongs to the *pallidicollis* species group. Judging from the shape of the penis, it is similar to *H. palawanensis* Yoshitomi and Satô, *H. javanicus* Yoshitomi and Satô, *H. baliensis* Yoshitomi and Satô, *H. manfredi* Yoshitomi and Satô, and *H. sarawakensis* Yoshitomi and Satô, but differs from them by the shape of the parameres projecting posteriorly and serrate in the inner margin.

The larva of this species is distinguished from the three previously known species of the larvae in the genus (*H. deflexicollis* [13], *H. satoi* [14], *H.* sp. [4]) by the following characteristics: (1) segment III of maxillary palpi somewhat short (about 1.5 times as long as segment I in *H. deflexicollis* and *H. satoi*); (2) the short setae on the lateral and posterior margins of tergite IX and sternite IX pectinate (simple setae in *H. satoi* and *H.* sp.).

Biological Notes. The type locality was a small stream in the Kinabalu National Park (Figure 11(b)). The stream was clear, and many aquatic insects were collected with this species.

Etymology. The species is named after Dr. H. Takizawa.

3.3. Hydrocyphon sagittiger sp.n. (See Figures 1(e) and 6)

Type Material. Holotype male (EUMJ): "(Indonesia) West Sumatra Batipuh 26. XI. 1974 T. Kobayashi," "Egyptian kidney bean."

Holotype male (EUMJ): "(Indonesia) West Sumatra Batipuh 26. XI. 1974 T. Kobayashi," "Egyptian kidney bean."

Male Description. Body oval, well convex dorsally, shiny, closely covered with yellowish-white setae. Coloration of body blackish-brown, but anterior part of head, lateral parts of pronotum, antennal segments I–V, and legs right-brown.

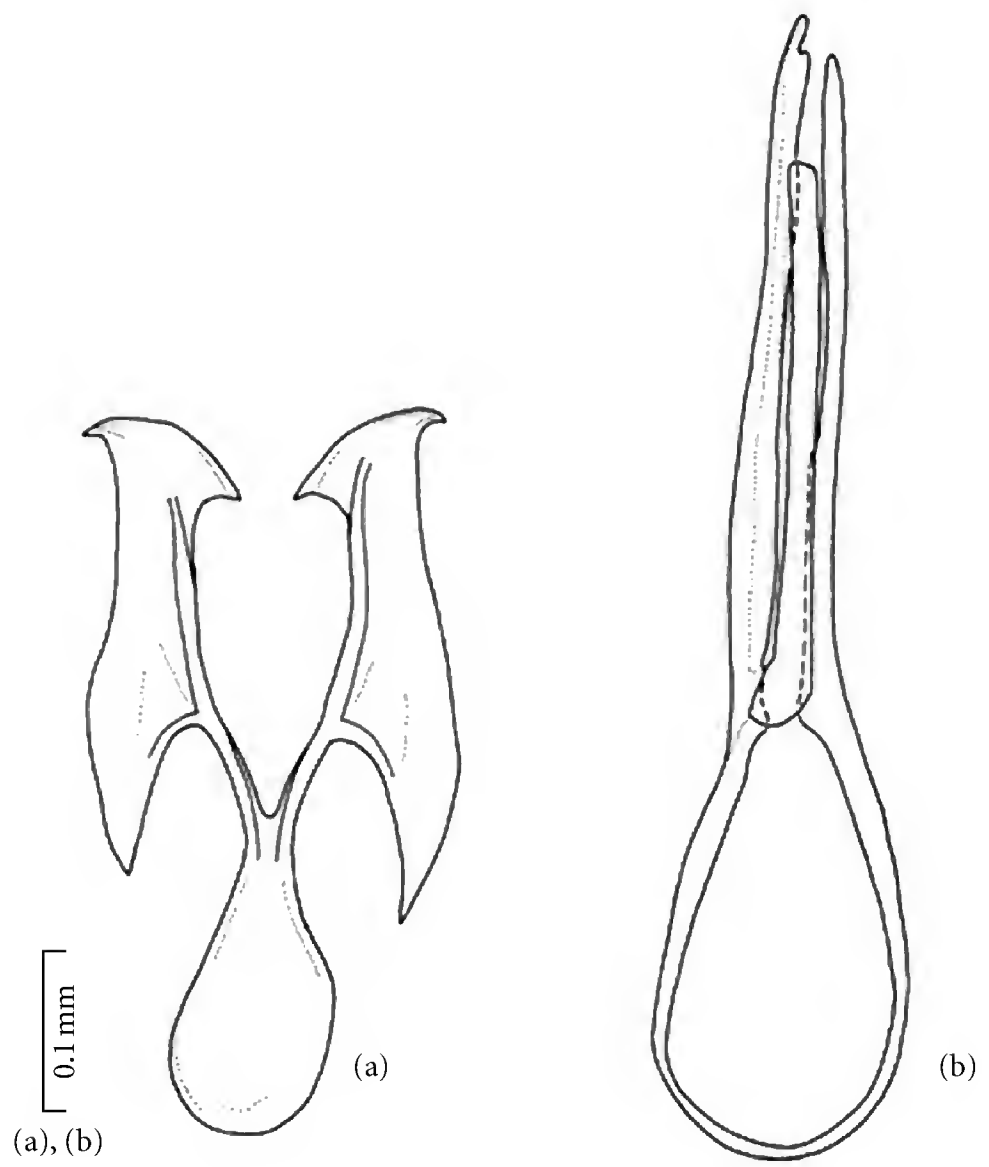

FIGURE 6: *Hydrocyphon sagittiger* sp.n., holotype, male. (a) Tegmen; (b) penis.

Head moderate in size, flat in dorsally, finely punctuate; clypeus short, straight in front margin; the distance between eyes about 2.1 times as long as the maximum diameter of an eye. Eyes moderate in size, prominent. Antennae short, reaching about proximal 1/8 of elytra. Pronotum punctuate as in head, slightly convex dorsally, depressed ventrally in lateral parts; front margin straight; antero- and postero-lateral corners obtuse; lateral and posterior margins gently arcuate; PW/PL 2.57. Scutellum small, equilateral-triangular. Elytra oval, strongly convex dorsally, broadest at the middle; humeral parts gently projecting dorsally; EL/EW 1.26; EL/PL 4.65; EW/PW 1.43; TL/EW 1.54.

Tegmen large, well sclerotized; proximal part long, peg-like; parameres wide, distinctly projecting anteriorly in antero-lateral corners, projecting subtriangularly in inner and outer corners of apices. Penis long, slightly asymmetrical, well sclerotized, about 1.5 times as long as tegmen; pala short, oblong, widest at proximal 1/3 of pala; parameroids very long, asymmetrical, almost straight, left one slightly longer than right one, excised at inner margin of left apex; trigonium consisting of a long lobe, straight, shorter than parameroids, obtuse at apex; median plate indistinct.

Female. Unknown.

Measurements. TL 2.09 mm; PW 0.95 mm; PL 0.37 mm; EL 1.72 mm; EW 1.36 mm.

Remarks. The species belongs to the *pallidicollis* species group, and is related to *H. jogjaensis* sp.n., *H. trilobus* Yoshitomi and M. Satô, 2005, and *H. subtrilobus* Yoshitomi and M. Satô, 2005. It differs from them by the following characteristics: inner corner of parameres projecting interiorly; left parameroid excised at apex; pala oblong.

Etemology. The species name refers to the shape of the apices of the tegmen.

3.4. Hydrocyphon serratibasialis sp.n. (See Figures 1(f) and 7)

Type Material. Holotype male (SEHU): "INDIA: KERALA Dhony Hills 180–450 m 7 DEC 1978 JAP-IND CO TR".

Male Description. Body oval, well convex dorsally, shiny, closely covered with yellowish-white setae. Coloration of head, scutellum, elytra, and ventral surface of thorax and abdomen blackish-brown; pronotum, legs, and antennae yellowish-brown.

Head moderate in size, slightly convex dorsally, finely punctuate; clypeus short, straight in front margin; the distance between eyes about 2.2 times as long as the maximum diameter of an eye. Eyes moderate in size, prominent. Antennae rather stout. Pronotum punctuate as in head, lightly depressed ventrally in lateral parts; front and lateral margins straight; antero-lateral corners about 120°; postero-lateral corners right-angle; posterior margin gently arcuate; PW/PL

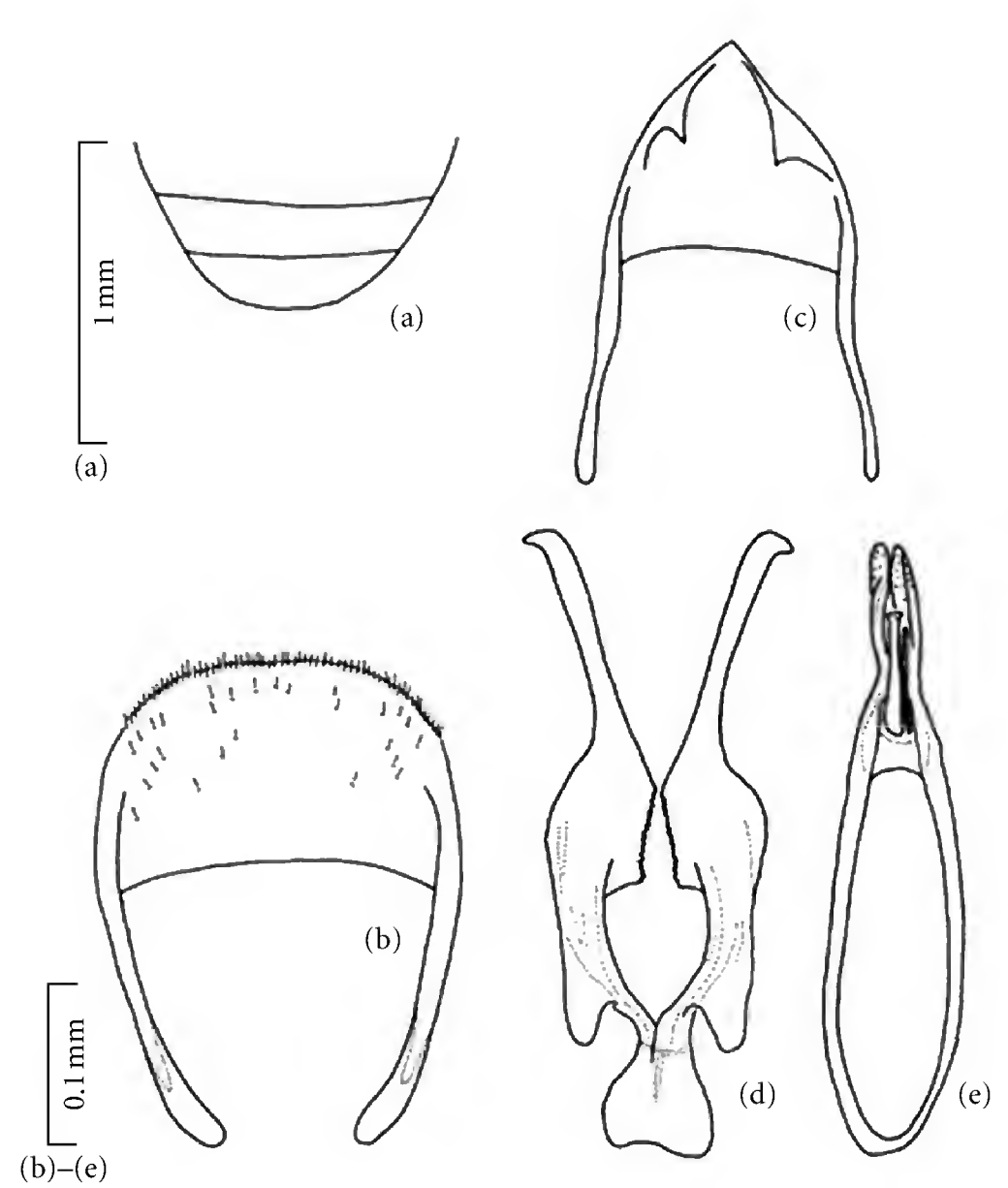

FIGURE 7: *Hydrocyphon serratibasialis* sp.n., holotype, male. (a) Sternites V–VII; (b) tergite VIII; (c) sternite IX; (d) tegmen; (e) penis.

2.78. Scutellum small, equilateral triangular. Elytra oval, strongly convex dorsally, broadest at the middle; humeral parts gently projecting dorsally; EL/EW 1.21; EL/PL 4.59; EW/PW 1.36; TL/EW 1.48.

Caudal margin of sternite VII gently arcuate. Tergite VIII moderately sclerotized, trapezoidal, bearing short spines along caudal margin, sparsely covered with short setae in caudal part, with a pair of slender apodemes, Sternites IX slightly sclerotized, upturned in postero-lateral parts, with a pair of long apodemes. Tegmen long, well sclerotized; proximal part short, expanded antero-laterally; parameres long, minutely serrate in mesal part of inner margin, distinctly protruding postero-laterally in apical parts, projecting anteriorly in antero-lateral corners. Penis asymmetrical, long, well sclerotized, about 0.9 times as long as tegmen; pala oblong, widest at basal 1/6; parameroids longer than trigonium, finely punctuate; trigonium consisting of two lobes, longer one forked, shorter one slender; median plate indistinct.

Female. Unknown.

Measurements. TL 2.07 mm; PW 1.03 mm; PL 0.37 mm; EL 1.70 mm; EW 1.40 mm.

Remarks. The species belongs to the *deflexicollis* species group, but is a distinct species having a characterized tegmen.

Etymology. The species name refers to the shape of the tegmen: "serrati−" = serrate + "basialis" = basal.

3.5. Hydrocyphon doiinthanonensis sp.n. (See Figures 1(g) and 8)

Type Material. Holotype male (EUMJ): "[North THAI] Maeo Khun klang 1350 m, Doi Inthanon 19. X. 1983 M. Sakai".

Male Description. Body oval, well convex dorsally, weakly shiny, closely covered with yellowish-white setae. Coloration of body brown, but antennae, apical part of femora, tibiae, and tarsi pale brown.

Head moderate in size, slightly convex dorsally, finely punctuate; clypeus short, straight in front margin; the distance between eyes about 2.3 times as long as the maximum diameter of an eye. Eyes moderate in size, prominent. Antennae rather stout. Pronotum punctuate as in head, slightly depressed ventrally in lateral parts; front and lateral margins straight; antero-lateral corners about 120°; postero-lateral corners almost right-angle; posterior margin gently arcuate; PW/PL 2.45. Scutellum small, equilateral triangular. Elytra oval, broadest at basal 1/4; humeral parts indistinctly projecting; EL/EW 1.28; EL/PL 4.63; EW/PW 1.48; TL/EW 1.55.

Caudal margin of sternite VII gently arcuate. Tergite VIII moderately sclerotized, trapezoidal, bearing short spines along caudal margin, sparsely covered with short setae in

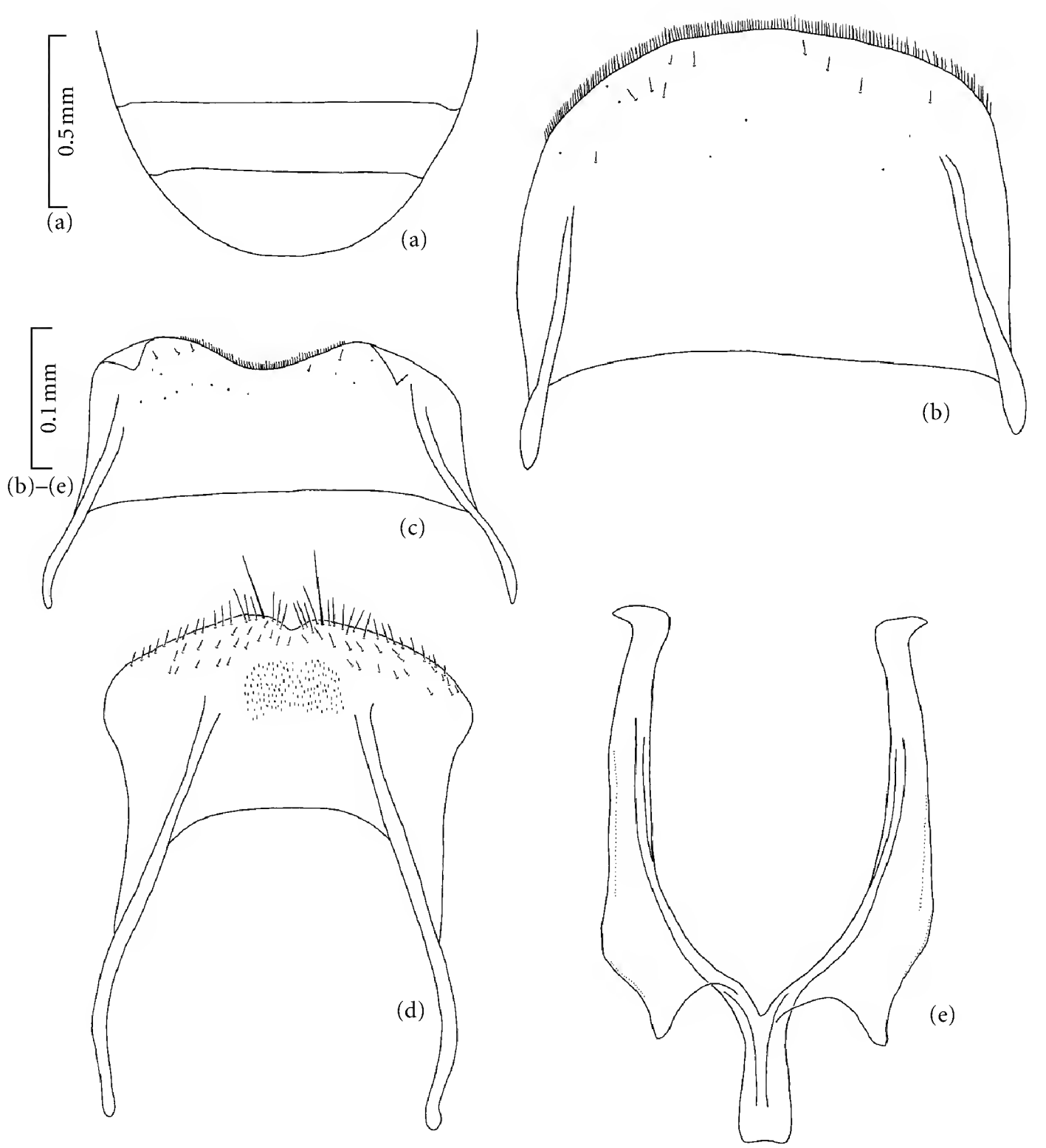

FIGURE 8: *Hydrocyphon doiinthanonensis* sp.n., holotype, male. (a) Sternites V–VII; (b) tergite VIII; (c) tergite IX; (d) sternite IX; (e) tegmen.

caudal part, with a pair of short apodemes. Sternite IX slightly sclerotized, bearing irregular setae in caudal part, with a pair of long apodemes. Tergite IX slightly sclerotized, trapezoidal, concave and bearing short spines in caudal margin, upturned in postero-lateral parts, bearing short setae in caudal part, with a pair of long apodemes. Tegmen large, well sclerotized; proximal part short, subparallel-sided; parameres long, projecting laterally in apices, projecting subtriangularly in anterior corners. Penis missing.

Female. Unknown.

Measurements. TL 2.25 mm; PW 0.98 mm; PL 0.40 mm; EL 1.85 mm; EW 1.45 mm.

Remarks. The species belongs to the *deflexicollis* species group. This species is distinguished from the previously known species by the concave posterior margin of the sternite and tergite IX and the shape of the parameres of the tegmen.

Etymology. The species is named after the type locality.

3.6. Hydrocyphon klapperichi sp.n. (See Figures 1(h) and 9)

Type Material. Holotype male (SMNS): "INDONESIEN: Sumatra, Prov. Aceh-Selatan, Babahrot 15–20. 8. 1983 leg. J. KLAPPERICH".

Paratypes 2 female (SMNS): same data as for the holotype.

Male Description. Body oval, convex dorsally, shiny, closely covered with yellowish-white setae. Coloration of body blackish-brown, but lateral parts of pronotum and legs paler.

Head moderate in size, flat in dorsal surface, finely punctuate; clypeus short, straight in front margin; the distance between eyes about 2.2 times as long as the maximum diameter of an eye. Eyes relatively large, prominent. Pronotum punctuate as in head, slightly depressed ventrally in lateral parts; front and lateral margins straight; antero-lateral corners 120°; postero-lateral corners right-angle;

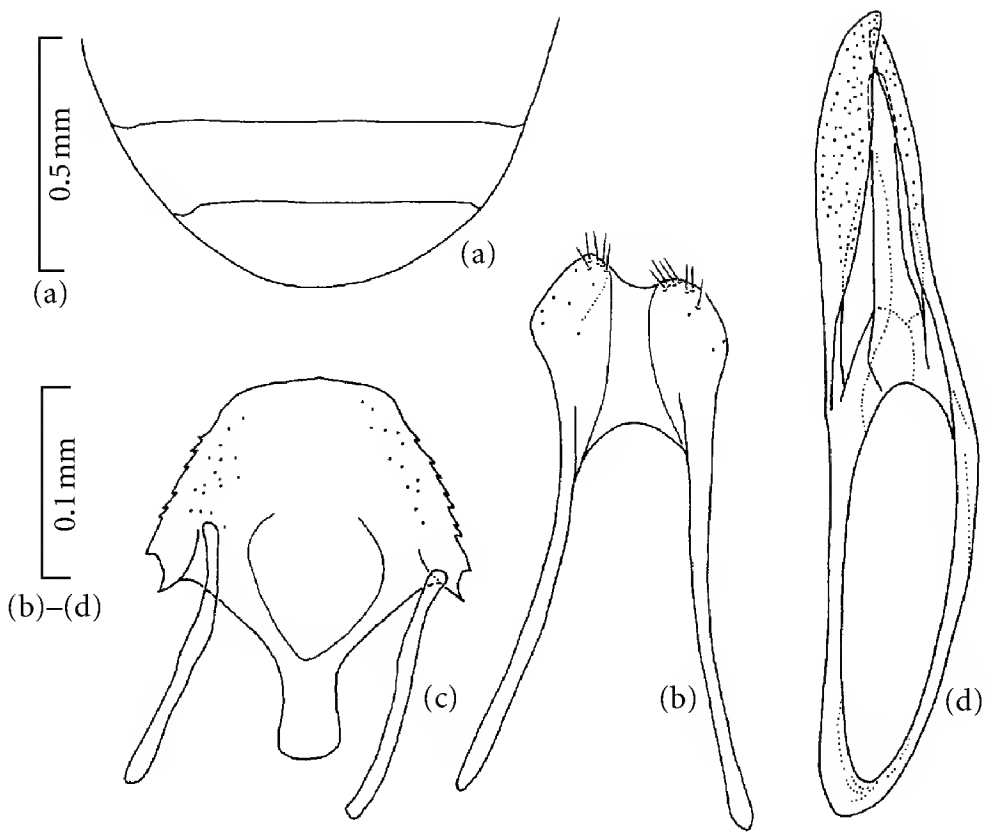

FIGURE 9: *Hydrocyphon klapperichi* sp.n., holotype, male. (a) Sternites V–VII; (b) tergite IX; (c) tegmen; (d) penis.

posterior margin gently arcuate; PW/PL 2.53. Scutellum relatively large, equilateral-triangular. Elytra oval, rather convex dorsally, broadest at basal 1/3; humeral part indistinctly projecting; EL/EW 1.20; EL/PL 4.27; EW/PW 1.41; TL/EW 1.48.

Caudal margin of sternite VII gently arcuate. Tergite VIII moderately sclerotized, trapezoidal, with a short apodemes. Tergite IX slightly sclerotized, bearing short setae in apical part, with a pair of long apodemes. Tegmen relatively large, moderately sclerotized; proximal part peg-like, short; parameres obscure, serrate at lateral margins, projecting and bifid in antero-lateral corners, punctuate; lateral projections very long, as long as parameres. Penis asymmetrical, long, well sclerotized, about 2.1 times as long as tegmen; pala oblong, gently tapered anteriorly; parameroids distinctly asymmetrical, closely punctuate, almost straight, left one wider and longer than right one; trigonium consisting of a long lobe, shorter than parameroids, obtuse at apex; median plate indistinct.

Female. Sexual dimorphism indistinct in external features, but body is somewhat larger; PW/PL 2.50–2.67 (2.58); EL/EW 1.46–1.47 (1.47); EL/PL 4.05–4.56 (4.30); EW/PW 1.10–1.17 (1.13); TL/EW 1.79–1.84 (1.81).

Measurements. Male ($n = 1$): TL 1.58 mm; PW 0.76 mm; PL 0.30 mm; EL 1.28 mm; EW 1.07 mm. Female ($n = 2$): TL 2.02 & 2.50 mm; PW 1.00 & 1.20 mm; PL 0.40 & 0.45 mm; EL 1.62 & 2.05 mm; EW 1.10 & 1.40 mm.

Remarks. The shape of the tegmen of this species is similar to that of the *mirabilis*, the *tamilensis*, the *kinabalensis*, and the *renati* species groups, but this species is easily distinguished from the latter by the serrate parameres and the shape of the penis. Judging from the shape of the penis (e.g., asymmetrical parameroids and single projection of trigonium), this species probably belongs to the *pallidicollis* species group.

Etymology. The species is named after Dr. J. Klapperich, who was the collector of the holotype.

3.7. Hydrocyphon deformis sp.n. (See Figures 1(i) and 10)

Type Material. Holotype male (SEHU): "INDIA: KERALA Dhony Hills 180–450 m 7 DEC 1978 JAP-IND CO TR."

Male Description. Body oval, well convex dorsally, strongly shiny, closely covered with yellowish-white setae. Coloration blackish-brown, but lateral parts of pronotum, mouth parts, antennae, and legs paler.

Head moderate in size, flat in dorsal surface, finely punctuate; clypeus relatively long, straight in front margin; the distance between eyes about 2.5 times as long as the maximum diameter of an eye. Eyes moderate in size, prominent. Antennae short and stout, reaching about proximal 1/3 of elytra. Pronotum strongly transverse, punctuate as in head, depressed ventrally in lateral parts; front and lateral margins straight; antero-lateral corners obtuse, postero-lateral corners almost right-angle; posterior margin arcuate; PW/PL 2.80. Scutellum relatively large, equilateral triangular. Elytra semicircular, well convex dorsally, broadest at basal 1/3; humeral parts slightly projecting dorsally; EL/EW 1.14; EL/PL 4.57; EW/PW 1.43; TL/EW 1.39. Legs relatively long.

Caudal margin of sternite VII gently arcuate. Tergite VIII moderately sclerotized, trapezoidal, bearing short setae and spines along caudal margin, with a pair of short apodemes. Tergite IX membranous, with a pair of long and slender apodemes. Sternite IX slightly sclerotized, oblong, bearing short setae in postero-lateral parts. Tegmen moderately sclerotized; proximal part peg-like, short; parameres short, obtuse at

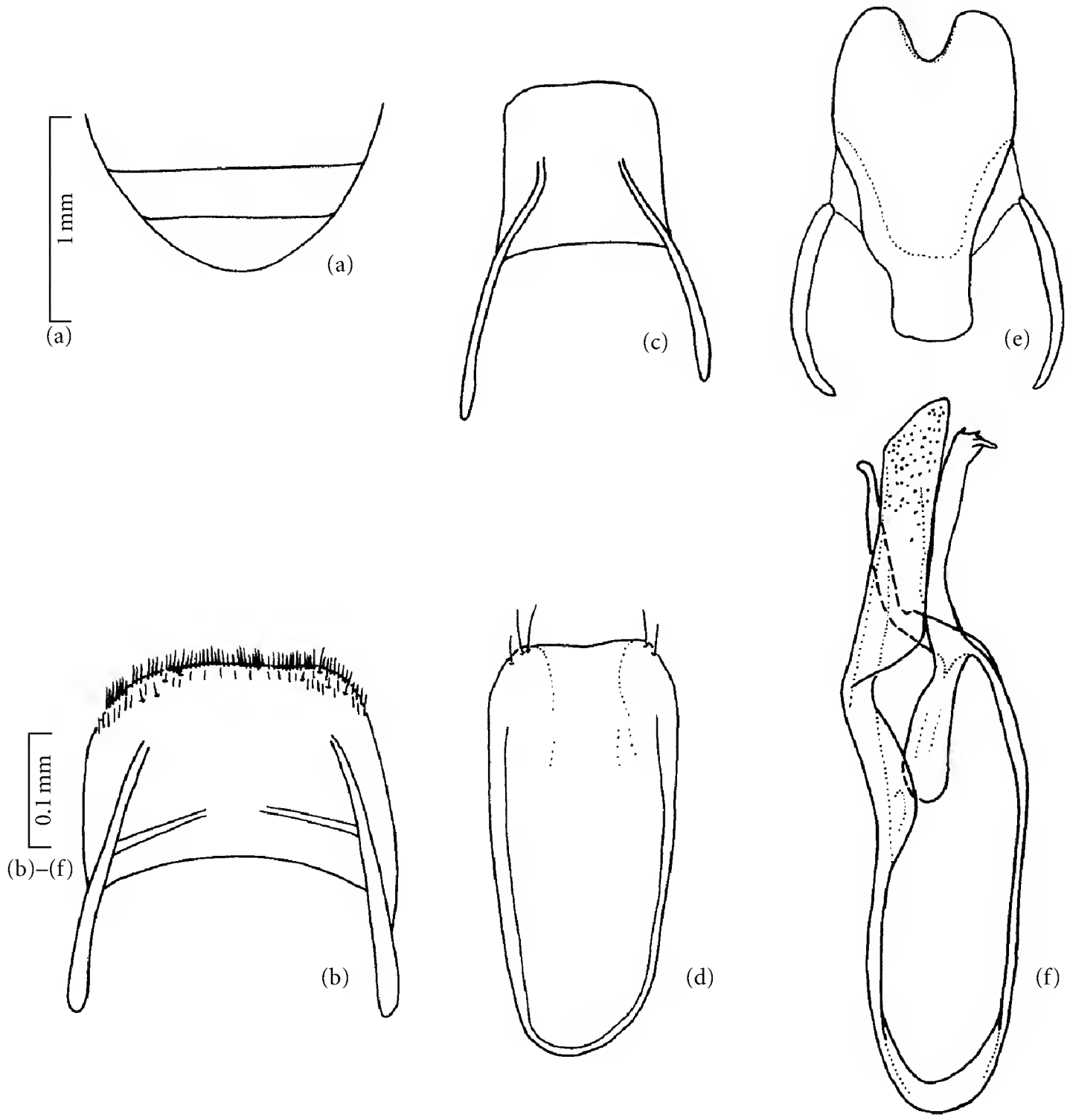

FIGURE 10: *Hydrocyphon deformis* sp.n., holotype, male. (a) Sternites V–VII; (b) tergite VIII; (c) tergite IX; (d) sternite IX; (e) tegmen; (f) penis.

(a) (b)

FIGURE 11: Habitats of *Hydrocyphon* spp. (a) Ngaglik, Yogyakarta (type locality of *H. jogjaensis* sp.n., 28. II. 2010, photo by H. Yoshitomi); (b) Kinabalu Park, Sabah (type locality of *H. takizawai* sp.n., 4. V. 2010, photo by H. Yoshitomi).

apices; lateral projections long. Penis asymmetrical, long, about 2.1 times as long as tegmen; pala oblong, subparallel-sided, arcuate in caudal margin; parameroids distinctly asymmetrical, left one wide and closely punctuate, diagonal in apical margin, right one distinctly curved inwardly, slender, with rather pointed apex; trigonium a little shorter than parameroids, serrate at apex; median plate short.

Female. Unknown.

Measurements. TL 1.95 mm; PW 0.98 mm; PL 0.35 mm; EL 1.60 mm; EW 1.40 mm.

Remarks. Judging from the shape of the penis in having an asymmetrical trigonium projection, this species belongs to the *pallidicollis* species group; however, the shape of the tegmen of this species is similar to that of the *mirabilis*, the *tamilensis*, and the *renati* species groups. This species is also similar to *H. kinabalensis* Yoshitomi and Satô, 2005 [2] in the shape of the tegmen and the left parameroid of the penis, but differs from it by the presence of trigonium and plate-like sternite IX.

Etymology. The species name refers to the shape of the tegmen.

4. New Combination of the Species

4.1. Hydrocyphon sieberi [15], Comb.n

Remarks. Judging from the original description and figures [15], this species clearly belongs to the *pallidicollis* species group of the genus *Hydrocyphon*. It is closely similar to *H. guangxiensis* Yoshitomi and Klausnitzer, 2003 [1], known from China, and differs from it by the shape of the right parameroid which has small projections at the inner margin of the apex (lacking projection in *guangxiensis*).

Distribution. India.

5. Additional Specimens Examined

5.1. Hydrocyphon sakaii Yoshitomi and Satô, 2003 [4]

Additional Specimens Examined. 9 Males (EUMJ), "(LAOS) Ban Saleui Xam Neua 30-31. III. 2005 J. Yamasako leg."; 1 female (EUMJ), "Mt. Phu Pan, 1500–1800 m, N20′11E 104′01 Houaphan Prov. N. E. Laos 21–25. V. 2004 T. Mizusawa"; 1 male (EUMJ), "Phu Pan (Mt.) alt. 1500–1800 m N20°11′/E104°01′ Laos 25. IV-5. V. 2004′′; 2 males (EUMJ), ditto, but "16–19. V. 2004, M. Sato leg."

Distribution. Laos.

5.2. Hydrocyphon wakaharai Yoshitomi and Satô, 2003 [4]

Additional Specimens Examined. 1 Male (EUMJ), "[N. Laos] Phu-Pan Alt. ca. 1600–1750 m Xam Neua Pref. Houapan province 21. V. 2005 T. Kurihara leg."; 3 males (EUMJ), "N-VIETNAM: Tam Dao 21°28′N 105°38′E 19. 5.–13.6., 800–1000 m leg. Malicky 1995," genit. s. nos. HY 857, 878, 882; 1 male, "Mt. Phu Bia Saisombun Laos 21-III-2005 M. Sato leg."

Distribution. Laos, Vietnam.

5.3. Hydrocyphon javanicus Yoshitomi and Satô, 2005 [2]

Additional Specimens Examined. 3 Males and 3 females (EUMJ), "(Indonesia) Ciburum alt. 1,600 m Mt. Gede, Jawa Barat VII. 27. 1977 Shinji Nagai leg."; 1 male (EUMJ), ditto but "20. VII. 1997."

Distribution. Indonesia (Java Isl.).

5.4. Hydrocyphon triforius Yoshitomi and Satô, 2005 [2]

Additional Specimen Examined. 1 Male (EUMJ), "Ban A Chia 890 m Lai Chau N. Vietnam 8-V-1995 Y. Nishikawa", genit. s. no. HY 1098.

Distribution. Malaysia, Thailand, Vietnam (new record).

5.5. Hydrocyphon tamilensis Yoshitomi and Satô, 2005 [2]

Additional Specimens Examined. 2 Males & 1 female (SEHU), "INDIA: TAMIL N. Coonoor 1700–1900 m 29 NOV 1978 JAP-IND CO TR."

Distribution. India.

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Review Article

Spider-Ant Associations: An Updated Review of Myrmecomorphy, Myrmecophily, and Myrmecophagy in Spiders

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This paper provides a summary of the extensive theoretical and empirical work that has been carried out in recent years testing the adaptational significance of various spider-ant associations. Hundreds of species of spiders have evolved close relationships with ants and can be classified as myrmecomorphs, myrmecophiles, or myrmecophages. Myrmecomorphs are Batesian mimics. Their close morphological and behavioral resemblance to ants confers strong survival advantages against visually hunting predators. Some species of spiders have become integrated into the ant society as myrmecophiles or symbionts. These spider myrmecophiles gain protection against their own predators, live in an environment with a stable climate, and are typically surrounded by abundant food resources. The adaptations by which this integration is made possible are poorly known, although it is hypothesized that most spider myrmecophiles are chemical mimics and some are even phoretic on their hosts. The third type of spider-ant association discussed is myrmecophagy—or predatory specialization on ants. A table of known spider myrmecophages is provided as is information on their biology and hunting strategies. Myrmecophagy provides these predators with an essentially unlimited food supply and may even confer other protections to the spiders.

1. Introduction

The majority of spiders are solitary generalist predators of insects [1]. Most spiders, as with most arthropod predators, are averse to ant predation because ants are generally aggressive, some are venomous, and most are simply noxious for a variety of reasons [2]. Nevertheless, hundreds of arthropod species live in some level of proximity or association with ants [3–5]. The present paper supplements a review I published in 1997 [5] identifying and describing the biology of spiders that are found in association with ants. In the earlier article, I summarized what was then known about the biology and identities of ant-mimicking, or myrmecomorphic, spiders as well as spiders living in close proximity to or living within ant colonies, known as myrmecophiles. That review included tables listing known spider myrmecomorphs and myrmecophiles. The purpose of the present paper is not to replicate information contained in the 1997 article but, instead, to provide a summary of the extensive theoretical and empirical work that has been carried out in recent years testing the adaptational significance of the various spider-ant associations. Additionally, I summarize instances of a different kind of spider-ant association—that of predator-prey relationships, or myrmecophagy—and provide a table of known species of spiders that feed on or specialize on ants.

2. Spider Myrmecomorphy

2.1. Morphological and Behavioral Adaptations. Morphological adaptations conferring mimetic resemblance to ants include color pattern similarities as well as more dramatic morphological changes such as abdominal constrictions and/or constriction of the cephalothorax, both of which give the illusion that the spider has more than two body parts [5–7] (Figures 1(a) and 1(b)). One recent paper demonstrated that some of these morphological adaptations may be synapomorphic for lineages [8], suggesting that at least some of the morphological adaptations associated with myrmecomorphy may be under phylogenetic constraint. Additional morphological adaptations seen in some spider myrmecomorphs include enlargement of the chelicerae or enlargement or other adaptations associated with the pedipalps or first legs. For example, males of some species of salticids in

(a) (b) (c)

FIGURE 1: Myrmecomorphy in spiders. (a) The model ant *Pseudomyrmex simplex* (Smith) and its mimic, (b) *Synemosyna petrunkevitchi* (Chapin) (Salticidae). Photos © Lyn Atherton, used by permission. (c) *Myrmarachne formicaria* (De Geer) (Salticidae) showing the enlarged chelicerae of the male. Photo © Jay Cossey/PhotographsFromNature.com, used by permission. Scale bars = 1 mm.

the genus *Myrmarachne* have greatly enlarged chelicerae that extend anteriorly [9] (Figure 1(c)). These large chelicerae are thought to have evolved via sexual selection [10]. Recent research demonstrated that male *Myrmarachne* with enlarged chelicerae mimic encumbered ants (worker ants carrying items in their mandibles) [11, 12]. In the myrmecomorphic species in the family Corinnidae, *Pranburia mahannopi* Deeleman-Reinhold, the first pair of legs of males has a thick brush of setae around the distal part of the femora. When the spider is disturbed or alarmed, it brings the femora together and the brushes give the illusion of an ant head (i.e., the spider behaviorally and morphologically acquires a third body part [13]).

Spider myrmecomorphs resemble the model ants to varying degrees of accuracy. Some myrmecomorphs are, at least to the human observer, nearly perfect mimics; others generally resemble ants but no specific model species in the vicinity of the spider can be identified. The latter are termed "imperfect" or "inaccurate" mimics [14, 15]. Some species of myrmecomorphic spiders are polymorphic mimics, mimicking multiple species of ants found in the habitat (see [5, Table 1], and [9, 16–20]). One species of jumping spider (Salticidae), *Myrmarachne bakeri* Banks, is polymorphic in color patterns and individual spiders can even change patterns during the course their lives, even after molting to maturity [20]. Individuals can change their patterns even under constant environmental conditions and feeding regime [20]. Other myrmecomorphs are transformational mimics, mimicking different species of ants during their different developmental stages (see [5, Table 1], and [9, 16–18, 21]).

In addition to morphological resemblance to ants, most spider myrmecomorphs are also behavioral mimics (see citations in [5]). This behavioral mimicry includes erratic movement, much more akin to the movement of ants than the movement of spiders, and lifting the first or second pair of legs when moving through the environment as an antennal illusion [22]. Myrmecomorphic salticid spiders also hunt their prey by lunging at and sometimes tapping the prey rather than by leaping on it as is common in most non-mimetic salticids [11, 22–24]. In other words, these spiders maintain their resemblance to ants even when hunting.

2.2. General Adaptive Significance of Myrmecomorphy. Myrmecomorphy has long been hypothesized to be an example of Batesian mimicry, conferring an adaptive advantage to the mimics against visually hunting arthropod predators that have either an innate or learned aversion to ants. Several studies have provided strong support for this hypothesis, demonstrating that myrmecomorphic spiders are less likely to be chosen as prey by visually hunting predators that would otherwise readily accept spiders [25–32]. In several of these studies, the predators used are naïve and have never encountered ants before, demonstrating that aversion to ants, at least in some arthropod predators, is innate rather than learned [27, 28, 30]. In order for myrmecomorphy to provide an adaptive advantage to the mimics, the mimics must live in close proximity to the models [33–38]. In addition, mimics should be rarer than models [15, 34, 36, 39, 40].

However, myrmecomorphic spiders, particularly those in the salticid genus *Myrmarachne*, often live in high concentrations within a given area. For example, *Myrmarachne melanotarsa* Wesolowska and Salm lives in aggregated groups in which their silken nest complexes are in close association with nests of their model ant, *Crematogaster* sp. [24]. Since ants live in often very large colonies, it has been hypothesized that aggregations of myrmecomorphs are an example of "collective mimicry" in which the myrmecomorphic spiders are, by living in aggregated groups, mimicking the colonial aspects of the models. Groups of mimics may be perceived by predators as more aversive than single individuals found in the habitat [24, 29]. A counter to this hypothesis is that the mimic may therefore outnumber the model in small areas of the habitat, making it more likely that predators will

sample and learn the patterns of the palatable mimics and making Batesian mimicry less effective [34]. In some visually hunting spider predators, such as the wasp *Pison xanthopus* (Brulle) (Sphecidae), individuals can develop search images for myrmecomorphic spiders and stock proportionally more mimics in their mud cells than would be expected if the wasp was randomly hunting spiders in the environment [41]. Therefore, some predators are capable of learning to search for myrmecomorphs. However, in a study of the mud-dauber *Sceliphron spirifex* (L.), Jocqué found no myrmecomorphic spiders among almost 600 spiders removed from mud nests, despite *Myrmarachne* species being common in the habitat suggesting that, at least for this wasp, ant mimicry does provide protection from visually hunting predators [42].

Yet, it has been pointed out that mimics can still confer protection against predators even when they are more abundant than the model if certain conditions exist: (1) if the model is very noxious, then the predators will avoid good mimics regardless of the relative proportions of models and mimics; (2) if the mimic has low nutritional value and is, therefore, not worth pursuing; (3) if very profitable alternative prey are present in which case the predator will avoid both model and mimic regardless of the relative abundance of each; or (4) if the relative perception of abundance is different, for example, if the predator perceives the model as more abundant than the mimic (perhaps because of the higher activity levels of the models) [37].

2.3. Evolution of Polymorphic Mimicry. In recent years, researchers have explored the adaptive basis and the conditions under which polymorphic mimicry might arise. Theoretically, a mimic species should converge on mimetic resemblance of the single model species found in that habitat, particularly for predators that learn to avoid the model [37]. Yet many instances of polymorphic mimicry among spider myrmecomorphs have been documented (see citations in Section 2.1). Several hypotheses have been proposed to explain the existence of polymorphic mimics. For example, Ceccarelli and Crozier [43] suggested that the evolutionary rates between different morphs of the salticid *Myrmarachne* and their presumed models differ [43]. These authors demonstrated that morphs of the mimics radiated rapidly leading to higher degrees of polymorphism and provided evidence of possible sympatric speciation. *Myrmarachne plataleoides* (O. P.-Cambridge) mimics the weaver ant *Oecophylla smaragdina* (Fabricius). Borges et al. [19] showed that the different color morphs of *M. plataleoides* may mimic different models in the habitat besides *O. smaragdina*. Males of each color morph showed greatest interest in the silk retreats of females of their own color morph. Disruptive selection may be maintaining the polymorphism in this population [19]. In addition, it has been proposed that polymorphic mimicry, in essence, provides a "moving target" for template learning among visually hunting predators that learn to avoid aversive prey [44]. Nelson [44] proposed that polymorphism in a myrmecomorphic species reduces the apparent number of mimics per model. Therefore, predators cannot easily distinguish palatable mimics from the unpalatable models because the characteristics of the prey are continuously changing. The new mimetic form will be advantageous since it is rare, but if this morph increases too much in frequency within the habitat, it may lose its mimetic protection and be selected against [37]. This selective process itself may generate selection for new morphs [37].

Sexual dimorphism can be considered a type of polymorphism. In many cases of sexually dimorphic spider myrmecomorphs, the male is more mimetic than the female, such as in species of the Corinnidae genus *Castianeira* and the Oonopidae genus *Antoonops* [13, 45]. Such sexual dimorphism may be adaptive if the sexes are different in ecology and are thus exposed to different predation pressures and selective forces [46]. Joron [46] provides a model supporting this mode of evolution and selection for sexual dimorphism among mimetic species. Although mimics gain protection from the resemblance to noxious species, they are often more conspicuous in their color markings than related species that have evolved cryptic coloration. Thus conspicuousness can be considered a cost of Batesian mimicry [47]. A palatable species may be evolutionarily maximizing its level of protection for the smallest cost (in terms of conspicuousness) and this evolutionary balancing act may lead to sexual dimorphism in which the more active sex (which, in spiders, is typically the males) evolves mimetic resemblance to noxious models whereas the other sex remains relatively more concealed and camouflaged behaviorally and morphologically [47].

In some species of the salticid genus *Myrmarachne*, the males and females are both mimetic but the males have extraordinarily long chelicerae. This sex mimics ants carrying an object in their mandibles [11, 12]. The large chelicerae of males are thought to have evolved via sexual selection [10]. These large chelicerae are an encumbrance to males during prey capture; however, they make males much more efficient than females in breaking into other spiders' silken retreats and feeding on eggs or juveniles [10]. Consequently, in this case of sexual dimorphism, both sexes have maintained mimetic resemblance to the models, although the male is mimicking a slightly different type of model ant (an encumbered ant). Any costs incurred from the dimorphism may be outweighed by benefits in opening up a different trophic niche for the males (oophagy).

2.4. Evolution of Imperfect or Inaccurate Mimicry. It is well documented that many mimics are imperfect in their mimetic resemblance to the model. These species generally resemble the putative models but are not accurate mimics [14, 31, 37, 47, 48]. Some authors propose that poor mimics are just on an evolutionary trajectory towards perfection. This hypothesis is discussed by Edmunds [49] and Gilbert [37]. Gilbert [37] refutes this hypothesis saying, "*In my view it is better to assume that poor mimetic patterns have evolved to an equilibrium state, rather than being in the process of being perfected by constant directional selection*" since there is no experimental or theoretical support for the hypothesis that imperfect mimics are just mimics on their way towards perfection.

Recently, authors have instead proposed various evolutionary scenarios that may select for imperfect or inaccurate mimicry rather than explain this phenomenon away as "evolution in progress." Many papers point out that if a model is extremely unpalatable, noxious, or difficult to capture, then even imperfect mimics will gain strong selective advantage from a general resemblance to this model and there may be no selective advantage or pressure for more accurate mimetic resemblance [34, 37, 39, 47, 50]. In fact, the fitness costs of close morphological resemblance (see Section 2.5) may select against accurate mimicry and may select for imperfect mimicry if either confers approximately the same selective advantage in terms of escape from predation. In a study by Duncan and Sheppard [50], the authors experimentally demonstrate that, when the model is very noxious, even imperfect mimics gain protection. However, when the model is only moderately distasteful, selection favors more accurate mimics. They showed that when the cost of making a mistake, attacking a distasteful model because it is mistaken for a palatable mimic, is high, the predator rejects a greater proportion of mimics and there is little selection for more accurate mimicry. When the penalty for making a mistake is low, tiny improvements in mimetic resemblance confer a selective advantage to the mimics, leading to more accurate mimicry [50]. In a study by Speed and Ruxton [47], the authors propose that if generalization by the selective agents (the predators) is narrow, selection towards accurate mimicry is predicted. If generalization by predators is relatively wide (e.g., in the case of a particularly noxious model), variations in mimetic forms may be selected for with both accurate and inaccurate mimics. Finally if generalization by predators is intermediate, then the rate of evolution selecting for accurate mimicry will be slow and polymorphic mimetic forms will be stable.

In situations in which the model either becomes rare or is weakly aversive and the incentive to attack and sample the models (by predators) is high, then close mimics may in fact be selected against. Kin selection among the mimetic population would select for less accurate mimics that diverge in their mimetic resemblance to the weakly defended model [15, 34, 37]. Inaccurate mimicry can also be favored in species with limited dispersal and high local abundance in which neighboring mimics are related (i.e., kin selection) [15].

A study by Kikuchi and Pfennig [39] provided experimental support for the hypothesis that evolution of accurate mimicry is a gradual process and depends on the relative abundance of the model. In this study, the authors found that in areas where the model was abundant, predators attacked cryptic (or camouflaged) prey, accurate mimics, and intermediate (or imperfect) mimics with the same low frequency. In other words, in areas where the model was abundant, predators generalize and imperfect mimics gain the same relative protection as more accurate mimics. In habitats where the model population was low, camouflaged species and mimics attained greater protection than imperfect mimics. Thus the authors showed that Batesian mimicry can evolve through gradual steps towards more accurate mimicry depending on conditions and context (particularly the abundance of models in the habitat) [39]. This study also suggests that mimics may have evolved from cryptic or camouflaged ancestors.

Accuracy of the mimetic resemblance may depend largely on the visual acuity of the selective agent. If predators with keen vision serve as the primary selective agents, then these predators may select for more accurate mimicry [34]. Then again, mimicry may be in the eyes of the beholder. Arthropods that humans view as poor mimics were perceived by pigeons, in an experimental test, as very good mimics [14]. Dittrich et al. [14] also showed that slight changes in the morphology of the mimic led to sometimes dramatic improvements, from the perspective of the selective agent, in perceived mimetic resemblance. They further pointed out that discrimination between a good and a poor mimic occurs via multiple features (e.g., color, form, size), not a single characteristic [14]. Other authors have also suggested that selection for increasingly better mimetic resemblance can, in fact, be a gradual process through directional selection [50, 51].

Related to the hypothesis that mimetic accuracy is dependent on the visual acuity of the selective agent is the multi-predator hypothesis, which proposes that inaccurate Batesian mimics evolved as a result of selective forces from a suite of predators [52]. For example, model averse predators select for more accurate morphological mimics in a given habitat while specialist predators on the model (e.g., ant predators or myrmecophages) select for inaccurate mimicry or for secondary defenses in the mimic [52]. Secondary defenses may include fast evasive movements by the mimics (quickly dropping all pretense of behavioral mimicry) or signaling the predator in such a way as to communicate its true identity [52]. If both kinds of predators are present in a habitat, there may be selection for inaccurate mimics or for polymorphic mimicry [52].

One hypothesis explaining imperfect Batesian mimicry that has gained some momentum in recent years is the multi-model hypothesis. If many potential model species live in a given habitat (e.g., many different species of ants), then it may be adaptive for the mimetic species to evolve a general, imperfect resemblance—a gestalt resemblance—to all of them than to evolve a specific morphological resemblance to a particular model [33, 37, 49]. For example, a general ant-mimicking spider in such a habitat can then have a much greater range than a spider that resembles only one of the potential models. If it is an accurate mimic, then its range is limited to the range of that one species in order to be an effective Batesian mimic. In one study, the authors found that some species of accurate ant mimics were found in association with a single model (measured as the closest ant collected where the spider was found). Some imperfect mimics (by human standards) were collected in proximity to more than one species of ant, conferring some support for the multi-model hypothesis [33]. However, in this same study, the author also found habitats in which accurate and inaccurate mimics did not associate with the models as predicted.

2.5. Trade-Offs Affecting the Evolution of Myrmecomorphy. A close morphological resemblance to ants makes myrmecomorphs more attractive to ant predators or myrmecophages. Thus myrmecomorphs are faced with an evolutionary trade-off: they gain protection from general arthropod predators but risk predation from a completely different suite of predators ([11, 12, 53] and citations above under discussion of multi-predator hypothesis). Many spider myrmecomorphs confront a threat from a myrmecophage by completely dropping their behavioral mimicry. These spiders will stop their erratic ant-like movement and run away, drop on a silk thread, signal to the predator in a spider-specific manner, or otherwise communicate their true identity to the predator [11, 52, 54]. This strategy is effective in allowing the spider to escape from the myrmecophage (or from ants that may confront it directly) [11, 24, 54].

Myrmecomorphs face other costs that may affect their fitness, including (1) constraint of the circadian rhythm of the mimic since it must be active at the same time of day as the model for the resemblance to be adaptive; (2) an imposed limit to the myrmecomorph's trophic niche because it would only have access to prey that lived in the same habitat as the model; (3) a possible detrimental or costly effect on mating or reproduction since many myrmecomorphs must mate in a sheltered location, where their non-ant-like behavior will not "give the game away" or may mate for a shorter duration than non-mimetic relatives for the same reason; (4) a lowering of fecundity with the abdominal narrowing or constrictions often associated with myrmecomorphy and the resultant decrease in the number of eggs a female can produce [37, 55]. It has been documented that narrower abdomens in female spiders limit the number of eggs that can be produced in comparison to non-mimetic relatives [9, 18, 56–61]. In addition, there may be a cost associated with alteration in the prey capture behaviors, such as those seen in myrmecomorphic salticids that lunge rather than jump upon their prey, which may be a much less effective prey capture strategy.

Nevertheless, if the primary predators demonstrate an innate, rather than learned, aversion to ants, the circadian rhythm of the myrmecomorphs may not be greatly affected and they can be active at any time of day. The limitation of trophic niches may not apply to general ant mimics since these spiders can exist, according to the multi-model hypothesis, across a potentially broad range of habitats. It does seem though that most spider myrmecomorphs do share the same habitat as their models and are active at the same time of day. It has even been pointed out that no species of wolf spider (family Lycosidae) has been reported to be an ant mimic because most lycosids are nocturnal and not active when visually hunting arthropod predators are most active [4]. Researchers investigating the inaccurate myrmecomorphs *Liophrurillus flavitarsis* (Lucas), *Phrurolithus festivus* (C. L. Koch) (both in the family Corinnidae), and *Micaria sociabilis* Kulczynski (Gnaphosidae) found that, in comparison to these species' closest relatives, the trophic niche of each was constrained by their resemblance to ants because they were limited to catching only small invertebrates found in the same habitat as the models. The circadian rhythms of these myrmecomorphs were also constrained because the myrmecomorphs were all diurnal (as were the models) but the closest relatives were nocturnal. However, the reproductive traits were not constrained since the fecundity of the inaccurate mimics was about the same as the non-mimetic relatives and the myrmecomorphs mated out in the open on bark, not dropping their behavioral mimicry when copulating [55].

The evolution of close morphological and behavioral mimicry of ants is costly and these costs should be measured as fitness components [37]. In addition, more studies should attempt to identify the operators or selective agents selecting for mimetic resemblance since the visual acuity of these selective agents (if they can be identified) may affect the accuracy of the resemblance. All these costs, trade-offs, and constraints should be taken into account when testing or modeling the adaptive significance of myrmecomorphy. The relative measures of the costs and benefits of mimetic resemblance may have a significant impact on the accuracy of the resemblance. If, for a particular species, the fitness costs of close mimetic resemblance due to lower fecundity greatly outweigh the benefits, then imperfect or inaccurate mimicry may be selected for. For example, in a habitat where the primary selective agent is a predator with low visual acuity, increased mimetic accuracy may impose a higher cost in terms of fecundity than is gained in terms of escape from predation. In small species of spiders in which greater mimetic resemblance would lead to dramatically lower fecundity due to a narrowing of the female's abdomen, dimorphic mimicry may be selected for and males may show greater mimetic resemblance than females. Too few models take into account fitness costs of mimetic resemblance and the relative effect such trade-offs may have on the evolution of imperfect, polymorphic, transformational, and dimorphic mimicry.

3. Spider Myrmecophily

3.1. Additional Records of Spider Myrmecophiles. Myrmecophiles are defined as ant guests, arthropods that have evolved close associations with ant species, often living alongside the ants or within the ant colonies [2, 3, 5, 62]. Some, but not many, of these myrmecophiles are also myrmecomorphs. Recent work (cited below) has found that, among spider myrmecophiles, some are also myrmecophages.

An extensive table of spider myrmecophiles was presented by Cushing [5]. Table 1 supplements this earlier table and provides records of spider myrmecophiles not included in the previous table. Not as much work has been carried out exploring the natural history, adaptations, or evolutionary significance of spider myrmecophiles as has been done with spider myrmecomorphs and myrmecophages. Nevertheless, some significant research has been conducted recently that expands our understanding of the biology of these interesting ant associates and how this unique lifestyle may have evolved in a group of arthropods that otherwise includes primarily free-living, solitary predators.

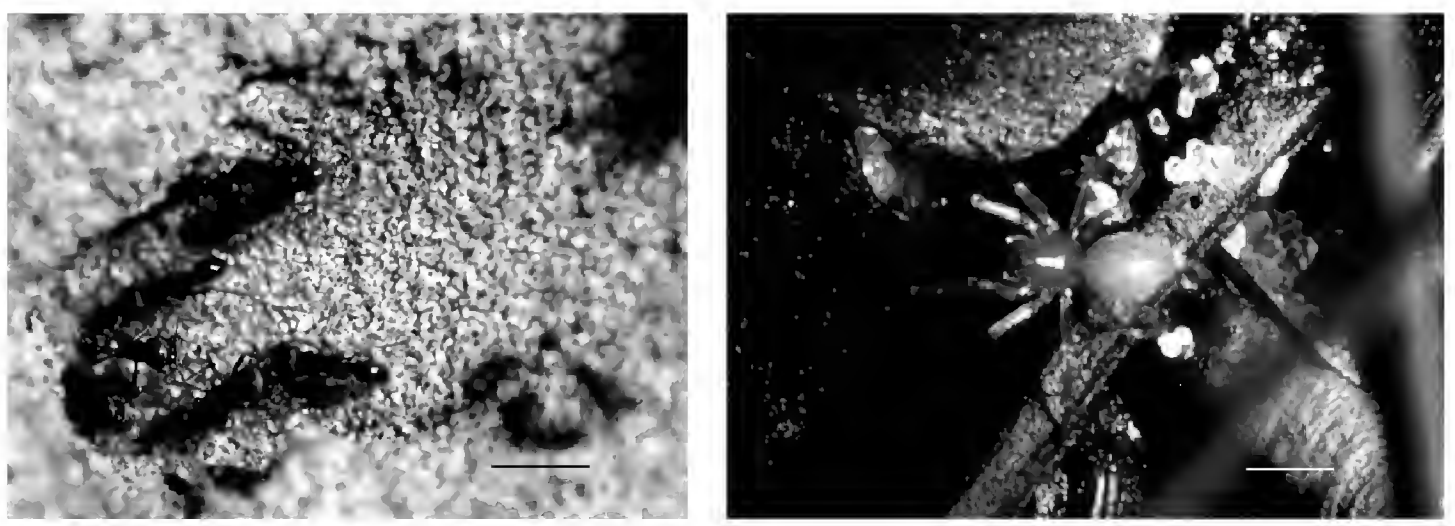
3.2. Adaptive Significance of Myrmecophily. An ant colony, as pointed out by Hölldobler and Wilson [2], can be considered

Table 1: Spider myrmecophiles found in association with or inside ant nests. This table is meant to supplement the table of Araneae myrmecophiles found in Cushing [5]. Spider taxonomy according to Platnick [63]; ant taxonomy according to http://antbase.org/.

Spider myrmecophile	Ant host	Notes on biology	References
Linyphiidae			
Diastanillus pecuarius (Simon)	*Formica* cf. *fusca* L. and *F. lemani* Bondroit	Found under stone near ants.	[64, 65]
Pseudomaro aenigmaticus Denis	*Lasius flavus* (Fabricius)	Associated with nests.	[65]
Syedra myrmicarum (Kulczynski)	*Manica rubida* (Latreille) and *Formica* sp.	Found under stone near ants.	[64, 65]
Oonopidae			
Dysderina principalis (Keyserling)	*Labidus praedator* (Smith) (publ. as *Eciton praedator*)	Found inside nests.	[66]
Gamasomorpha maschwitzi Wunderlich	*Leptogenys processionalis distinguenda* (Emery) (publ. as *L. distinguenda*)	Found inside nests. Chemical mimic. Phoretic. Follows emigration trails of hosts. Builds webs inside nest.	[65, 67–69]
Gamasomorpha wasmanniae Mello-Leitão	*Eciton* sp.	Found inside nests.	[70]
Xestaspis loricata (L. Koch) (publ. as *G. loricata*)	*Myrmecia dispar* (Clark)	Found inside nests.	[71]
Salticidae			
Cosmophasis bitaeniata (Keyserling)	*Oecophylla smaragdina* (Fabricius)	Lives inside nest. Is chemical mimic of ant. Feeds on ant larvae by using tactile mimicry.	[72–76]
Phintella piatensis Barrion and Litsinger	*O. smaragdina*	Lives in proximity to ants.	[77]
Theridiidae			
Eidmannella pallida (Emerton)	*Atta sexdens* (L.)	Lives in old fungus chambers of nest.	[78]

an isolated ecosystem. Arthropods symbiotic with ant hosts typically experience a stable microclimate, plentiful food (either in the form of other symbionts, the hosts themselves, or other resources brought into the colony by the hosts), and protection from their own predators and parasites [5, 68, 77]. The degree of integration into the colonies varies greatly from species with just a loose affiliation or association with the ant nests to symbionts that spend their entire lives within the ant nests and fail to thrive when removed from this habitat [5, 79]. These symbionts can have a neutral, a positive, or a negative influence on the host colonies depending on their natural history. If the effect of the myrmecophile on the host is costly enough, there should be selection for the host to recognize and attack or remove these guests from the nest [69]. For example, the myrmecophile *Masoncus pogonophilus* Cushing (Linyphiidae) feeds on collembolans and other symbionts found in the colonies of the harvester ant, *Pogonomyrmex badius* (Latreille) [80] (Figure 2). Therefore, this spider may have a slightly negative effect on the colonies of these ants since the primary prey of the spiders, collembolans, graze fungal spores found inside the nest chambers, particularly the seed storage chambers [80], and thus keep fungal infestations low. However, populations of these spiders are so small within any given colony that their net effect on the host's success is probably negligible [79, 80]. Some evidence suggests that hosts can recognize and will attack these symbionts, particularly those introduced from a neighboring nest [81, Cushing pers. obs.]. The myrmecophilic spider *Gamasomorpha maschwitzi* (Wunderlich) (Oonopidae) is found inside the nests and bivouacs of the army ant, *Leptogenys distinguenda* (Emery), where it apparently feeds on insects captured by the hosts. Therefore, this myrmecophile has a negative impact on host fitness as a kleptoparasite on the host's prey. However, as with *M. pogonophilus*, the abundance of spiders within any given colony is so low that its negative impact is likely negligible and these spider guests are either ignored or treated with only very low levels of aggression [67, 68]. Sometimes spiders are even groomed by the host ants [69]. The spider *Attacobius attarum* (Roewer) (Corinnidae) (originally published as the clubionid *Myrmecques attarum*) lives with *Atta sexdens* (L.) where it feeds on ant larvae and pupae [82] and thus also has a negative impact on host colonies. The hosts are known to antennate the spiders but do not show any aggression towards these myrmecophiles [82].

It has been noted that certain types of ant colonies are more open to invasion by myrmecophiles than others. Characteristics of host colonies that are most open to invasion by myrmecophiles include: colonies with multiple queens (polygynous colonies), colonies with multiple nest sites (polydomous colonies, which are often also polygynous), and very large colonies [83]. These societies tend to be more "loose, flexible, and dynamic" than monogynous colonies and tend to have less social cohesion leading to increased



(a) (b)

FIGURE 2: Myrmecophily in spiders. (a) The host ant *Pogonomyrmex badius* (Latreille) at the nest entrance. (b) The myrmecophilic spider, *Masoncus pogonophilus* Cushing on the surface, walking along the emigration trail of the host ant. Scale bar in (a) = 8 mm, scale bar in (b) = 1 mm. Photos © author.

vulnerability to invasion by myrmecophiles [83]. In general, myrmecophile populations tend to occur in one of the following distinct patterns: (1) a myrmecophilic species is found in many colonies at certain locations throughout a host species' range but not at other locations (i.e., high infestation but low transmission), (2) a myrmecophilic species is found throughout the host's range but only within a few colonies at any given locality (i.e., low infestation but high transmission), or (3) the myrmecophile is found in only a few colonies at any one locality and not throughout the host's range (i.e., low infestation and low transmission) [83]. Population size of myrmecophiles is often quite low within a colony, but this depends on the type of myrmecophile. Spider myrmecophiles that have been studied in any depth, in general, tend to have small populations within a colony [67, 68, 79]. Intraspecific aggression between spider myrmecophiles within a colony has been reported [69] and may be one factor in keeping populations small.

3.3. General Adaptations Facilitating Integration into Colonies. Close integration within ant colonies seems to be more common in certain families, such as the Linyphiidae and Oonopidae [5]. These spiders have several characteristics (morphological and behavioral) that may serve as preadaptations to a symbiotic lifestyle inside ant nests [67]. For example, both families include very small spiders (typically less than 5 mm); the species are often found in moist, humid microhabitats such as leaf litter, under rocks or logs, or under bark; and many species in these families (particularly oonopids) have morphological adaptations such as hard sclerotized scuta covering their abdomens that may provide some protection against attacks by host ants. Witte and colleagues point out that some species of oonopids may scavenge insect remains in the webs of other spiders [67]. All these behavioral and ecological characteristics may preadapt spiders to a myrmecophilic lifestyle within ant colonies. Smaller body sizes allow them to "sneak" inside the nests and become integrated. Protective scuta (and small sizes) may provide some protection against attacks from the hosts. A scavenger lifestyle may be considered a preadaptation to stealing food (insects or ant brood) from workers. The constant temperature and humidity of an underground ant nest may be an attractive environment to species otherwise restricted to similar temperature and humidity regimes.

Once integrated into colonies, spider myrmecophiles certainly have evolved dramatic host-specific adaptations allowing them to become even more integrated into various aspects of the host's life cycle. These adaptations, in turn, place severe constraints on the geographical distribution of these inquilines or ant guests; the symbionts are restricted to the range of that host species [83]. This may explain why such inquilines are very localized or rare and may be subject to frequent extinctions [83]. Adaptations common to myrmecophiles include evasive devices such as behaviors, morphological structures, or chemical signals used to appease hosts or to mimic hosts; protective morphological structures such as sclerotized cuticular "shields" or plates; mechanisms to communicate with hosts via chemical cues, tactile cues, or even auditory cues [83].

3.4. Chemical Mimicry. Among spider myrmecophiles, besides the preadaptations mentioned above, many have evolved the capacity to absorb, biosynthesize, or otherwise mimic the host ant's cuticular hydrocarbon colony odor. To survive inside the host colony, the guest must be considered a nest mate by the hosts and should, therefore, have somehow acquired the chemical odor of the hosts via either biosynthesis of the key compounds or by passively acquiring the chemical cues [84]. Thus far, no research has definitively documented glandular secretions that spider myrmecophiles might use to biosynthesize the compounds. If such glands are documented, then it is likely that the association between the host and the myrmecophile is an ancient association and the myrmecophile and host coevolved [85, 86]. However, biosynthesis may evolve rapidly in myrmecophile populations if the compounds biosynthesized can be easily manufactured by co-opting an already existing chemical pathway or if the guest can re-purpose an already existing compound [86].

The chemical signature of ant colonies may change over time [2]. Thus intruders (guests) into colonies must be able

to update their profiles constantly in order to avoid detection and attack. If the myrmecophile's chemical profile does not match the host's closely enough then it will become more difficult for the guest to approach the host in order to update its profile, making social integration into the colonies a "well-balanced and potentially fragile system" [69]. Myrmecophiles can acquire colony odors by rubbing against the host ants, associating with nest materials, or by eating the ant's brood (larvae or pupae) [84]. All these mechanisms are seen in spider myrmecophiles. It may be that these myrmecophiles do not need to acquire an exact chemical match to the host's hydrocarbon profile, but need only one or two key constituents that are biologically most important in nest recognition and acceptance by the hosts [86].

For example, the oonopid, *G. maschwitzi*, found with the army ant, *L. distinguenda*, has a cuticular hydrocarbon profile that includes only compounds also seen in the host ant's profile but not all the compounds seen in the host's profile [69]. These spiders crawl on top of workers, moving their legs actively over the cuticle of the host, perhaps as an adaptation to acquire the host's chemical odor [68, 69, 78]. The hydrocarbon profile of the myrmecophilic spider matches that of the host's to a high degree; however, colony-specific matching was not evident [69]. Nevertheless, ants of *L. distinguenda* from different colonies did not show high levels of intercolony aggression; therefore, it may not matter that the myrmecophile's profile lacks these colony-specific compounds but just generally matches the gestalt odor of the species (i.e., has key chemical constituents that identify it as an ant and a member of the same species) [68, 69]. Research has also demonstrated that the phoresy displayed by *G. maschwitzi* may also function as a behavioral mechanism for the spider to acquire food (ant larvae, pupae, or insects being carried by the workers) via kleptoparasitism [68]. The spider riding on the back of the ant snatches the food item directly from the host's mandibles. In fact, these spiders have not been observed to hunt prey on their own [67] so this kleptoparasitic lifestyle may be another example of extreme adaptation related to their symbiotic life with these ants.

The salticid *Cosmophasis bitaeniata* (Keyserling) lives inside the colonies of the weaver ant, *Oecophylla smaragdina* (Fabricius), where it feeds on the larvae of the host ant [72–75]. The spider is more often found in and around older nests that have lots of larvae [72]. The spider touches the antennae and head of minor workers with its front legs, stimulating the workers to release the larva that the worker is carrying [72]. The spider otherwise avoids direct contact with the worker ants [72, 75]. The spider is a chemical mimic of the host [73–76]. It has been shown that the spider acquires the colony specific hydrocarbon profile by handling and eating the ant larvae [74, 76]. The hydrocarbon profile of the spider is colony specific but does not match the profile of the major workers [75]. Larvae from different colonies do not elicit aggressive responses from the host; thus spiders that mimic the hydrocarbon profile of the larvae rather than the workers may be more easily accepted by both their own hosts as well as those of neighboring colonies [76].

The spider *Attacobius attarum* that lives inside the nests of the leaf cutter ant, *Atta sexdens* (L.) rides on the dorsa of workers and alates [78, 82, 87]. The spiders may disperse to new colonies via the alates [78, 82, 87]. *Attacobius attarum*, like *G. maschwitzi* and *C. bitaeniata*, is a kleptoparasite; the spider feeds on ant larvae and pupae and can steal the brood directly from the mandibles of workers [82]. The ants antennate the spiders and the spiders reciprocate by "antennating" the ants with their front legs, possibly providing mimetic tactile cues [82]. No aggression towards these kleptoparasites has been reported [82].

The theridiid spider, *Eidmannella pallida* (Emerton) (published as *Eidmannella attae*), also lives with *A. sexdens* where it is found in unused fungus chambers that the ants use to store refuse and dead ants [78]. Likewise, the linyphiid, *M. pogonophilus*, lives in seed chambers and empty chambers of the seed harvester ant, *P. badius* [79, 80]. Both these spider myrmecophiles may acquire host colony odor passively via the nest materials. Neither has been reported as phoretic, as kleptoparasitic, or as a predator of the hosts or their brood. Thus passive integration and acquisition of colony odor is likely for these symbionts.

3.5. Ability to Follow Chemical Cues of the Hosts. *Cosmophasis bitaeniata* can distinguish between nestmate and non-nestmate major workers and shows less tendency to try and escape when confined with nestmates, demonstrating that these myrmecophiles are not only chemical mimics but are also able to interpret chemical cues provided by the hosts [74]. Data suggests that the ability to interpret chemical signals of the hosts may be a general characteristic of spider myrmecophiles that are closely integrated into ant colonies. Research on *M. pogonophilus* and *G. maschwitzi* showed that spiders are able to follow trail pheromones laid by the ants [67, 68, 79, 80]. In controlled tests, Witte et al. found that *G. maschwitzi* is sensitive to high concentrations of naturally laid ant trail pheromones [67]. I found *M. pogonophilus* in the emigration trails of *P. badius* when the hosts emigrated to new nest sites [79, 80] (Figure 2(b)).

Spider myrmecophiles may use ant trail pheromones as a means of dispersing to new colonies. In a given habitat, it is not uncommon to find spider myrmecophiles in all or nearly all the nests of a given host, even if the host is not polygynous or polydomous [68, 79]. Thus in at least these instances, dispersal to new colonies must be occurring. Only one study has attempted to examine the population structure of a myrmecophilic spider, *M. pogonophilus*, which was found in nearly all colonies of *P. badius* in a given habitat (i.e., 10 colonies out of 12 that were excavated) [79]. *Pogonomyrmex badius* colonies are established by single inseminated queens [88] that can live for at least 15 years [89]. I hypothesized that spider populations might be considered metapopulations [90], made up of isolated demes, or local populations, with very low per-generation migration between populations resulting in low genetic diversity between individuals within populations (i.e., myrmecophiles within an ant nest) and higher genetic heterogeneity between populations (i.e., between populations of spiders found in different colonies) due to genetic drift [79]. Instead, I found that genetic diversity among individual spiders within populations (within a

colony) was greater than the genetic diversity between populations from neighboring ant nests suggesting that spiders do disperse to new nests frequently enough to maintain high intra-population differentiation and low inter-population differentiation [79]. Although tests of the spiders' ability to follow trail pheromones (naturally laid and artificial trails) were inconclusive, I further hypothesized that spiders were able to locate new nests by following trail pheromones. They were found to emigrate with their hosts to new nest sites (see above), thus they may, during emigration, get "side-tracked" onto the foraging trail of a neighboring *P. badius* colony [79].

3.6. Life Cycle of Spider Myrmecophiles. Very little is known about the life cycle of any spider myrmecophile. Even for one of the best studied species, *G. maschwitzi*, no spiderlings have ever been detected in the emigration trails nor inside the nests [67, and Volker Witte, pers. communication]. *Masoncus pogonophilus* builds prey capture webs inside nest chambers and females deposit small silken egg sacs each containing up to seven eggs in depressions in the walls of the chambers [80]. The salticid, *C. bitaeniata* also deposits its egg sacs within the nest chambers of *O. smaragdina* [72]. A *G. maschwitzi* female was collected with one large egg in the abdomen and another with five smaller eggs [67]. Both *M. pogonophilus* and *C. bitaeniata* have female biased sex ratios [72, 80].

3.7. Future Directions. A great deal more research needs to be done to understand the basic biology of spider myrmecophiles. Questions and directions for future research include the following.

(i) How closely integrated are spider myrmecophiles with their host ants?

(ii) How do these spiders reproduce inside the ant colonies or does reproduction occur outside the nests?

(iii) How do they disperse to colonize other nests?

(iv) Is chemical integration a widespread phenomenon among spider myrmecophiles?

(v) Can any spider symbiont biosynthesize chemical compounds that act to appease or mimic the hosts?

(vi) Are spider myrmecophiles generally able to interpret the chemical signals of their hosts?

(vii) Is there evidence of a co-evolutionary relationship between symbionts and hosts?

(viii) How closely related are spider myrmecophiles within a colony and do these patterns of relatedness explain the female-biased sex ratios seen in some species?

4. Spider Myrmecophagy

4.1. Species Records. Spiders, like other arthropod predators, generally avoid preying upon ants. However, ants have been documented as part of the diet for well over 100 species of spiders (Table 1). Fossil evidence of spider myrmecophagy dates back 30–50 mya in Baltic amber specimens including one containing an inclusion of spider silk with an ant that had been fed upon as well as another showing a spider with an ant in its chelicerae [91]. Myrmecophagic spiders exist on a continuum from euryphagous to stenophagous predators [92]. Huseynov et al. [92] propose five categories of spider myrmecophages: (1) non-acceptors of ants (the majority of spider species); (2) reluctant acceptors that do prey on ants but prefer other prey; (3) indifferent acceptors that feed indiscriminately on ants and other prey; (4) facultative ant choosers that prefer ants to other prey; (5) obligatory ant choosers that feed exclusively on ants (unless severely food deprived). In Table 2, the various spider myrmecophages that have been documented from the literature are categorized as (R) Reluctant acceptors, (I) Indifferent acceptors, (F) Facultative ant choosers, or (O) Obligatory ant choosers based upon information about their biology provided in the literature. If researchers have only documented that the particular species eats ants but provide no other information about the hunting behavior or prey preference of the spiders, the species is categorized as (Unk) Unknown. However, these spiders are likely to turn out to be either reluctant or indifferent acceptors of ants in the diet. Details of the predatory biology of spider myrmecophages are also included in the table.

4.2. Evolutionary Costs and Benefits of Myrmecophagy. Spider myrmecophagy is a high risk hunting strategy. Risks for myrmecophages include being attacked by the prey, living in close proximity to dangerous prey, being attacked when mating, having the prey attack and destroy one's eggs if nesting and oviposition occur close to the ant nests [58, 143, 175]. However, a spider that evolves strategies for overcoming an ant's defenses and aggression faces relatively little competition for a nearly unlimited food resource [114, 143] (Figure 3(a)).

One study demonstrated that myrmecophagic spiders may actually derive protection against attacks from their own prey: when myrmecophagic, myrmecophilic, myrmecomorphic, and non-ant associating salticids were trapped with ants, the myrmecophagic spiders showed the highest survival rate followed by the myrmecomorphs and myrmecophiles, suggesting that ant associates may signal the ants in such a way that the ants show little aggression towards these spiders [176]. Thus not only are myrmecophagic spiders obtaining a nutrient rich, unlimited food supply through their specialized diet, but they may also be deriving protection from the ants, just as myrmecophilic and myrmecomorphic spiders do.

Although it has not been suggested that spider myrmecophages are chemical mimics of ants, as has been demonstrated for spider myrmecophiles, there is some evidence that certain species of myrmecophages may either be releasing chemical compounds that appease their potential prey or may be able to "read" chemical cues released by ants. For example, Lubin suggested that the thomisid, *Tmarus stoltzmanni* Keyserling, may use its 1st and 2nd pairs of legs to detect chemical or tactile cues from the ants [148]. *Habronestes bradleyi* (O. P.-Cambridge) (Zodariidae) waves its front legs around when hunting and, when the legs are amputated, the spider has a difficult time locating ant prey

Table 2: Spider myrmecophages. *Categories (defined in text) include R: Reluctant myrmecophage; I: Indifferent acceptor; F: Facultative ant predator; O: Obligatory ant predator; Unk: cannot be determined from information about their biology presented in the literature (these are most likely R or I myrmecophages). Spider taxonomy according to Platnick [63]; ant taxonomy according to http://antbase.org/.

Spider myrmecophage	Category of myrmecophage*	Notes on biology	References
Araneidae			
Metepeira gosoga Chamberlin and Ivie	Unk	Author suggests that spiders may feed on ants found only on cholla where spider is also found.	[93]
Metepeira sp.	Unk	Reported feeding on *Crematogaster opuntiae* Buren.	[93]
Deinopidae			
Deinopis sp.	Probably I	Throws web over ants passing below.	[94]
Eresidae			
Seothyra sp.	F	Lives in silk lined burrows. Mouth of burrow covered by prey capture web. Captures mostly ants. Male spider runs on ground during day and is myrmecomorph and behavioral mimic of *Camponotus* sp. and mutillid wasps (dimorphic mimicry).	[95]
Gnaphosidae			
Callilepis nocturna (L.)	May be F	Feeds on *Formica* spp. and *Lasius* spp. Actively searches for ants and may enter nests to hunt workers. Approaches ant and bites on base of antenna. Antennae seem to act as stimulus to trigger attack.	[96–98]
Linyphiidae			
Frontinella communis (Hentz)	I	Occasionally preys on ants.	[99]
Oecobiidae			
Oecobius annulipes Lucas	O	Main food is *Plagiolepis pygmaea* (Latreille) but other ants (e.g., *Lasius flavus* (Fabricius)) accepted in lab. Bites at base of antenna. Swaths ant in silk and encircles it. Sometimes uses last pair of legs as well as spinnerets to direct silk over prey. Reduced chelicerae and enlarged gnathocoxae may be adaptations to myrmecophagic lifestyle.	[100]
O. cellariorum (Dugès)	O	Feeds on *Plagiolepis pygmaea* (Latreille). Bites at base of antenna.	[100]
O. templi O. P.-Cambridge	O		[100]
Oonopidae			
Triaeris stenaspis Simon (publ. as *T. patellaris*)	Unk	Reported attacking *Cyphomyrmex costatus* Mann.	[101]
Oxyopidae			
Oxyopes apollo Brady	Unk	Eats ants.	[102]
O. globifer Simon	I/F	Ants constitute large % of prey.	[99, 102]
O. licenti Schenkel	Unk	Eats ants.	[102]
O. salticus Hentz	Unk	Eats ants.	[102]
O. scalaris Hentz	I	Occasionally eats ants.	[99, 102]
O. sertatus L. Koch	Unk	Eats ants.	[102]
Peucetia viridans (Hentz)	Unk	Eats ants.	[103]
Pholcidae			
Crossopriza lyoni (Blackwall) (publ. as *Crossopriza stridulans*)	Unk	Feeds on fire ants, *Solenopsis invicta* Buren.	[104]
Salticidae			
Aelurillus aeruginosus (Simon), *A. cognatus* (O. P.-Cambridge), and *A. kochi* Roewer	F	Prefer ants over other prey. Innately recognize ants even if ants are not moving. Attack from front unless ant is passing (then switch to rear attack). Use different hunting behavior for ants than for other prey. If hungry, show no preference for ants over other prey.	[105]

Table 2: Continued.

Spider myrmecophage	Category of myrmecophage*	Notes on biology	References
Aelurillus m-nigrum Kulczyński	F	Prefers ants over other prey; 85% of diet in field consists of ants. Uses different hunting behaviors for ants than for other prey: lunges, attacks from front, bites, releases, bites again.	[92]
Aelurillus spp.	F	Species in genus prefer ants over other prey. Use different hunting behaviors for ants than for other prey.	[106]
Anasaitis canosa (Walckenaer) (publ. as *Corythalia canosa* or as *Stoidis aurata*)	F	Prefers ants over other prey. Uses different hunting behaviors for ants than for other prey: attacks from front, holds forelegs away from struggling ant. Also stilts body off ground.	[107, 108]
Anasaitis spp.	F	Species in genus prefer ants over other prey. Use different hunting behaviors for ants than for other prey.	[106]
Chalcotropis spp.	F	Use different hunting behaviors for ants than for other prey: some attack from rear, some head-on, then lunge, bite, release, and wait.	[106, 109]
Chrysilla lauta Thorell	F	Prefers ants. Uses different hunting behaviors for ants than for other prey: attacks from rear, bites gaster (not appendages), retreats and waits, may lunge and strike several times. When ant quiescent, spider approaches, bites again, and carries it away.	[110]
Chrysilla spp.	F	Species in genus prefer ants over other prey. Use different hunting behaviors for ants than for other prey.	[106]
Cosmophasis sp.	Unk	Feeds on ants and is myrmecomorph.	[59]
Euophyrs spp.	F	Use different hunting behaviors for ants than for other prey: some attack from rear, some attack head-on, then lunge, bite, release, and wait.	[106]
Evarcha albaria (L. Koch)	I/F	Robs ants of their prey and of their brood (eggs and larvae) that workers carry (kleptoparasites).	[111]
Habrocestum pulex (Hentz)	Some F Some I	Some individuals prefer ants over other prey; some prefer other prey over ants. Myrmecophagic individuals use different behaviors for ants than for other prey: lunge or leap onto petiole or thorax, bite, release, repeat (up to 6 times). Keep front legs off ground away from ant. Reported preying on *Crematogaster* spp.	[112–114]
Habrocestum spp.	F	Species in genus prefer ants over other prey. Use different hunting behaviors for ants than other prey.	[106]
Hasarius adansoni (Audouin)	Probably I	Will feed on ants.	[115]
Hentzia palmarum (Hentz) (publ. as *Eris marginata*)	Unk	Reported feeding on workers of *Myrmica* sp.	[113]
Icius sp.	Unk	Reported feeding on small brown ants.	[113]
Menemerus fulvus (L. Koch) (publ. as *Menemerus confuses*)	I/F	Robs ants of their prey and of their brood (eggs and larvae) that workers carry (kleptoparasites).	[111]
Myrmarachne foenisex Simon	F	Regularly feeds on weaver ant (*Oecophylla*) larvae. Also mimics weaver ants.	[59]
Natta horizontalis Karsch (publ. as *Cyllobelus rufopictus*)	F	Prefer ants. Uses different hunting behaviors for ants than for other prey: attacks from rear, bites gaster (not appendages), retreats, and waits, may lunge and strike several times. When ant quiescent, spider approaches, bites again, and carries it away.	[110]
Natta spp.	F	Species in genus generally prefer ants. Use different hunting behaviors for ants than for other prey: attack from rear, bite gaster (not appendages), retreat and wait, may lunge and strike several times. When ant quiescent, spider approaches, bites again, and carries it away.	[106, 110]

Table 2: Continued.

Spider myrmecophage	Category of myrmecophage*	Notes on biology	References
Phidippus johnsoni (Peckham and Peckham)	I	Occasionally eats ants.	[99, 116]
Plexippus setipes Karsch	I/F	Robs ants of their prey and of their brood (eggs and larvae) that workers carry (kleptoparasites).	[111]
Siler cupreus Simon (publ. as *Silerella vittata*)	F/O	Eats ants. Spider population increases in areas infested with Argentine ants, *Linepithema humile* (Mayr). Also robs worker ants of brood including eggs, larvae, and pupae being carried by workers (kleptoparasitism).	[117–120]
Siler semiglaucus (Simon)	F	Prefer ants. Uses different hunting behaviors for ants than for other prey; bites gaster (not appendages), retreats and waits, may lunge and strike several times. When ant quiescent, spider approaches, bites again, and carries it away.	[110]
Siler spp.	F	Use different hunting behaviors for ants than for other prey: some attack from rear, some from head-on, lunge, bite, release and wait.	[106, 109]
Tutelina formicaria (Emerton)	F	Also myrmecomorph. Preys on red and black ants.	[121]
Tutelina similis (Banks)	F	Preys primarily on ants and is also a myrmecomorph. Uses different hunting behaviors for ants than for other prey: bites quickly, releases, retreats, carries paralyzed prey to safe area.	[99, 113]
Tutelina spp.	F	Other species of *Tutelina* found on mound of *Pogonomyrmex salinus* Olsen (publ. as *P. owyheei*) feeding on worker ants.	[113]
Xenocytaea spp.	F	Species in genus prefer ants over other prey. Use different hunting behaviors for ants than other prey.	[106]
Zenodorus durvillei (Walckenaer), *Z. metallescens* (L. Koch), and *Z. orbiculatus* (Keyserling)	F	Prefer ants over other prey. Feed on ants caught in other spider's webs—but only if spiders can approach safely without getting caught. Ambush ants; hang upside down and lunge at ant while releasing dragline. Repeatedly bite larger ants. Do not hold onto injured ant.	[106, 108]
Zenodorus spp.	F	Species in genus prefer ants over other prey. Use different hunting behaviors for ants than other prey.	[106]
Scytodidae			
Scytodes sp.	Unk	Feeds on fire ants, *Solenopsis invicta* Buren.	[104]
Theridiidae			
Achaearanea spp.	Unk	Feed on "carpenter ants." Ants become entangled in gum footed sticky thread attached to substrate. Movement of ant causes thread to snap and ant is lifted off ground.	[93]
Argyrodes sp.	Unk	Reported feeding on *Pogonomyrmex rugosus* Emery.	[93]
Asagena fulva (Keyserling) (publ. as *Steatoda fulva*) and *A. pulcher* (Keyserling) (publ. as *S. pulcher*)	Unk	Feed on *Pogonomyrmex badius* (Latreille) and *P. subnitidus* Emery. When ant workers captured in webs, major workers (patrollers) may attempt to free them but become caught in webs themselves.	[93, 122]
Cryptachaea riparia (Blackwall) (publ. as *Theridion saxatile* and as *Acaeoraneae riparia*)	F	Captures ants with above-ground web that has sticky threads attached to substrate. Webs built in areas of high ant activity or traffic. Greater than 88% of diet made up of ants (mostly *Formica* spp.). Ant gets tangled in sticky silk, struggling causes line to snap, ant is suspended, spider responds to vibrations, bites ant several times in legs and antennae while wrapping in silk, cuts paralyzed ant, and carries it to sand-covered tube retreat.	[123, 124]
Dipoena punctisparsa Yaginuma	Unk	Feeds on small ants in genus *Lasius*.	[125]

Table 2: Continued.

Spider myrmecophage	Category of myrmecophage*	Notes on biology	References
Enoplognatha ovata (Clerck) (publ. as *Theridion lineatum* or *T. lineamentum*)	Unk	Feeds on *Pogonomyrmex barbatus* (Smith). Builds webs in grass near colony. Ants crawling up into grass or passing below get entangled.	[126]
Euryopis californica Banks	I/F	Reported feeding on *Pogonomyrmex rugosus* Emery.	[93]
Euryopis coki Levi	I/F	Preys on *Pogonomyrmex salinus* Olsen (publ. as *P. owyheei*). Spider captures ant on the mound by trapping ant against ground with sticky silk. Bites on leg. Ant swings off ground on thread. When paralyzed, spider drags it away using a web sling attached to the ant and to the spinnerets.	[127]
Euryopis episinoides (Walckenaer) (publ. as *E. acuminata*)	I/F	Feeds on ants. Attacks *Crematogaster* ants and transports each attached to spinnerets.	[128]
Euryopis formosa Banks	I/F	Captures and carries workers of *Pogonomyrmex salinus* Olsen. Carries ant across ground. One attack described: spider bit gaster, released ant, moved to front and waited, reapproached paralyzed ant, climbed onto ant and began dragging across ant nest using web sling.	[129]
Euryopis funebris (Hentz)	F/O	Reported feeding on *Camponotus castaneus* (Latreille). Throws adhesive silk over ant passing by on tree trunk and fastens it to tree. Encircles ant, throwing silk. Bites leg. Cuts paralyzed ant free and carries it to crack or crevice or drops on line to feed.	[130, 131]
Euryopis scriptipes Banks	I/F	Feeds on ants.	[132]
Euryopis texana Banks	I/F	Female reported preying upon moving line of small ants.	[133]
Other *Euryopis* spp.	I/F	Prey on ants. Throw adhesive silk over ants and fasten to trees.	[131–133]
Latrodectus corallinus Abalos	Unk		[93, 134]
Latrodectus hesperus Chamberlin and Ivie	Probably I	Feeds on *Pogonomyrmex rugosus* Emery. Builds web on colony mound over foraging trail. Spider throws silk on ant that gets caught in gum threads. Spider approaches ant from above, bites posterior femur, retreats, returns after ant paralyzed, and pulls ant to retreat or to hidden part of web. Also feeds on other species of ants.	[93]
Latrodectus mactans (Fabricius)	I/F	75% of prey in cotton fields in Texas made up of fire ants, *Solenopsis invicta* Buren. Also reported feeding on *Pogonomyrmex badius* (Latreille) and *P. barbatus*.	[89, 126, 135]
Latrodectus mirabilis (Holmberg)	Unk	Feeds on *Acromyrmex* spp. and *Camponotus* spp. Builds webs over colony entrances.	[93, 134]
Latrodectus pallidus O. P.-Cambridge	F	Primary prey are ants. Feeds on *Monomorium semirufus* (*nomen dubium*, but probably *Messor semirufus* (André)). Females build webs over foraging trails. Capture ants from above with trip line attached to substrate and pull prey into retreat. Spiders can also descend to ground and catch ants running on trails.	[136–138]
L. quartus Abalos	Unk	Feeds on *Acromyrmex* spp. and *Camponotus* spp. Builds webs over colony entrances.	[93, 134]
Latrodectus revivensis Shulov	Unk	Remains of *Messor* sp. found in webs.	[136]
Latrodectus tredecimguttatus (Rossi)	Unk	Remains of *Messor* sp. found in webs.	[136, 137]
Latrodectus spp.	Unk	Members of genus may generally be myrmecophages. Reported feeding on *Monomorium* sp. and *Messor semirufus* (André).	[136–138]
Parasteatoda tepidariorum (C. L. Koch) (publ. as *Achaearanea tepidariorum*)	Unk	Feeds on fire ants, *Solenopsis invicta* Buren.	[107]

Table 2: Continued.

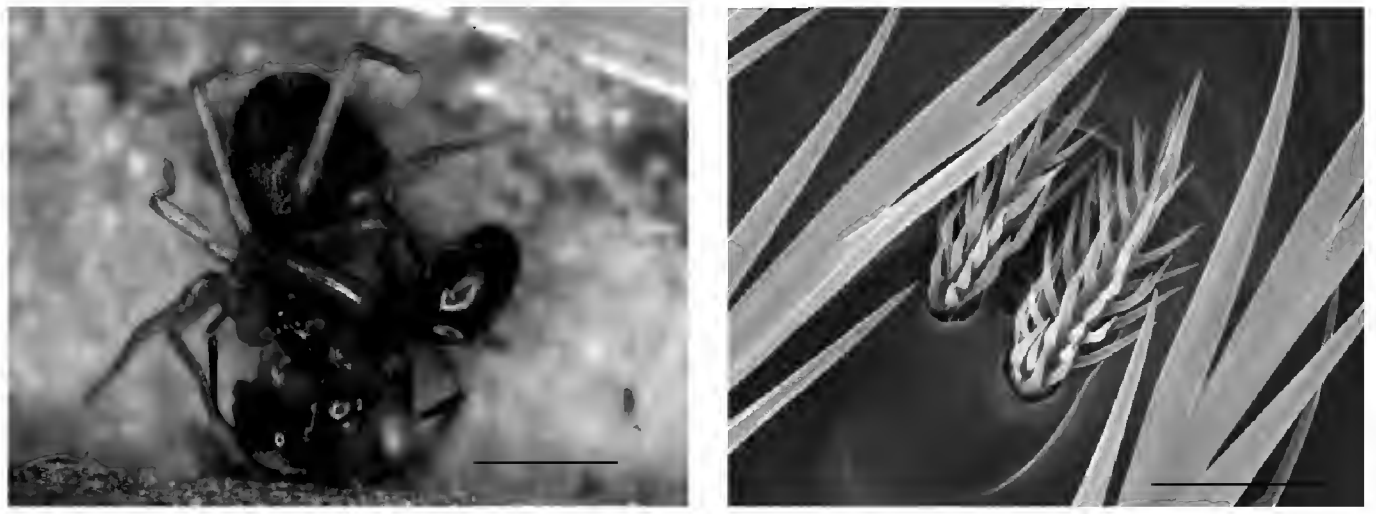
Spider myrmecophage	Category of myrmecophage*	Notes on biology	References
Phycosoma mustelinum (Simon) (publ. as *Dipoena mustelina*)	Unk	Captures various species of ants of wide range of sizes.	[125]
Steatoda albomaculata (De Geer)	I	Feeds on ants; ant remains found in webs.	[139]
Steatoda fulva (Keyserling)	I/F	Reported building webs near nest entrance of colonies of *Pogonomyrmex badius* (Latreille).	[122]
S. triangulosa (Walckenaer)	I	Feeds on fire ants, *Solenopsis invicta* Buren.	[104]
Yaginumena castrata (Bösenberg and Strand) (publ. as *Dipoena castrata*)	Unk	Mostly feeds upon *Camponotus* sp. and *Lasius* sp. and most individual spiders feed upon single type of prey. The larger the spider, the larger the ant it can attack.	[125]
Thomisidae			
Amyciaea albomaculata (O. P.-Cambridge)	O	Myrmecomorph of *Oecophylla smaragdina* (Fabricius) (publ. as *O. virescens*). Adult spiders with eye spots on abdomen. Juvs. yellow and mimic other species of yellow ants (transformational mimics). Spider waits near foraging trail of ant, attacks from behind, bites back of body, drags paralyzed ant to edge of vegetation, drops down to feed.	[140]
Aphantochilus rogersi O. P.-Cambridge (publ. as *Cryptoceroides cryptocerophagum*)	O	Also a myrmecomorph of *Cephalotes pusillus* (Klug) (publ. as *Zacryptocerus pusillus*). Attacks from behind. Holds dead ant as "protective shield." Females oviposit near ant nest and defend egg sacs against worker ants.	[141–143]
Aphantochilus spp.	Unk	Feed on cephalotine ants.	[57, 141–143]
Bucranium spp.	Unk	Feed on cephalotine ants. Hold dead ants as protective shield against attacks from other ants.	[57, 141–143]
Mecaphesa californica (Banks) (publ. as *Misumenops californicus*)	Unk	Feeds on *Pogonomyrmex rugosus* in vegetation near ant nests.	[93]
Mecaphesa coloradensis (Gertsch) (publ. as *Misumenops coloradensis*)	Unk	Feeds on alate females of *Pogonomyrmex maricopa* Wheeler and *P. desertorum* Wheeler after they have removed their wings and while resting on bushes waiting for temperatures to drop in order to dig new nest chambers.	[144]
Mecaphesa lepida (Thorell) (publ. as *Misumenops lepidus*)	I	Occasionally feeds on ants.	[99]
Misumenops argenteus (Rinaldi)	Probably I	17% of prey are ants; mostly ants that get caught in trichomes of plant *Trichogoniopsis adenantha* (OC), where spider spends most of its time.	[145]
Runcinioides argenteus Mello-Leitão (publ. as *Misumenops argenteus*)	Unk	Includes ants in diet.	[146]
Saccodomus formivorus Rainbow	May be F or O	Builds a basket-like web that appears to attract wandering *Iridomyrmex* ants. Spider also uses behavioral tactics-tapping ant with its own legs before attacking.	[4, 147]
Thomisus onustus Walckenaer	I	42.8% of diet consists of ants.	[147]
Tmarus stoltzmanni Keyserling	O	Feeds exclusively on ants; but only those without stings such as dolichoderine and formicine ants. Uses frontal attacks. May have sensory structures on 1st or 2nd pair of legs to detect chemical or tactile cues from ants.	[148]
Other *Tmarus* sp. (from Australia)	Unk	Includes ants in diet.	[148, 149]
Xysticus californicus Keyserling	Unk	Attacks harvester ants in California (cites unpubl. work of Snelling).	[148, 149]
X. loeffleri Roewer	R	Ants comprise only a minor part of diet.	[150]
Other *Xysticus* spp.	I/F	30–35% of diet of some spp. of *Xysticus* comprised of ants. One spider seen preying on *Pogonomyrmex salinus* Olsen. Spider seen on back of ant where it rode around, biting ant until paralyzed. Spider bit at base of petiole.	[129, 150]

Table 2: Continued.

Spider myrmecophage	Category of myrmecophage*	Notes on biology	References
Zodariidae			
Diores spp.	Probably F or O	Feed on ants.	[151]
Habronestes bradleyi (O. P.-Cambridge)	O	Spider also myrmecomorph. Waves front legs around when hunting ants. When legs are amputated, spider has difficult time locating prey (*Iridomyrmex purpureus* (Smith)).	[152–154]
Lachesana insensibilis Jocqué	I	Polyphagous but will eat ants smaller than themselves. Uses different hunting behaviors for ants than for other prey: bites, releases, re-approaches, bites again.	[155]
Lachesana tarabaevi Zonstein *and Ovtchinnikov*	F	Preys mostly on harvester ants in genus *Messor* and on isopods.	[156]
Pax islamita (Simon)	I	Polyphagous but will eat ants smaller than themselves. Uses different hunting behaviors for ants than for other prey: bites, releases, re-approaches, bites again.	[155]
Trygetus sexoculatus (O. P.-Cambridge)	O	Paralysis latency longer for male and juvenile attacks than for female attacks.	[157]
Trygetus spp.	O	Paralysis latency longer for male and juvenile attacks than for female attacks.	[155, 157]
Zodariellum asiaticum (Tyschchenko)	O	Specializes on formicine ants. Attacks other kinds of ants readily but there is shorter paralysis latency for formicine ants suggesting biochemical specificity of venom for certain kinds of ants.	[155]
Zodariellum spp.	Probably all O	Feed on ants.	[155]
Zodarion cyrenaicum Denis	O	Shows cooperative foraging behavior. But some individuals steal prey from others (kleptoparasitism). Paralysis latency longer for male and juvenile attacks than for female attacks.	[157–159]
Zodarion frenatum (Simon)	O	Feeds on *Cataglyphis bicolor* (Fabricius). Locates nests at night (maybe via odor cues?). Sometimes builds retreats near nest. Digs open closed nest entrances, which triggers ants to come out and repair. Spider sometimes enters nest. Bites ant's legs and carries paralyzed ant away from nest. Also kills ants in morning when they emerge from nest.	[158, 160, 161]
Zodarion germanicum (C. L. Koch)	O	Myrmecomorph as well as myrmecophage. Waves 1st legs as antennal illusion. Holds dead ant in chelicerae and presents dead ant to approaching live ant while "antennating" live ant with its own forelegs. Presumably presenting both odor and tactile cues to living ant to deceive it and avoid attack. Attacks *Cataglyphis bicolor* (Fabricius).	[162, 163]
Zodarion jozefienae Bosmans	O	Females and juveniles actively hunt ants. Mature males are kleptoparasites on females' prey (spend energy on mate searching, not prey capture). Sexual size dimorphism (females larger).	[161, 164, 165]
Zodarion lutipes (O. P.-Cambridge)	O	Paralysis latency longer for male and juvenile attacks than for female attacks.	[157]
Zodarion nitidum (Audouin)	O	Paralysis latency longer for male and juvenile attacks than for female attacks.	[157]
Zodarion rubidum Simon	O	Myrmecomorph as well as myrmecophage. Waves 1st legs as antennal illusion. Holds dead ant in chelicerae and presents dead ant to approaching live ant while "antennating" live ant with its own forelegs. Presumably presenting both odor and tactile cues to living ant to deceive it and avoid attack.	[163, 166–168]

Table 2: Continued.

Spider myrmecophage	Category of myrmecophage*	Notes on biology	References
Zodarion spp.	O	All species obligate myrmecophages. Species also imperfect myrmecomorphs. Documented hunting various species. Do not survive well on non-ant diet. Seem to be behaviorally adapted to hunt ants and seem to have evolved nutritional limitations (non-ant prey do not provide required nutrients). Attack from rear, bite legs, retreat, may repeat, re-approach, pick up, and carry away paralyzed ants. Move front legs while hunting. Have femoral organ that may secrete chemical involved in prey capture.	[49, 98, 151, 157, 158, 160, 161, 166, 168–174]



(a) (b)

Figure 3: Myrmecophagy in spiders. (a) *Zodarion rubidum* Simon eating an ant. (b) Femoral organ on *Z. rubidum*. Note the pore openings in the chitin between the two specialized setae of the femoral organ. Scale bar in (a) = 1 mm, scale bar in (b) = 10 μm. Photo of spider © author, SEM of femoral organ © Catherine Tuell, used by permission.

suggesting that the spider may have organs on its front legs that pick up chemical cues from ants [152, 153]. When these spiders detect chemical cues left by ants, they adopt prey capture posture and behavior [153]. Zodariid spiders in the genus *Zodarion* have a structure on the dorsolateral distal tip of the first femora called the femoral organ (Figure 3(b)). The organ consists of pores surrounded by specialized setae with secretory cells beneath the cuticle [171]. It is hypothesized that the femoral organ may release chemicals that somehow subdue the ants upon which the spiders prey (the setae may facilitate dispersion of the secretion) [171]. *Zodarion rubidum* Simon (and other species in the genus) move their front legs around while moving through the environment, similar to the antennal illusion of myrmecomorphs. The spiders seem to use the legs (perhaps via the femoral organs) to pick up cues about ants and conspecifics the spiders may encounter [166]. Recent work by Pekár and Jiroš [177] tested whether various species of myrmecomorphs including one myrmecophage, *Zodarion alacre* (Simon), were also chemical mimics of ants. They found little overlap in the chemical signature of the spiders and ants. Only a weak similarity in profiles was seen for the myrmecophage. The authors hypothesized that the femoral organ of *Zodarion* may be used to synthesize the compounds responsible for the similarity.

The family that includes the most specialized (stenophagous) myrmecophages is the Zodariidae (Table 2). Plesiomorphic representatives of this family, *Lachesana insensibilis* Jocqué and *Pax islamita* (Simon), are polyphagous but will eat ants and hunt ants differently from other prey [155]. Thus these plesiomorphic representatives of zodariids have behavioral preadaptations for hunting ants [155]. Pekár hypothesized that obligatory myrmecophagy may be a derived behavior because, within the Zodariidae, it is only seen in more recent taxa; primitive representatives of the family seem to be polyphagous [98].

4.3. Specialized Hunting Behaviors of Spider Myrmecophages. The majority of reluctant or indifferent myrmecophages will accept ants in the diet but typically show no specialized hunting behavior for these potentially dangerous predators, whereas the majority of facultative and obligatory myrmecophages have evolved specialized hunting strategies to subdue ants with minimum risk to themselves. It has been pointed out that "*when predators evolve prey-specific capture behaviour for use against dangerous prey, they also tend to evolve distinct preferences for these dangerous prey*" [114, 178]. Hunting dangerous but abundant and/or high quality prey seems to select for behavioral plasticity in hunting behavior [105]. Such behavioral flexibility, or using different hunting strategies depending on the identity of the prey and on the circumstances, is common to both myrmecophagic and araneophagic spiders [11, 105, 179].

Many myrmecophagic spiders, particularly facultative or obligatory predators, live in close proximity to ant colonies, often building their webs directly over nest entrances or foraging trails or establishing retreats close to or adjacent to nest mounds [44, 93, 122, 124, 126, 127, 130, 131, 134, 136–138, 140, 143, 147, 158, 160, 161, 180]. In addition to living in close proximity to their prey, these spiders also show specialized hunting behaviors as predicted for stenophagous predators hunting dangerous prey. Web-building myrmecophages (largely in the family Theridiidae, see Table 2) often build webs directly over ant foraging trails where they extend sticky silk strands down to the substrate. When an ant contacts the sticky strand, the ant is catapulted into the air and into the aboveground portion of the web where the spider waits [4, 93, 124, 127, 136–138, 140]. The spider then typically bites the ant one or more times and, each time, the spider retreats until the ant is paralyzed or moribund [93, 130, 131]. The spider then typically carries the ant to a secluded retreat to feed or may even drop on a line to feed [93, 124, 130, 131, 136–138] (possibly to avoid detection from worker ants that may be attracted to alarm pheromones released by the captured ant). When catching non-ant prey, theridiids and other web building spiders do not typically retreat after biting the prey and may or may not carry the paralyzed prey to a different part of the web.

Non-web-building spiders, such as zodariids and salticids (the other families with large numbers of myrmecophagic species), show similar specialized hunting behaviors when attacking ants. For example, zodariids typically attack quickly from the rear of an ant, bite a leg, retreat, and may repeat this sequence several times until the ant is paralyzed. The spider then lifts the moribund ant and carries it to a secluded place to feed (Table 2 and [98, 158, 162, 167, 168]). It has been suggested that the paralyzed ant is used as a shield and a decoy to protect the zodariid from attacks by living ants; the paralyzed ant provides pheromone cues to a curious worker ant that passes by and may provide tactile cues as well [163, 166, 167]. Additional tactile cues are provided by the zodariid, which holds and waves its first pair of legs in front of its body like antennae [163]. The crab spider, *Aphantochilus rogersi* O. P.-Cambridge (Thomisidae), also uses the paralyzed ant as a shield, presumably protecting it from attacks by living ants [142, 143].

Many salticids lunge, rather than jump, at ant prey, then quickly bite, release, and bite again, each time retreating. Even nonmyrmecomorphic ant-eating salticids hunt ants by lunging. This is quite different from the usual stalk and pounce behavior shown to non-ant prey. Myrmecophagic salticids are much more cautious in their approach of ants and much more deliberate in where they bite the prey; some nearly always position themselves in front of the ant and bite the petiole or thorax [92, 105, 106, 108, 109, 114]. Others nearly always attack ants from the rear, lunging at the gaster (not the appendages), but always retreating and waiting until the ant is quiescent before carrying it away [99, 106, 109, 110]. Many salticids keep their front legs extended off the ground when attacking an ant, away from the ant's mandibles [108, 113]. The salticids do not show these behaviors when hunting non-ant prey. The salticids, *Zenodorus durvillei* (Walckenaer), *Z. metallescens* (L. Koch), and *Z. orbiculatus* (Keyserling), are all facultative myrmecophages that feed on ants caught in other spiders' webs, but only if there is a safe way to capture these prey [106]. These species of *Zenodorus* will walk across a line of detritus to the captured ant or will even hang upside down above the ant and lunge at the prey caught in the web [106]. Some spider myrmecophages, particularly *Callilepis nocturna* (L) (Gnaphosidae), and species of *Oecobius* (Oecobiidae) aim for the ant's antenna when hunting then retreat and wait as is seen in nearly all other species of myrmecophages [96, 97, 100].

4.4. Nutritional Costs of Myrmecophagy and a Stenophagous Diet. It has recently been demonstrated that at least some obligatory myrmecophages do not survive well on an ant-poor diet; some even starve rather than hunt non-ant prey [173]. Thus obligatory myrmecophages show both behavioral limitations (i.e., spiders are reluctant to hunt non-ant prey) and nutritional limitations (i.e., non-ant prey do not provide required nutrients for survival) [173]. In fact, in order to obtain the necessary nutrients for survival, these spiders selectively consume particular parts of the bodies of their ant prey suggesting that "*specialist predators can use a behavioral strategy to balance nutrient intake by selective exploitation of different prey body parts*" [174]. These authors found, for example, that *Zodarion rubidum* preferentially fed on the foreparts of the ant body, which were richer in proteins, than on the gaster, which is higher in lipids but also contains possible toxins such as formic acid. These obligatory myrmecophages may take their specialization a step further by feeding primarily on one or two types of their preferred prey. For example, *Zodarion* species possess more effective venoms against particular groups of ants, such as formicine ants rather than myrmicine ants [151, 157, 170]. *Zodarion germanicum* (C. L. Koch) does better, in terms of growth and survival, on a diet that includes the preferred formicine ants than on a diet restricted to myrmicine ants [172].

5. Discussion

Research on spider myrmecomorphs has demonstrated, unequivocally, that these spiders are Batesian mimics and that the mimicry confers strong adaptive advantages to their survival. Some research has also tested how and why polymorphic and imperfect mimicry evolved. Future research on myrmecomorphic spiders should focus on the costs, trade-offs, and constraints inherent in the evolution of close morphological (and behavioral) resemblance to ants. These factors may have a significant impact on the accuracy of the resemblance. It is also important to identify the selective agents involved in this type of mimetic resemblance since the characteristics of the selective agents (e.g., the visual acuity of the selective agents and whether there is more than one actor in the drama) may explain the phenomena of polymorphic and imperfect mimicry.

Research on spider myrmecophiles has not been extensive in the years since the first review article. Nevertheless, the research that has been carried out, particularly on the species

Gamasomorpha maschwitzi and *Cosmophasis bitaeniata*, is fascinating and demonstrates that the biology of these symbiotic spiders is closely linked to the lifestyle and biology of the host ants. From my earlier review article [5], and from Table 1, it is clear that many more species of myrmecophilic spiders can be studied and details of their biology explored. In the section on spider myrmecophiles, I suggest additional directions for future research such as: What adaptations are involved in colony integration? How do myrmecophiles disperse to neighboring colonies? Do all spider myrmecophiles mimic colony odors? To what extent can myrmecophiles interpret the chemical cues released by the hosts? What is the population structure of spider myrmecophiles (i.e., is the spider population within a single nest made up of close relatives)?

I also provide a summary of what is known about spider myrmecophages and present an extensive table listing all (I hope) records of spider myrmecophages from the literature. Recent research on these specialist predators has revealed the evolutionary costs and benefits of this stenophagous diet. It has also highlighted the extraordinary morphological and behavioral adaptations that have evolved enabling spiders to specialize on such dangerous prey.

Although spiders and ants seem unlikely co-evolutionary partners given ants' territorial aggressiveness and spiders' solitary lifestyles, it is clear that hundreds of species of spiders have evolved close relationships with ants. The information on spider myrmecomorphs, myrmecophiles, and myrmecophages included herein supplements information presented in the 1997 review [5]. The present paper includes the first comprehensive summary of the extensive research on myrmecophagic spiders. In addition, it presents an overview of the research carried out since 1997 that examines the evolutionary costs and benefits of the various spider-ant associations. One of my primary goals has been to provide ideas for new or expanded avenues of research on these fascinating arthropod relationships.

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Research Article

On the Genus *Paragoniastes* Comellini, 1979, with Description of a New Species from Ilhéus, Brazil (Coleoptera, Staphylinidae, Pselaphinae)

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The types of the species of the *Goniacerine* genus of Pselaphinae *Paragoniastes* Comellini are revised. *Paragoniastes parki* Comellini, 1979, is synonymized with *P. besucheti* Comellini, 1979 (*P. besucheti* = *P. parki* syn. nov.), and *P. uesci* Cuccodoro & Kurbatov sp. nov. is described from the Brazilian state of Bahia. These taxa are described, illustrated, and keyed. Additional characters pertaining to the genus are given.

1. Introduction

Members of *Paragoniastes* are small predaceous rove beetles of the pselaphine tribe Goniacerini inhabiting the forest leaf litter. The genus was erected by Comellini to accommodate *Goniastes westwoodi* Raffray, 1890 from "Brésil" and three new species from the southern Brazilian states of Santa Catarina and Parana (*P. besucheti*, *P. parki*, and *P. raffrayi*).

In the frame of a survey of the pselaphine fauna of the Brazilian state of Bahia, we collected several *Paragoniastes* at the campus of Universidade Estadual de Santa Cruz, Ilhéus. Comparison of these specimens with the types of *Paragoniastes* housed in the Muséum d'histoire naturelle, Geneva, indicated not only that they were a new species but also that the holotype and unique specimen of *P. parki* is conspecific with the types of *P. besucheti*. The new species (*P. uesci*) is the first record of the genus in the Brazilian Nordeste.

The species are described and keyed, and their habitus is figured. We present drawings of the aedeagi of all the species of *Paragoniastes*, including that of *P. westwoodi*. We also mention some additional features pertaining to the genus, such as the system of meso- and metasternal foveae, and the conformation of the second visible abdominal sternite.

2. Material and Methods

All the specimens mentioned in this study (167 specimens) have been examined. These are housed in the Muséum d'histoire naturelle (MHNG), Geneva, Switzerland, in the Muséum National d'Histoire Naturelle (MNHN), Paris, France, and in the Universidade Estadual de Santa Cruz (UESC), Ilhéus, Brazil. In the future, the material deposited in UESC will be deposited in the Museu de Zoologia da Universidade de São Paulo (MZSP), Brazil.

The label data of the type of *P. westwoodi* are reproduced literally between " ", with additional information pertaining to labels, or localities between [ ], with | as a separator between each individual label.

Measurements are defined as follows: body length is measured from anterior outline of head (i.e., apical margin of labrum) to apex of abdomen; head width (HW) is the distance between outer outline of head just behind eyes;

head length (HL) is the medial distance between tip of frontoclypeus and occipital margin; pronotal length (PL) is the medial distance between anterior and posterior margins of pronotum; pronotal width (PW) is the maximal distance between lateral outline of pronotum; elytral length (EL) = elytral sutural length; elytral width (EW) is the maximal width of the elytra taken together. Antennal articles are measured in dorsal view, their length axially (without basal stalk), and their width at their maximal width.

The abdominal tergites and sternites are numbered according to Chandler [3] in Arabic (visible position) and Roman (morphological position) numerals; they are counted from tergite 1 (IV) and sternite 1 (III). Terminology of surface sculpturing follows Harris [4]. The aedeagi and other body parts illustrated here were mounted in Canada balsam on acetate slides and drawn using a drawing tube mounted on a compound microscope. The habitus figures are composites taken using a digital camera mounted onto a Leica MZ Apo dissecting microscope and processed using Automontage software.

3. Taxonomy

Key to the species of *Paragoniastes*

(1) (a) Center of pronotum areolate—*P. besucheti* Comellini.
(b) Center of pronotum covered with longitudinal ridges slightly diverging anteriorly—2.

(2) (a) Elytra smooth, without marked discal striae—*P. westwoodi* Raffray.
(b) Elytra scabriculate, with marked discal striae—3.

(3) (a) Elytra lacking humeral stria. Mesofemora bearing posteriorly a conspicuous subbasal tooth-like process—*P. raffrayi* Comellini.
(b) Elytra with humeral stria. Mesofemora bearing posteriorly at most an obsolete subbasal tooth-like process—*P. uesci* sp. Nov.

3.1. Paragoniastes Comellini, 1979.

> *Paragoniastes* Comellini, 1979: 681; type species: *Paragoniastes raffrayi* Comellini (by original designation).

Additional Characters. Habitus as in Figures 1(a), 1(b), 1(c), 1(d), 2(a), and 2(b). Maxillary palpi as in Figure 3(e). Pronotum with shallow medial antebasal fovea and pair of well-marked lateral antebasal depressions (without true lateral antebasal foveae). Elytron with sutural and lateral striae on entire elytral length; usually present is internal discal stria and external discal stria, and occasionally humeral stria; when present, discal and humeral striae evanescent subapically, internal discal and humeral striae reaching basal margin, and external stria evanescent subbasally. Prosternum with pair of lateral procoxal foveae; medial carina absent. Mesosternum (Figure 3(b)) scabriculate, except prepectus smooth; the latter larger than mesosternal shield, medially carinate and laterally concave to allow accommodation of procoxae; pair of lateral foveae in connection with pair of promesocoxal foveae. Mesocoxal cavities separated. Metasternum (Figure 3(b)) scabriculate, markedly delimited from mesosternum; with pair of lateral mesocoxal foveae and medial metasternal fovea. Legs with second tarsomeres slightly shorter than third; single tarsal claw. Abdominal tergites each 1–5 bearing four macrosetae, tergites 1–3 each with medial ridge. Abdominal sternite 1 (Figure 3(d)) very short, visible only on mesal portion; sternite 2 (Figure 3(d)) bearing pair of deep transverse cavities densely covered by pubescence, each with basolateral and mediobasal foveae. Aedeagus with basal bulb of median lobe membranous and two symmetrical parameres.

Comments. Particularly notable in the genus is the structure of the lateral and promesocoxal foveae, which are unusually connected with each other, suggesting that the promesocoxal foveae might result from an extreme bifurcation of the lateral foveae. The membranous basal bulb of the aedeagus can easily collapse and its shape is thus not discriminant. Sexual dimorphism appears to affect only the medial area of the abdominal sternites. The presence of mesofemoral spines in both sexes is particularly notable, as it is usually a sexually dimorphic feature in Pselaphinae.

3.1.1. Paragoniastes raffrayi Comellini, 1979. See Figures 1(a), 1(b), and 3.

> *Paragoniastes raffrayi* Comellini, 1979: 682.

Material examined (holotype and 156 paratypes, all in MHNG): "Brésil, Santa Catarina, Nova Teutonia, F. Plaumann, vi.1972", 1 macrophthalmus male (holotype), 13 microphthalmus males and 12 females; same data, but xii.1967, 3 microphthalmus males and 1 female; same data, but xii.1969, 1 female; same data, but i.1970, 2 females; same data, but ii.1970, 1 macrophthalmus male and 2 females; same data, but v.1970, 1 macrophthalmus male; same data, but iii.1972, 1 female; same data, but x.1972, 1 macrophthalmus male, 2 microphthalmus males and 2 females; same data, but xii.1972, 2 females; same data, but ii.1973, 1 macrophthalmus male and 1 female; same data, but xi.1974, 2 macrophthalmus males and 5 females; same data, but iv.1976, 21 macrophthalmus males, 34 microphthalmus males and 48 females.

Description. Body (Figures 1(a) and 1(b)) 1.75–1.80 mm long. Head 1.2-1.3 times longer that wide (without eyes). Antennae (holotype) with scape 1.1-1.2 times longer than pronotal width; 2nd article somewhat transverse; 3rd 1.7-1.8 times longer than wide, 2.6–2.8 times longer than 2nd and 1.4-1.5 times longer than 4th; 4th 1.1-1.2 times longer than wide; 5th 1.6 times longer than wide and about as long as 3rd. Pronotum as long as wide, covered with longitudinal ridges slightly diverging anteriorly, except posterior quarter scabriculate. Elytra scabriculate, without transverse carinulae. Elytral internal and external discal striae well marked; humeral stria absent, or at most obsolete on basal quarter.

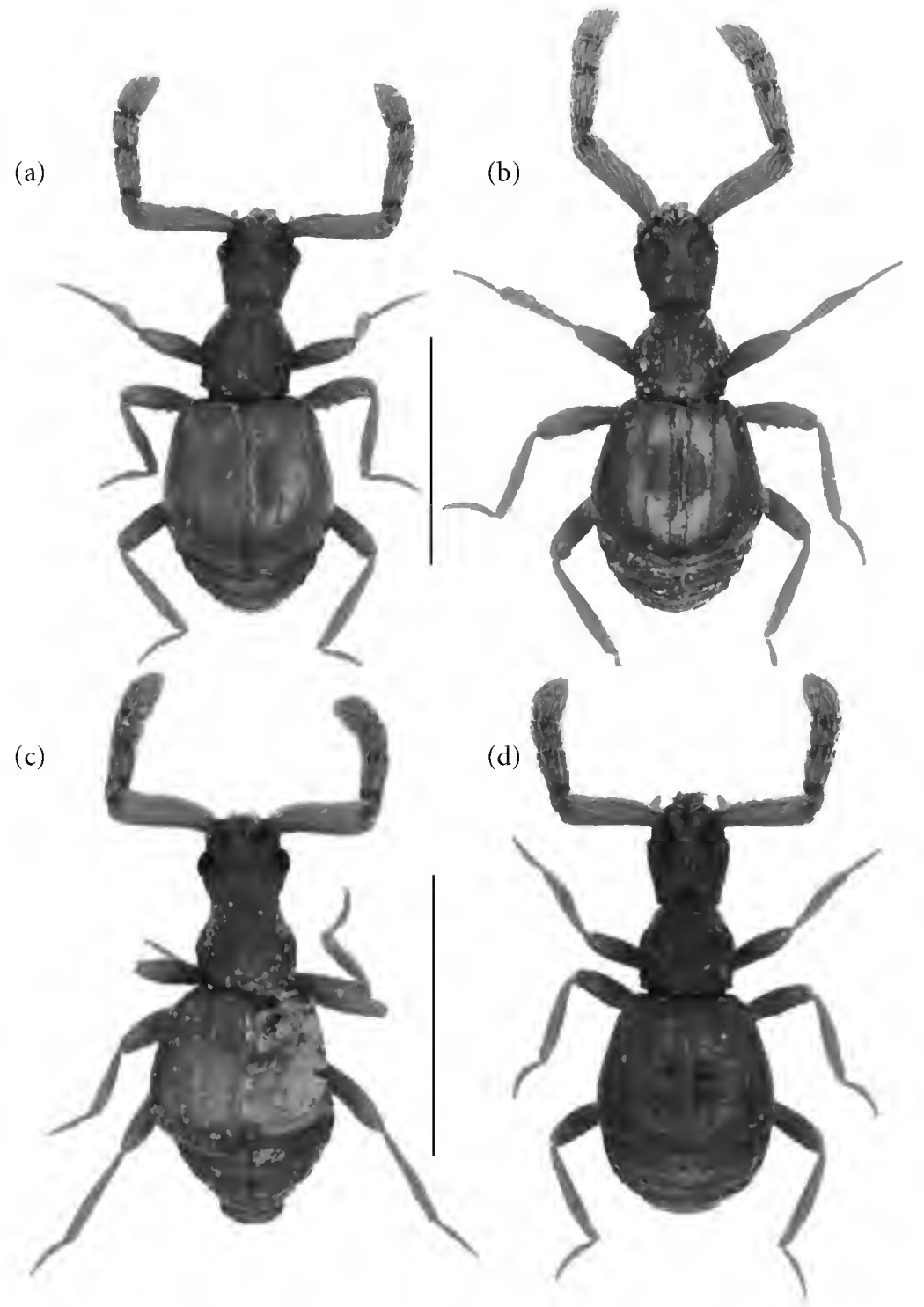

FIGURE 1: (a) and (b) *Paragoniastes raffrayi*, male, macrophthalmus (a) and microphthalmus (b). (c) and (d): *P. besucheti*, male, macrophthalmus ((c): holotype of *P. parki*) and microphthalmus ((d): holotype). Scale bars: 1 mm.

Presence of 13 setae arranged along internal discal striae; strial setae about as long as interval between them, and more than three times longer that interstrial setae, the latter almost indistinct. Mesofemora bearing posteriorly a conspicuous subbasal tooth-like process.

Measurements (holotype): HL = 0.43 mm; HW = 0.34 mm; PL = 0.40 mm; PW = 0.40 mm; EL = 0.71 mm; EW = 0.75 mm.

(i) Male. Eyes of macrophthalmus individuals with 28–30 facets, microphthalmus individuals with 5–8 facets. Abdominal sternites 2–5 medially depressed. Aedeagus (Figures 3(a) and 3(c)) 0.20–0.21 mm long.

(ii) Female. Eyes with 5 facets.

Distribution. *Paragoniastes raffrayi* is apparently restricted to the south Brazilian state of Santa Catarina, where it occurs in sympatry with *P. besucheti*.

Comments. Within the specimens examined, the sex ratio is 50%, and 35% of the males are macrophthalmus. *Paragoniastes raffrayi* is easily distinguished from its congeners by the presence of marked discal striae in combination with the almost completely evanescent humeral stria. The presence of mesofemoral spines in both sexes is particularly notable, as it is usually a sexually dimorphic feature in Pselaphinae.

3.1.2. Paragoniastes besucheti Comellini, 1979. See Figures 1(c), 1(d), 4(a)–4(d).

Paragoniastes besucheti Comellini, 1979: 686.

Paragoniastes parki Comellini, 1979: 685 **syn. nov.**

Material examined (6 specimens, all in MHNG): "Brésil, Santa Catarina, Nova Teutonia, F. Plaumann, xii.1976", 1 microphthalmus male (holotype of *P. besucheti*); same data, but xi.1976, 1 macrophthalmus male (holotype of *P. parki*) and 1 microphthalmus male (paratype of *P. besucheti*); "Brésil, [Parana], Rondon, <24°38′S; 54°07′E> F. Plaumann, iii.1965" 2 microphthalmus males and 1 female (paratypes of *P. besucheti*).

Description. Body (Figures 1(c) and 1(d)) 1.45–1.50 mm long. Head 1.2-1.3 times longer than wide (without eyes).

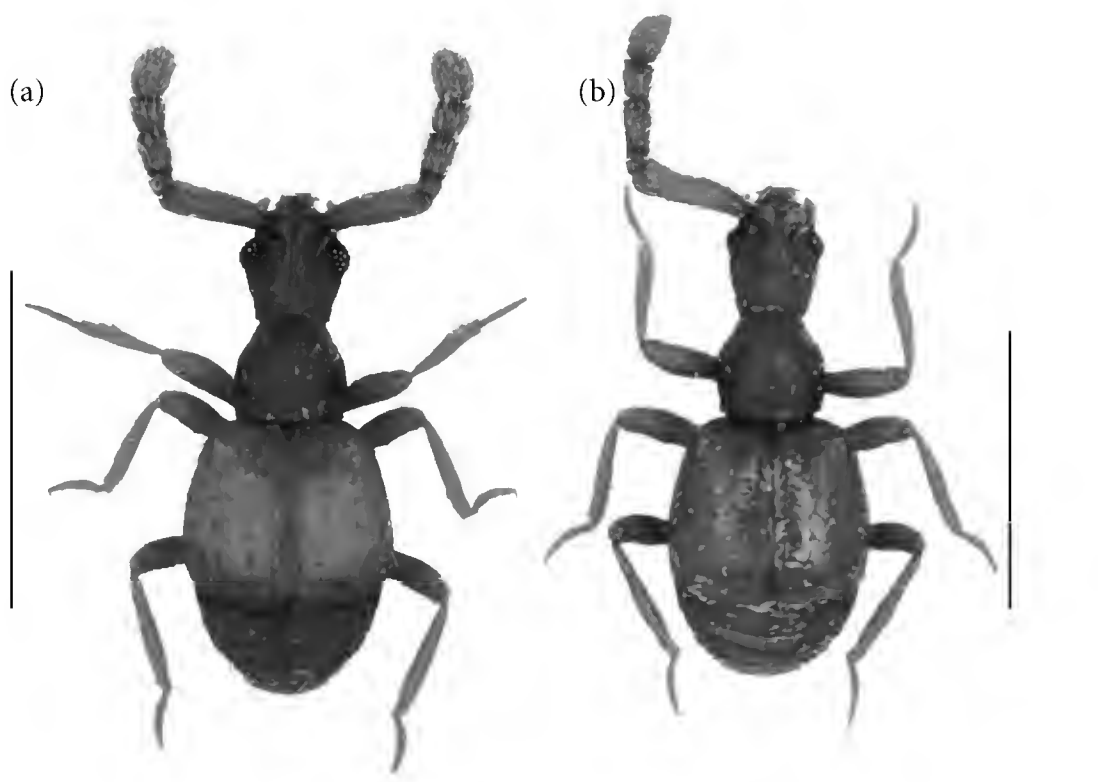

FIGURE 2: (a) *Paragoniastes uesci*, male, macrophthalmus (paratype). (b) *P. westwoodi*, male, macrophthalmus (holotype). Scale bars: 1 mm.

Antennae (holotype) with scape 1.1-1.2 times longer than pronotal width; 2nd article as long as wide; 3rd 1.6-1.7 times longer than wide, 1.8-1.9 times longer than 2nd and 1.3-1.4 times longer than 4th; 4th somewhat longer than wide; 5th 1.6-1.7 times longer than wide and 1.2-1.3 times longer than 3rd. Pronotum slightly wider than long, areolate, except anterior quarter covered with longitudinal ridges slightly diverging anteriorly. Elytra smooth, irregularly covered with transverse carinulae. Elytral internal and external discal striae well-marked; humeral stria present on more that three quarters of elytral length. Presence of 13 setae arranged along internal discal striae; strial setae about as long as interval between them, and about as long as interstrial setae. Mesofemora bearing posteriorly at most obsolete subbasal tooth-like process.

Measurements (holotype): HL = 0.37 mm; HW = 0.30 mm; PL = 0.32 mm; PW = 0.34 mm; EL = 0.54 mm; EW = 0.60 mm.

(i) Male. Eyes of macrophthalmus individuals with 34 facets, microphthalmus individuals with 3–5 facets. Abdominal sternites 2–4 medially depressed, 2-3 each with spinose medial process consisting of few agglomerated setae projecting from small medial tubercle, these tubercles not contiguous. Aedeagus (Figures 4(a), 4(b), 4(c) and 4(d)) 0.27-0.28 mm long.

(ii) Female. Eyes with 4-5 facets.

Distribution. *Paragoniastes besucheti* occurs in the south Brazilian states of Santa Catarina (in sympatry with *P. raffrayi*) and Paraná.

Comments. This species is the only member of the genus to possess an areolate pronotum. Close examination of the holotypes *P. besucheti* and *P. parki* indicated that the apparent differences between Comellini's aedeagal drawings of these two species result from misinterpretation of details and deformations of these structures on the microscope slides where the aedeagi were mounted.

3.1.3. Paragoniastes uesci Cuccodoro & Kurbatov **sp. nov.** See Figures 2(a), 4(e), and 4(f).

Holotype (macrophthalmus male, in UESC): "Brazil, Bahia, Ilhéus, Universidade Estadual de Santa Cruz (UESC), 80 m <14°39′S, 39°10′W> L. Pereira de Oliveira, M. Santana Fonseca & G. Cuccodoro, 31.vii.2011, sifting leaf litter in the forest of the campus".

Paratypes (2): same data as holotype, 1 macrophthalmus male in MHNG and 1 female in UESC.

Description. Body (Figure 2(a)) 1.50 mm long. Head 1.2-1.3 times longer than wide (without eyes). Antennae (holotype) with scape as long as pronotal width; 2nd article as long as wide; 3rd 1.4-1.5 times longer than wide, 1.9-2.0 times longer than 2nd and 1.3-1.4 times longer than 4th; 4th as long as wide; 5th 1.3-1.4 times longer than wide and 1.2-1.3 times longer than 3rd. Pronotum slightly wider than long, entirely covered with longitudinal ridges slightly diverging anteriorly. Elytra scabriculate, irregularly covered with transverse carinulae. Elytral internal and external discal striae well-marked; humeral stria present on more that three quarters of elytral length. Presence of 10 setae arranged along internal discal striae; strial setae about as long as interval between them, and about two times longer than interstrial setae. Mesofemora bearing posteriorly at most an obsolete subbasal tooth-like process.

Measurements (holotype): HL = 0.36 mm; HW = 0.29 mm; PL = 0.32 mm; PW = 0.34 mm; EL = 0.57 mm; EW = 0.62 mm.

(i) Male. Eyes of macrophthalmus individuals with 30–32 facets (microphthalmus individuals unknown). Abdominal sternite 2 with small medioapical tubercle bearing short setae directed forward; sternites 3–5

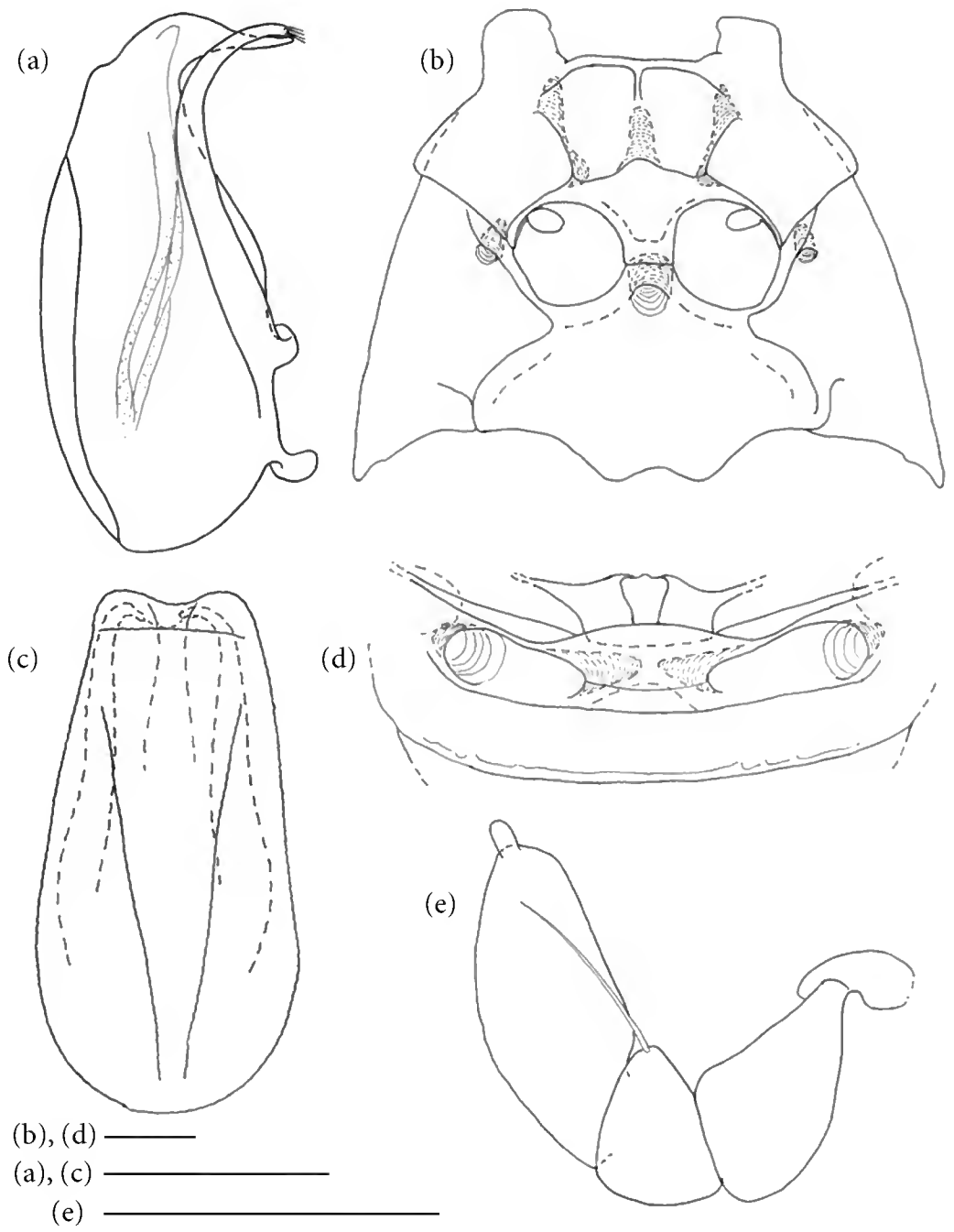

FIGURE 3: *Paragoniastes raffrayi*. (a) and (c): Aedeagus in lateral (a) and dorsal (c) views. (b) Mesosternum and metasternum, female, ventral view, pubescence omitted. (d): Medial area of abdominal sternites 1-2, female, ventral view, pubescence omitted. (e) Left maxillary palpus, female, dorsal view. Scale bars: 0.1 mm.

medially depressed. Aedeagus (Figures 4(e) and 4(f)) 0.25–0.26 mm long.

(ii) Female. Eyes with 1 facet.

Distribution. This species is known so far only from the state of Bahia and represents the first record of the genus in the Brazilian Nordeste.

Comments. Paragoniastes uesci is the only member of the genus with the pronotum entirely covered with longitudinal ridges slightly diverging anteriorly in combination with long and well-marked elytral humeral striae.

Etymology. The epithet *uesci* is an acronym for the Universidade Estadual de Santa Cruz, Ilhéus, campus where the new species was discovered.

3.1.4. Paragoniastes westwoodi (Raffray, 1890). See Figures 2(b), 4(g), and 4(h).

Goniastes westwoodi Raffray, 1890: 209.

Material examined (holotype, macrophthalmus male, in MNHN): "Brésil [handwritten on white rectangular label] | Muséum Paris, 1917/col. A. Raffray [typewritten on green rectangular label] | Type [typewritten on red rectangular label] | *G. westwoodi* [handwritten]/A. Raffray det. [typewritten on white rectangular label] | *Goniastes westw* [handwritten on green rectangular label] | *Goniastes westwoodi* Raffray type | *Paragoniastes westwoodi* (Raffray) type [handwritten on white rectangular label]".

Description. Body (Figure 2(b)) 1.80 mm long. Head 1.4-1.5 times longer than wide (without eyes). Antennae (holotype) with scape 1.1-1.2 times longer than pronotal width; 2nd article as long as wide; 3rd 1.7-1.8 times longer than wide, 1.9-2.0 times longer than 2nd and 1.4-1.5 times longer than 4th; 4th 1.1-1.2 times longer than wide; 5th 1.5-1.6 times longer than wide and 1.1-1.2 times as long as 3rd. Pronotum as long as wide, covered with longitudinal ridges slightly diverging anteriorly, except posterior quarter scabriculate. Elytra smooth, irregularly covered with transverse carinulae. Elytral internal and external discal striae evanescent; humeral stria absent, or at most obsolete on basal quarter. Presence of 13 setae arranged along internal discal striae; strial setae about as long as interval between them, and about as long as interstrial setae. Mesofemora bearing posteriorly at most an obsolete subbasal tooth-like process.

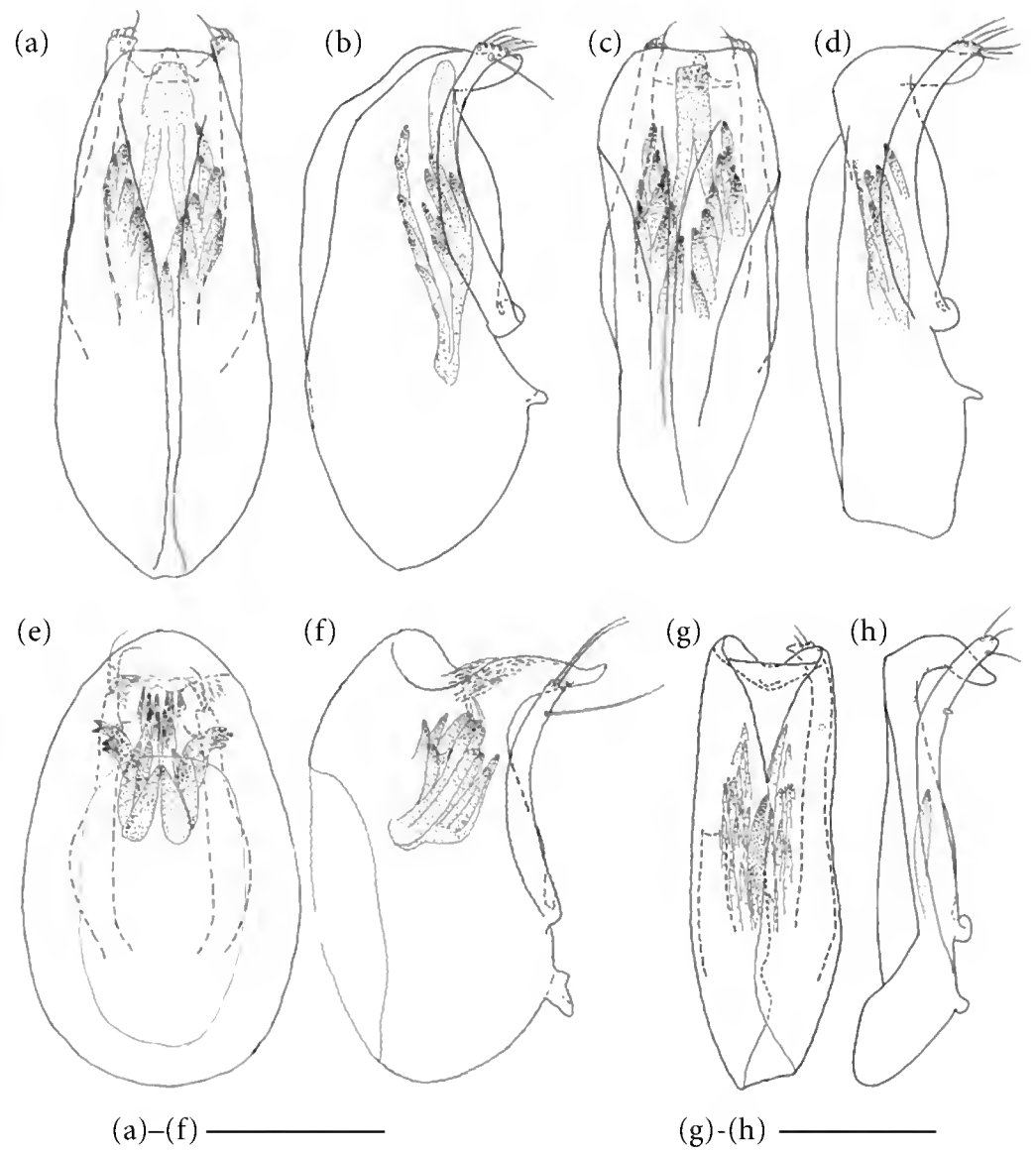

FIGURE 4: (a–b) *Paragoniastes besucheti* ((a) and (b) holotype; (c) and (d) holotype of *P. parki*). (e) and (f) *P. uesci* (holotype). (g) and (h) *P. westwoodi* (holotype, feft paramere broken). Aedeagus in dorsal (a, c, e, and g) and lateral (b, d, f, and h) views. Scale bars: 0.1 mm.

Measurements (holotype): HL = 0.49 mm; HW = 0.34 mm; PL = 0.41 mm; PW = 0.41 mm; EL = 0.72 mm; EW = 0.77 mm.

(i) Male. Eyes of macrophthalmus individual with 34 facets (microphthalmus individuals unknown). Abdominal sternites 2-3 not depressed, each with small medial tubercle bearing short setae directed backward, these tubercles contiguous; sternite 4 medially depressed. Aedeagus (Figures 4(g) and 4(h)) 0.35 mm long.

(ii) Female. Unknown.

Distribution. The only information available on the distribution of this species is that it comes from Brazil.

Comments. Paragoniastes westwoodi is the only member of the genus with evanescent discal striae.

Acknowledgments

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Review Article

Trait-Mediated Indirect Effects of Phorid Flies on Ants

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This paper provides a synthesis of the ecological impact of phorid fly parasitoids on ants. We find the most important impact of phorids on ants to be trait-mediated effects. Phorids diminish the foraging activity of ants, frequently reducing the number and average size of foragers and reducing the amount of food retrieved by a colony. However, ants' coping mechanisms include changing foraging site and time. Phorids can also affect competition, especially through changes in the ability of the host to win in exploitative competition. Factors such as microclimate, resource size, and habitat complexity interact with phorids to change their effect on competition. By being highly specific and attacking ants high in the competitive hierarchy, phorids can alter the linear nature of the competitive transitivity, and by reducing the number of foragers, they can change the discovery-dominance tradeoff that is observed in some ant communities. Trait-mediated effects of phorids also cascade to other trophic levels. As an example, we discuss the trait-mediated cascade of phorids on the *Azteca instabilis* system in coffee. In this system, by reducing the foraging activity of *A. instabilis*, phorids reduce the direct and indirect biological control impact of the ant in the coffee agroecosystem.

1. Introduction

The best-studied family of ant parasitoids, Phoridae, has been recognized as an important mediator of ant community structure [1]. Indeed, over the past decades, there have been many studies on the impacts of phorid attacks on ants, from the effects on ant foraging activity, size of foragers, and amount of food retrieval, to the effects at the community level involving several interacting species at different trophic levels. What is clear from this literature is that the main consequences of phorid attacks on ants are not direct density effects but rather effects mediated by changes in the behavior of ants, the so-called trait-mediated indirect effects (TMIEs) [2]. Although many reviews have been written about TMIE generally [2–4], there has not been a review on how phorid flies impact ant communities through trait-mediated indirect interactions (TMIIs). Since phorid parasitoids attack mainly workers and parasitism rates tend to be very low, their direct impact on the colony is minor [1, 5]. However, attacking phorid flies elicit ant defensive behaviors that can have repercussions at the community level. These trait-mediated effects have been shown to be important for understanding invasibility of ants [6] as well as the role of ants in biological control of agricultural pests [7].

In this paper we provide a synthesis of studies on the ecological impacts of phorids on ant assemblages and ecological networks focusing on TMII. The literature review focuses on studies published over the last ten years, since Feener's review [1]. However, we also use some of the older literature to support our conclusions. We first examine the effects of phorid parasitoids on ant foraging activity, including number of foragers, forager sizes, and amount of food retrieved. We then examine the evidence for the hypothesis that phorid parasitoids alter the outcome of competition among ants. More specifically, we examine evidence for the impact of phorid flies on exploitative and interference competition and for how parasitoids may alter competitive dominances among ant assemblages [1, 8–11]. Finally, we present evidence for trait-mediated effects that transcend ant assemblages and result in changes in the broader community including ant prey, ant mutualists, and

the predators of the mutualists. For this last section, we focus on our own work of *Azteca instabilis* in coffee plantations.

2. Direct Density Effects of Phorid Parasitization on Ants

Studies examining direct mortality due to phorid parasitism on ants have reported very low parasitism rates. For example, *Pseudacteon* parasitism on *Solenopsis geminata* has been reported to be only 3% [12], while *Apocephalus* parasitization on *Pheidole* has been reported to be 5% [13, 14]. Parasitization rates of ants in their introduced ranges can be even lower. Morrison and Porter [5] reported a 0.058% of average parasitism rate per colony of *Solenopsis invicta*, a host ant species of *Pseudacteon tricuspis* Borgmeier in a time-span of two years study, in northern Florida. In his review, Feener [1] also indicated that the effects of density-mediated interaction between phorid parasitoids and host ants are normally very low, with 1-2% as the likely magnitude of the effect of direct phorid parasitism on host ant density reduction. Since most phorid flies that parasitize ants are highly host specific, use ant pheromones to find their host [15], and attack workers while foraging, retrieving food resources, or performing other tasks outside their nest, they are bound to elicit specialized defensive behavior that can result in TMIE [1]. Most of the research on the effects of phorid flies on ants has focused on these trait-mediated interactions between phorids and their ant hosts.

3. Trait-Mediated Effects on Ant Foraging Activity, Resource Acquisition, and Defense

3.1. Number of Foragers. One of the first recognized TMIE of phorids on ants was their effect on foraging activity [16]. Most ant colonies show behavioral responses to attacks by phorids. The most common behavioral response is a reduction in the number of foragers. Most of the studies show that the number of foragers or ants recruited to a resource is reduced significantly in the presence of phorids (Table 1) [16–37]. In the case of *Azteca instabilis*, this activity reduction results from two actions on the part of the individual workers. First, some ants go inside their nest, and second, some ants acquire a defensive posture and stop moving [7]. This defensive stationary posture has also been observed in *S. geminata* [21]. It has been shown that some species of *Pseudacteon* that parasitize *A. instabilis* and *Solenopsis* species require movement of the host in order to oviposit [15, 38]. The combination of these two actions on the part of *A. instabilis* and *S. geminata* workers results in a reduction of 50% of the foraging activity in these two species [21, 30, 31]. Reductions in ant foraging activity in the presence of phorid flies have also been reported for *Linepithema* [32, 39], *Pheidole* [13, 14], and *Atta* [17, 40]. Ants can also respond to phorid attacks by increasing foraging activity during periods of time when phorids are not active, for example, at night. This seems to be the case for several species of the leaf cutter ants in the genus *Atta* [16, 17, 26], for *Linepithema* [39], and for *A. instabilis* (de la Mora, unpublished data).

3.2. Size of Foragers. Phorid parasitoids also influence the size of foragers (Table 1). The pattern of worker size selection by ovipositing female phorid flies has been described for a few species of *Pseudacteon* on *Solenopsis* [12, 41–43], and *Neodohrniphora* on *Atta* [44, 45]. Mathis and Philpott [15] discuss ant size as a factor in host acceptance by phorid species. Differences in host size preferences within the fire ants are seen as an effective niche partitioning when several species attack the same host [36]. However, in general, phorid parasitoids tend to prefer larger than average workers. In these cases, the response of the ants to the presence of phorids is to reduce the average size of the foragers [17, 23, 25–28, 40, 46, 47]. Morrison and Gilbert [43] reported that the size of the emergent phorid was positively related to the size of the host worker with females emerging from a larger host. If ant colonies respond to phorid attacks by changing the size distribution of foragers, this can alter the phorid's sex ratio and can potentially affect the efficiency of phorid parasitoids in biological control of invasive ants [43].

3.3. Acquisition of Food Resources. The reduction in the number and size of foragers can have an effect on the ability of ants to obtain and defend food resources [16, 17, 22–28] (Table 1). Laboratory and field studies have reported up to 50% reduction in food acquisition by *S. invicta* in the presence of phorid flies [21, 23, 40]. In laboratory experiments, Mehdiabadi and Gilbert [22] showed that the presence of only one phorid fly per 200 workers of *S. invicta* reduced the number of large size workers 50 days later. In the same experiment, they demonstrated that the reduction in foraging and size of foraging workers resulted in a nearly twofold reduction of protein colony consumption. Reduction in the amount of food consumed in the presence of phorid flies has been reported for other ant genera including *Linepithema* [24] and *Pheidole* [20]. However, in another laboratory experiment with *S. invicta* and its *Pseudacteon* parasitoids, Morrison [23] showed that in control trials, where no phorids were present, food retrieval was intermediate to that of the phorid-no-phorid trials, suggesting that ants are foraging more in the no-phorid trials (of the phorid-no-phorid trials) to compensate for the reduction in food retrieval from the phorid-present trials. This kind of compensation can happen in the field if the ants forage more during periods of no-phorid activity, as discussed previously, or shift to forage underground when phorids are present, something that has been shown to happen in *Solenopsis* [48]. Furthermore, in a laboratory experiment, Ramirez et al. [49] reported that reduction in food retrieval was not observed when the trials were left running for a period of 72 hours. These experiments suggest that in the long run and under field conditions ants that are attacked by phorid parasitoids compensate for potential losses in the amount of food retrieved by foraging more at times when phorids are not active.

Table 1: Ant response to phorid parasitism.

Research retrieval			
Ant spp.	Phorid spp.	Measured ant response to phorid parasitism	References
Atta cephalotes	*Neodohrniphora curvinervis*	Reduced resource retrieval	[16]
Atta sexdens	*Neodohrniphora* sp.	Reduced number of loaded ants	[17]
Atta sexdens	*Neodohrniphora* sp.	Reduced resource retrieval	[18]
Azteca instabilis	*Pseudacteon* sp.	Increased time for foragers to carry away resource	[19]
Pheidole diversipilosa	*Apocephalus* sp. 8	Increased resource turnover rate by competitor	[20]
Solenopsis geminata	*Pseudacteon browni* and *P. bifidus*	Reduced resource retrieval	[21]
Solenopsis invicta	*Pseudacteon tricuspis*	Reduced colony protein consumption	[22]
Solenopsis invicta	*Pseudacteon tricuspis*	Reduced resource retrieval	[23]
Solenopsis richteri	*Pseudacteon* sp. (multiple)	Reduced number of workers at resource	[24]
Worker size			
Ant spp.	Phorid spp.	Measured ant response to phorid parasitism	References
Atta	*Neodohrniphora erthali*	Increased number of hitchhikers	[25]
Atta cephalotes	*Neodohrniphora curvinervis*	Sent out workers in smaller size	[17]
Atta cephalotes	Unreported	Sent out workers in smaller size	[26]
Atta laevigata	*Apocephalus attophilus*	Sent out workers in smaller size	[27]
Atta sexdens	*Neodohrniphora* sp.	Reduced forager mass	[17]
Solenopsis invicta	*Pseudacteon tricuspis*	Sent out workers in smaller size	[22]
Solenopsis invicta	*Pseudacteon tricuspis*	Sent out workers in smaller size	[23]
Solenopsis invicta	*Pseudacteon* sp.	Altered ratio of worker size	[28]
Solenopsis richteri	*Pseudacteon* sp.	Sent out workers in smaller size	[24]
Ant activity			
Ant spp.	Phorid spp.	Measured ant response to phorid parasitism	References
Atta laevigata	*Apocephalus attophilus*	Altered number of foragers	[27]
Atta sexdens	*Myrmosicarius grandicornis*	Reduced number of loaded ants	[29]
Atta sexdens	*Neodohrniphora* sp.	Increased number of unloaded workers returning to nest & decreased number of loaded workers returning to nest	[17]
Atta sexdens	*Neodohrniphora* sp.	Altered number of foragers	[17]
Azteca instabilis	*Pseudacteon* sp.	Reduced ant activity	[30]
Azteca instabilis	*Pseudacteon* sp.	Reduced number of ants	[31]
Linepithema humile	*Pseudacteon* sp.	Altered number of foragers	[32]
Pheidole titanis	*Pseudacteon* sp.	Reduced ant activity	[14]
Solenopsis invicta	*Pseudacteon tricuspis*	Reduced number of exposed ants	[20]
Solenopsis invicta	*Pseudacteon tricuspis*	Altered recruited ant size	[22]
Solenopsis invicta	*Pseudacteon tricuspis*	Altered forager size	[34]
Solenopsis invicta	*Pseudacteon tricuspis*	Reducers number of ants at baits	[23]
Solenopsis invicta	*Pseudacteon tricuspis*	Reduced number of foragers	[35]
Solenopsis invicta	*Pseudacteon* sp.	Reduced number of ants at baits	[28]
Solenopsis richteri	*Pseudacteon* (multiple spp.)	Reduced number of ants at baits	[24]
Solenopsis richteri	*Pseudacteon* (multiple spp.)	Reduced various ant activity	[24]
Solenopsis saevissima	*Pseudacteon* sp.	Reduced ant activity	[36]
Solenopsis sp.	*Pseudacteon* sp.	Altered number of foragers	[37]

3.4. Compensatory Factors. Other factors can help host species compensate for the negative trait-mediated effects of phorid parasitoids. For example, habitat complexity in the form of leaf litter provides refuge from parasitoids for soldier caste of *Pheidole diversipilosa* and *P. bicarinata* resulting in an increased number of foraging soldiers even in the presence of phorid parasitoids [50]. Habitat complexity, thus, allows these two species to balance foraging success with the

avoidance of parasitism. Likewise, the size and distribution of resources can have similar effects. In a field experiment, Wilkinson and Feener [51] demonstrated that the presence of multiple large resources allows colonies of *P. diversipilosa* to redistribute soldier ants from sites that have phorid flies to sites that do not have phorids, therefore maintaining overall numbers of foraging soldiers at the same levels as found in the absence of phorid parasitoids.

There is a gap in our knowledge about how many ant hosts mitigate the threat of phorid parasitism by altering regimes, altering posture of exposure, or by foraging on other resources. It is possible that we may be overestimating the population level impact of phorid parasitoids on ants by focusing on day time interactions or by not measuring other population level parameters such as density, occupancy, and colony migration.

4. Phorid Parasitoids and Competition within Ant Assemblages

The kinds of behavioral changes described in the previous section can have important consequences for ant community structure. Since phorid parasitoids tend to be highly specific and attack only one or very few species of ants within a community, they can alter the competitive interactions and change ant community structure [1]. This effect can be especially important when the host ant is a competitive dominant species, which in the best-studied cases of ant-phorid interactions they frequently are, probably because dominant ant species are evolutionarily more conspicuous [1].

When phorid parasitoids are present, host species are faced with a tradeoff between defending themselves against parasitism and maximizing their competitive abilities. The outcome of this tradeoff is not always clear. Based on the evidence of the TMIE of phorid parasitoids on host ants, it is tempting to conclude that phorid parasitoids reduce the competitive ability of host species. However, this is not always the case. Indeed, competitive interactions among ants are complex and influenced by a variety of factors, and therefore, a generalized outcome of the effects of phorid parasitoids on ant competitive interactions is highly unlikely. What we see in the literature is a reflection of that complexity.

4.1. Exploitative and Interference Competition. Ants of different species engage in exploitative and interference competition with each other [20, 21, 34, 52–54]. Exploitative competition occurs when the removal of a limiting resource by one species makes it unavailable for other species, while interference competition involves direct aggressive interactions between individuals of different species. The presence of phorid parasitoids has been shown to influence both of these types of competition but this is, in no way, a universal phenomenon. For example, in laboratory experiments, phorid parasitoids were found to increase the exploitative competitive ability of *Forelius mccooki*, a competitor of the host species, *S. invicta*. However, phorids did not affect the direct aggressive interactions between the two species [34]. Furthermore, although the competitor of the host species increased the number of foragers by a factor of two in the presence of phorid flies, that did not translate into higher colony growth. Similar results have been reported in field experiments. A study of the competitive interactions between *S. geminata* and *S. invicta* in the presence of phorid parasitoids of *S. geminata* found that the host species retrieved 50% less food than the nonhost species in the presence of phorid flies [21]. Much the same as in the lab experiment, in the field, phorid parasitoids had no effect on the interspecific aggression between *S. geminata* and *S. invicta* and did not affect the outcome of these interactions at resources. In the case of *A. instabilis*, phorids also seem to influence exploitative but not interference competition [31, 55]. In field experiments, competitors of *A. instabilis* were able to access bait resources 12 times more often in the presence of phorids and were able to take over baits only when phorid parasitoids of *A. instabilis* were present. However, in most cases, *A. instabilis* did not lose competitive interactions with other species [55]. The lack of an effect in the interference competition interactions between host and nonhost species could be due to the behavioral response of the ants engaged in the fight or a change in behavior of the phorid parasitoids. For example, *S. geminata* has been seen to ignore attacking phorids when engaged in fights with *S. invicta* [21]. But, phorid parasitoids have also been observed to lose interest or be distracted by ants that are engaged in active fighting with other ants. Feener [13] presented the first evidence for the TMIE of phorids on interference competition between the host species *Pheidole dentata* and its competitor, *Diplorhoptrum texanum* (referred to as *Solenopsis texana*). It is reported that parasitism by phorids was the factor that most strongly influenced the turnover of resources from *P. diversipilosa* to its competitors [20]. The same study also reports that phorid parasitoids reduce exploitative competitive abilities of *P. diversipilosa* [20]. On the other hand, Orr et al. [53] report that phorid parasitoids seldom influence exploitative competition between two *Linepithema* species and their nonhost competitors in Brazil. This field study joins others that have not been able to detect clear effects of phorids on ant competition [56].

4.2. Factors That Interact with Phorid Parasitoids to Affect Competition. The lack of a clear pattern on the effect of phorid's TMIE on ant competition has to do with the many other factors that are involved in determining the winners and losers of both exploitative and interference competition. Among the potential factors, here we will discuss four, for which there is some evidence in the literature: feedback loops caused by ant chemical pheromones, size and distribution of resources, habitat complexity, and abiotic factors such as temperature and humidity.

Phorid flies are known to use ant kairomones released by their host ant to locate them [15]. When an ant encounters a competitor, it is more likely to release alarm pheromones that can be used by their phorid parasitoids to find them more easily, causing a positive feedback that may result in a higher turnover rate of resources from host species to their competitors [20]. There are at least two cases where these

kinds of positive feedbacks have been documented. The parasitoid *Apocephalus* sp. discovers faster and arrives in greater numbers at recruitment events where its host species, *P. diversipilosa*, is engaged in competitive conflict than to recruitment events where the host is foraging alone or does not experience conflict [20]. Likewise, parasitoids of two species of *Linepithema* arrived significantly faster at resources where the host was with another ant species than when it was alone [39]. Furthermore, the rate at which phorid flies arrive at baits depends on the competitor species present and the type of response it elicits from *Linepithema*. Phorid parasitoids arrived faster at baits when the competitor elicits a chemical response versus baits where the competitor elicits primarily physical aggression [39]. If host ants engaged in direct competition with other species elicit faster and stronger responses from their phorid parasitoids than those that do not encounter competitors, phorids can have an even stronger effect on competition through this positive feedback. Moreover, if the feedback mechanism works for some competitors and not others, as in the case of *Linepithema* and its competitors, the impact of phorids on community structure and colony energetic will depend on these behavioral responses and will be different in different community contexts.

The effects of resource size and distribution and habitat complexity were discussed in the previous section in the context of compensation mechanisms for acquiring resources under the pressure of phorid attacks. These factors can also buffer the impacts of phorids on competitive interactions between host species and nonhost species [10, 20, 50, 51]. For example, habitat complexity, by allowing continued foraging even when phorids are present, can influence the competitive success of the host species [50]. Likewise, widely distributed resources may allow host species to redistribute their foragers to resources not monitored by phorids and continue succeeding in exploitative competition [51]. Recruitment to large resources, on the other hand, could increase the number of phorid attacks but the effect of resource size has not been well explored in the literature. In general, ants that recruit to resources tend to recruit more and larger workers to larger resources [20, 24]. Since phorid parasitoids show a density-dependent response to ants [55, 57], higher numbers of ants at a resource will attract higher numbers of phorid parasitoids. Therefore, a higher proportion of large resources at a particular site could represent a liability for those host species that recruit to large resources, which is the case for most species attacked by phorids. However, if a higher proportion of larger resources also results in greater availability of large resources to hosts, ant hosts would be able to switch to resources not monitored by parasitoids [50].

Temperature and humidity affect not only ants but also phorids [58–61]. These two variables could interact to lead to very different competitive outcomes under varying environmental conditions. For example, parasitoid habitat preferences (see [15]) have been shown to cause major differences on parasitism pressure on host ants and their interactions with competitors [11]. In laboratory experiments, Ramirez et al. [49] demonstrated that changes in humidity interact with the presence of phorid parasitoids to alter the competitive outcome of encounters between the invasive *S. invicta* and the native species *S. xyloni*. They attributed the lack of establishment and spread of *S. invicta* in New Mexico to these interactions.

4.3. Competitive Dominance Hierarchies and Species Coexistence. Interspecific competition can have profound effects on the abundance, composition, and distribution of species. Communities structured by competition can be organized in a variety of ways that can greatly influence species coexistence and, therefore, the maintenance of diversity within a community. Competitive communities that are organized in a linear transitive dominance hierarchy will tend to have low species diversity because, at equilibrium, the competitive dominant species will exclude all others. On the other hand, intransitive hierarchies, a situation in which the competing species cannot be ranked in a perfect competitive hierarchy, can promote diversity [62–67]. Interspecific competition has been identified as an important factor in structuring ant communities, especially among ground foraging omnivorous ants that forage more or less for the same resources [52, 68–76]. However, to date, no competitive intransitivity has been convincingly demonstrated for any ant community. Rather, ant communities have been described to be organized in transitive dominance hierarchies [10, 11, 52, 69, 77, 78]. A question then emerges as to how ant communities are able to maintain species diversity under conditions of transitive dominant hierarchies. TMIE mediated by phorid parasitoids can provide a partial answer to this question [10, 13, 20], although other factors such as environmental variation [56, 75, 79] and size of resources [10, 51, 80, 81] have called into question the generality of the transitive dominance hierarchies among ant communities.

By being highly specific and attacking ants that tend to be high in the competitive hierarchy, phorids can alter the linear nature of the competitive transitivity. In a study of the ant community in pine-oak woodlands in Arizona, LeBrun [10] describes several distinct dominance hierarchies within the ant assemblage. However, the linearity of the dominance hierarchies was determined by the size of the resource and the presence of phorid parasitoids. When competing for fixed resources or for small nonfixed resources in the absence of phorids, the assemblage exhibits significant linear dominance hierarchies. In contrast, in the presence of phorids for both fixed and small resources, this linearity breaks down [10]. For example, on fixed resources, phorids caused the second dominant species to drop to the second most subordinate, and the third species dropped to the forth position. These changes in the ranking of species dominance generated more indeterminacy in the outcome of individual paired interactions reducing the asymmetries underlying the dominance in the transitive hierarchy. It has been shown, at least theoretically, that when interactions take place locally, which is the case for ants competing for food resources, an increase in symmetry favors diversity [66], providing a potential mechanism for the maintenance of diversity in ant communities.

An alternative competitive structure to the dominant hierarchy that has been described for ant assemblages is the so-called dominance-discovery tradeoff [11, 68, 69, 72, 82–87]. The tradeoff implies that while some species are good at dominating resources, others are good at discovering the resource [54, 69, 74, 83, 85]. This tradeoff can also lead to species coexistence in much the same way that the competition-colonization tradeoff [88] and the virulence-transmission tradeoff [89] lead to species coexistence [10, 90]. Phorid-induced TMIE can play an important role in maintaining species within the dominance-discovery tradeoff curve, therefore promoting diversity [85]. For example, examining the same ant assemblage in pine-oak woodlands in Arizona, LeBrun and Feener found that the elimination of phorid parasitoids caused host species to become too dominant for their level of discovery ability, breaking down the dominance-discovery tradeoff. It has been proposed that this could be a mechanism for the spread of invasive ant species in areas where they are released from parasitoid pressure [1, 85]. However, examining the competitive interactions among an assemblage of ground foraging ants and *S. invicta* in its native range, Feener et al. [11] did not find strong support for this hypothesis. Rather, they proposed that both interspecific competition and TMIE of phorid parasitoids affect the success of *S. invicta* in its native range, but that these factors vary dramatically in different regions.

Overall, these studies suggest that phorid parasitoids can have a strong effect on competitive interactions within ant assemblages but these effects are, in no way, consistent and are mediated by many other factors that vary in time and space, such as microclimate, habitat heterogeneity, and size and abundance of resources.

5. Trait-Mediated Cascades: The Case of *Azteca instabilis* in the Coffee Agroecosystem

Ants are an important component of ecosystems in most regions of the world. Since they frequently constitute a great part of the animal biomass in ecosystems, are taxonomically diverse, and act as ecosystem engineers [91–93], they tend to interact with many other organisms. Given the strong TMIE of phorids on ants, and given the wide range of ecological interactions that ants form with other organisms, it should come as no surprise that these TMIEs cascade into other trophic levels of an ecological community. The best-documented case of these sorts of phorid-mediated cascading effects can be found in the *A. instabilis* system. For more than ten years, we have been studying the ecological interactions surrounding this ant species in coffee plantations in southern Mexico (for a review see [7]). Here we will describe the pivotal role that phorid parasitoids play in shaping these interactions.

Azteca instabilis is a dominant arboreal ant with a wide distribution in the Americas, from Brazil to Mexico [94]. On coffee plantations it is found nesting in shade trees and foraging on both shade trees and coffee plants. This species forms spatial clusters of nests that have a high genetic relatedness (Remfert, unpublished data). The clusters appear to be the result of self-organization emerging from the internal dynamics of the system—short distance dispersal to adjacent trees and density-dependent mortality [95]. One of the main resources for *A. instabilis* in the coffee plantations is honeydew from *Coccus viridis*, the green coffee scale [96]. This mutualism plays an essential role in the distribution of the scale insect, which is a potential pest in coffee [96]. *Azteca instabilis* has been reported to prey on a variety of herbivores in coffee plantations contributing to the control of potential insects pests [7, 19, 96–100]. The effect of *A. instabilis* on deterring herbivores is not only through the direct action of preying or removing herbivores from plants, but also through an indirect effect in which some herbivores avoid plants that have been foraged on by *A. instabilis*, but were no actual ants where present when the herbivores arrive [99]. Additionally, it has been shown that through a complex network of ecological interactions *A. instabilis* is a keystone species that contributes to the regulation of insect pests and diseases in coffee [7, 101–103]. *Azteca instabilis* also competes with other arboreal ant species, especially twig-nesting species [104], and influences the abundance and diversity of ground nesting and arboreal ant species (Perfecto and Vandermeer, in review; Ennis, unpublished data) and spiders (Marin, unpublished data). The mutualism between *A. instabilis* and the scale insects consists of protection of scales from parasitoids and predators, especially the coccinellid beetle, *Azya orbigera* [30, 101], and removal of sooty mold (Jha et al., in review).

Philpott et al. [19] published the first documented case of a phorid parasitoid attacking *A. instabilis*. At that time it was thought that only one species of *Pseudacteon* was responsible for the attacks. However, recently (see [105] in this issue) three species have been described attacking *A. instabilis*. *Pseudacteon* spp. have strong TMIE on *A. instabilis* [19, 31, 55], as reported previously. More importantly, these TMIEs cascade to other trophic levels within the community, with important implications for the biological control of insect herbivores and diseases of coffee [7, 101].

By reducing *A. instabilis* foraging activity, phorids disrupt the ability of the ants to remove insect pests from coffee [19]. In laboratory experiments, it was shown that phorids essentially cancel the ability of *A. instabilis* to deter coffee berry borer attacks on coffee fruits [100]. Likewise, we have demonstrated that the presence of phorids reduces the ability of ants to attack, carry away, and force off plants lepidopteran caterpillars that could be potential pests in coffee [19].

Higher-order cascading trait-mediated indirect effects have also been documented for this system [7, 30, 101]. The protection that the ants offer to their scale mutualist is the first level trait-mediated indirect effect-the ants disrupt the ability of the predatory beetle to kill and consume scale insects. By causing a reduction in the foraging activity of *A. instabilis*, phorids disrupt the ability of the ant to protect its mutualist, the green coffee scale [30]. This is the second order trait mediated indirect effect (Figure 1). When phorids are present, they essentially cancel the protective effect of ants against adults of *A. orbigera*, the coccinellid predator. In laboratory experiments, in the presence of ants and phorids

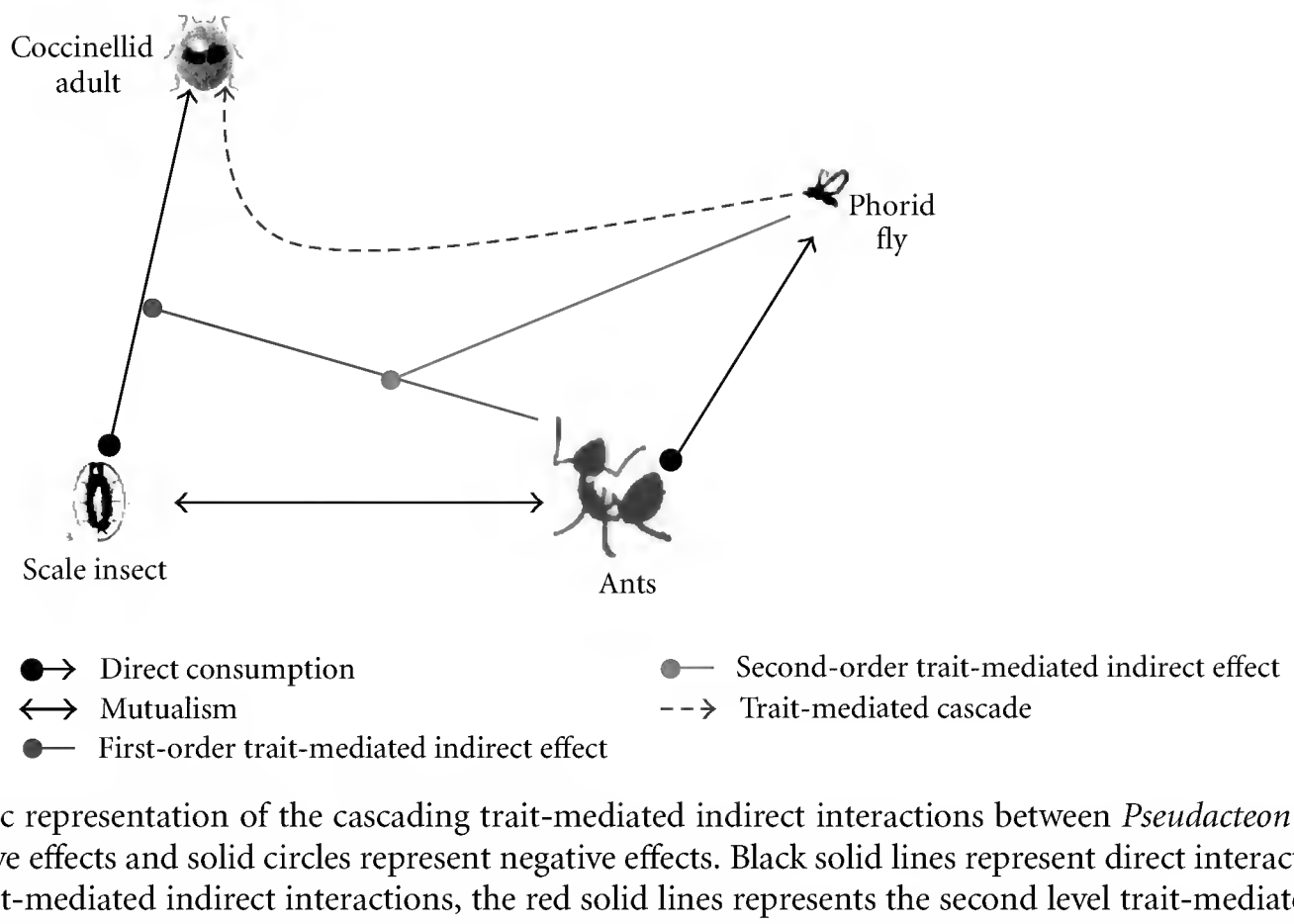

FIGURE 1: Diagrammatic representation of the cascading trait-mediated indirect interactions between *Pseudacteon* spp. and *Azya orbigera*. Arrows represent positive effects and solid circles represent negative effects. Black solid lines represent direct interactions, the blue solid line represents first level trait-mediated indirect interactions, the red solid lines represents the second level trait-mediated indirect interactions, and the dashed purple line represents the resulting cascading trait-mediated indirect interaction between the phorid flies and the coccinellid beetle.

adults of the predatory beetle were able to gain access to the scale and consume the same quantities as when no ants (and no phorids) were present. In other words, through these higher order cascading trait mediated indirect effects, the phorids facilitate the coccinellid beetle (Figure 1).

The complexity of this network of interactions increases when the larval stage of the coccinellid beetle is considered. The larva of *A. orbigera* is covered by waxy filaments that protects it from ant predation [106]. This means that larvae of the main predator of the scales are able to live in patches of high ant activity where the scale is abundant. Furthermore, the ants repel parasitoids in the vicinity of the scale insects, including any parasitoids of the coccinellid beetle, essentially protecting coccinellid larvae [106]. The presence of phorids could, potentially, eliminate this unintended protective effect of the ants on the coccinellid larvae, by reducing ant patrolling on clusters of scales. However, this interaction has not been yet documented.

Our research also shows that gravid female beetles of *A. orbigera* are able to eavesdrop on the "phorid-alert pheromones" (Hsieh, unpublished data) and oviposit under green coffee scales or other clandestine microsites that workers of *A. instabilis* and natural enemies of *A. orbigera* would have difficulty finding, removing, and predating. The natural history and interactions between *Pseudacteon* spp., *A. instabilis,* and *A. orbigera* can well explain why we can find high abundances of *A. orbigera* in the coffee agroecosystem. Since this is the main predator of the green coffee-scale, and it seems to require patches of *A. instabilis* for the successful development of its larvae, it can be argued that the maintenance of the *Azteca*-green coffee scale mutualism is essential for the successful biological control of the green scale at the level of the entire farm [7, 95].

Theoretically and empirically, parasitism in spatially distinct patches has been suggested to be an important driver of spatial self-organization of host-parasitoid dynamics [107]. The *Azteca* system in the coffee agroecosystem adds empirical evidence to the theory of spatial self-organization in host-parasitoid systems. We proposed that *Pseudacteon spp.* contributes to the spatial pattern formation of *A. instabilis* by acting as a density-dependent control mechanism [95]. Given the fact that the coccinellid beetle is able to capitalize on the trait mediated interaction between *Pseudacteon* spp. and *A. instabilis*, we suggest that adding trait-mediated cascades to theoretical models would increase our understanding of how complex systems might contribute to spatial self-organization and system stability. Furthermore, the *A. instabilis-Pseudacteon* spp.-*A. orbigera* system illustrates how trait-mediated cascades effect biological control in a spatially explicit complex ecosystems.

6. Conclusion

Phorid fly parasitoids influence ants mainly through trait-mediated indirect interactions. The presence of phorid flies results in a reduction of foragers, a change in the average size of foragers, mainly toward the smaller sizes, shifts in the time and places of foraging to avoid encounters with phorids, and reduction in the amount of food retrieved. These effects, independently or in combination, have important consequences for the way ants interact with other ant species and with other members of the interacting network within a community.

Through these TMIEs phorids can have important effects on competitive interactions among ants. When phorid parasitoids are present, host species respond behaviorally and

can impact their competitive abilities. However, since competitive interactions among ants are complex and influenced by a variety of factors, the outcome of the effects of phorid parasitoids on ant competitive interactions is highly variable. Phorids have been shown to reduce exploitative competitive abilities of some host species but not others. Likewise, they have been shown to affect the interference competition between host and nonhost, but this effect is not widespread among studies. It has been shown that phorid parasitoids can break a competitive hierarchy within ant assemblages by attacking the most competitive dominant species within the hierarchy. Phorids also can influence the dominance-discovery tradeoff that is found in some ant assemblages. The alteration of the competitive structure of ant assemblages could be important in understanding invasibility of ants to ranges where their phorid parasitoids are absent.

Trait-mediated effects of phorids on ants can also transcend the ant assemblage and have cascading effects on other trophic levels and other organisms linked to the host ant species through complex ecological networks. For example, phorid parasitoids can also influence the impact of ants on herbivores. If the host species is an important predator of an herbivore, the presence of phorids can release these herbivores from predation pressure from ants. This could be important in agroecosystems where ants have been shown to be important predators of insect pests. The study of *A. instabilis* in coffee plantations presents an excellent case study of these cascading trait-mediated indirect interactions and shows that they could be important in maintaining biological control.

Many areas of research remain open in the study of ant-phorid interactions. In particular there are very few studies that link TMIEs of phorids to population level consequences in ants and other organisms. Making and testing predictions regarding the TMIEs of phorids on population density, occupancy, colonization, and migration patterns across landscapes should be priority of future studies. The *Azteca* system described previously represents a step in the right direction to fill this gap in our knowledge of ant-phorid interactions. However, this system is only one example of the many complex ecological networks that could be influenced by phorid parasitoids. Future studies should focus on these kinds of complex ecological networks and on trait-mediated cascading effects that would be important in understanding the role of ants when they are embedded in complex ecological networks.

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Research Article

Diploid Male Production of Two Amazonian *Melipona* Bees (Hymenoptera: Apidae)

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The diploid male has already been recorded for *Melipona* Illger, and herein, in *Melipona seminigra merrillae* Cockerell and *Melipona interrupta manaosensis* Schwarz. This paper was carried out at the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, AM, Brazil. We produced and monitored 31 new colonies of *M. s. merrillae* and 32 new colonies of *M. i. manaosensis*. We sampled 2,995 pupae of *M. s. merrillae* and 2,020 of *M. i. manaosensis*. In colonies with a 1 : 1 sex ratio, male diploidy was confirmed by cytogenetic analysis and workers' behavior. We estimated 16 sex-determining alleles in *M. s. merrillae* and 22 in *M. i. manaosensis*. In colonies of *M. i. manaosensis* in a 1 : 1 sex ratio, workers killed the males and the queen that produced them soon after they emerged, as predicted. This behavior was not registered for *M. s. merrillae*, and sex ratios did not stay 1 : 1, indicating polyandry for this species.

1. Introduction

The haplodiploid mechanism of sex determination, or arrhenotoky, is a characteristic of Hymenopteran insects (ants, bees, wasps, and sawflies) and is widespread in invertebrate orders. It has independently evolved at least 17 times [1, 2]. In this sex determination mechanism, one fertile female (queen) lays fertilized and unfertilized eggs, which develop into diploid females and haploid males, respectively [1–6]. The diversity of sex-determining mechanisms that insects have evolved include heterogamy, haplodiploidy, paternal genome loss, X-chromosome elimination, and complementary sex determination (CSD) [5, 7–9]. Since Whiting [9], diploid males have been described for many arrhenotokous species [10–20]. In these species, the diploid male production (DMP) depends on the allelic composition at the gene *csd* (*complementary sex determiner*) [21, 22]. Under CSD, animals that are hemizygous at the *csd* locus become haploid males, whereas diploid individuals could develop into females or males when they are heterozygous or homozygous, respectively. The production of diploid offspring in a 1 : 1 sex ratio occurs between males and females (queen) that share one allele at the *csd* locus [9, 16, 23, 24]. According to previous studies, when diploid males are viable, they are fully sterile. In many species, these males are killed by workers in the larval phase or soon after they emerge [10, 13, 25–29]. If viable and fertile, diploid males produce diploid sperm and lead to triploid female offspring, which would be a "reproductive-dead end" because these females are sterile [4, 30, 31]. The paradigm of genetic load associated with DMP was not confirmed in some vespids [6, 19, 32].

In colonies of eusocial insects, the negative effect of DMP leads to a loss of half of the worker force per generation [16, 33, 34]. Therefore, these species have evolved high polymorphism at the *csd* locus to avoid the impact of sterile diploid males [29].

In the parasitoid wasp *Habrobracon hebetor* (Braconidae), 9–20 sex-determining alleles were recorded [9, 35, 36].

For *Apis mellifera,* Adams et al. [23] estimated 18.9 sex-determining alleles and Tarpy et al. [37] reported 8–27. For stingless bees, 20 sex-determining alleles were estimated in *Melipona compressipes fasciculata* [13], 24 in *Melipona scutellaris* [16], 22 in *Melipona interrupta manaosensis* (this work), and 16 in *Melipona seminigra merrillae* (this work). In native and introduced populations of the fire ant *Solenopsis invicta,* Ross et al. [38] reported 115–120 sex-determining alleles.

Similar to the self-incompatibility loci in plants, the high polymorphism of the *csd* gene is maintained by a strong selection pressure [22, 23, 39–42]. If k is the effective number of sex alleles in a panmictic population, the probability of a matched mating is 2/k, and the number of diploid individuals that is expected to be male is 1/k [23, 39, 40, 43]. Therefore, in natural populations, the expected frequency of diploid males is low [40], although inbreeding usually results in alterations.

Molecular studies in *Apis mellifera* showed the *csd* gene chromosome localization [44] and isolated and identified this primary signal of sexual development [24]. This gene has not yet been mapped or isolated in stingless bees, but diploid males have been recorded for some *Melipona* species [11, 13, 16, 25].

Since the number of mates increases the genetic variability and so the number of sex-determining alleles, females' mating frequencies, are an important parameter in studies of mating systems. Queens' mating frequencies in both solitary and social Hymenoptera, range from exclusively monandrous (queen mates once) [45–47] to extreme polyandry (queen mates more than six times) [45, 48–52].

Mating frequency in queens of bees is also variable. Most bee species are solitary with queens mating once, which is supported by chemical and ecological studies [53, 54]. However, many solitary bees mate multiply [48]. Thus, more studies on the mating system in the solitary bees are needed [53]. Studies on mating frequency in the genus *Bombus* (bumble bees) showed monandry for most species [55]. In the stingless bees studied so far, single mate seems to be a rule [45, 56, 57]. However, cases of mating with two males or more have been reported [16, 58]. Despite being rare in eusocial Hymenoptera, polyandry has been well documented in ants (genus *Atta*), wasps (genus *Vespula*) and in the advanced eusocial bees (genus *Apis*) [51].The genus *Apis* evolved to extreme polyandry, with mating frequency and effective paternity extremely variable among species and in some cases within the same species [37, 59–61]. The lower levels of polyandry were recorded for *Apis florea* (queens mate with 5–14 males [59]), and the higher levels were recorded for *Apis dorsata* (queens mate with 47–102 males [62]).

Research on the sex-determination mechanism of *M. s. merrillae* and *M. i. manaosensis* was carried out, and diploid males were seen in both species. The genetic diversity was calculated through diploid male frequency. The workers' behavior in colonies producing diploid males (in a 1 : 1 sex ratio) was registered daily. The expected behavior of *Melipona* workers was based on previous studies [11, 13, 16, 25] and was validated in *M. i. manaosensis.* However, the same behavior did not occur in *M. s. merrillae,* according to this study.

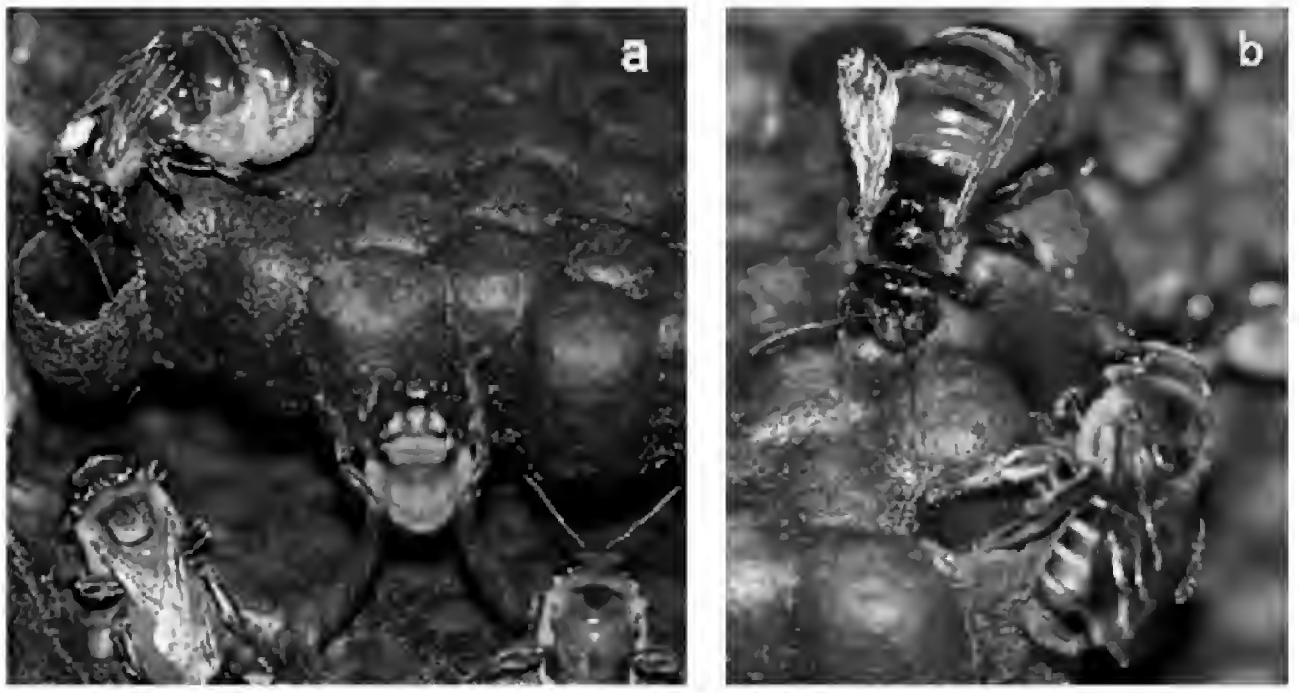
2. Materials and Methods

Thirty-one new colonies of *M. s. merrillae* (Figure 1(a)) and thirty-two new colonies of *M. i. manaosensis* (Figure 1(b)) were produced. This was performed by the reproduction of 63 colonies in excellent conditions from the meliponary of the Grupo de Pesquisas em Abelhas (GPA) of the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, AM, Brazil, during 2007 and 2008.

The manipulated and reproduced colonies were in good condition when they presented large brood combs in different developmental stages, had access to provisions (honey and pollen pots surrounding the brood cells), and there was a massive population of adult workers (Figure 2(a)–(d)) [63]. The original colonies from which the additional colonies were derived were called the "mother" colonies. The bees were reared in standard boxes that facilitated the generation from the "mother" colony. As a result of the formation of this generation, one of the new colonies became an orphan (without a queen bee), which can last for a few days until a new queen is mated and established. The new queens were marked on the pronotum with a white spot of nontoxic ink (Figure 1(a)). After mating confirmation by the first oviposition, the subsequent egg laying was monitored. Each of the 63 new colonies was surveyed 40 days after the start of oviposition. To verify a 1 : 1 sex ratio, combs with 30 to 100 cells from the new queens' first brood were removed from the colony and reared in a temperature-controlled chamber at 28°C to complete the development into pupa e. From these, 2,995 individual pupae of *M. s. merrillae* and 2,020 of *M. i. manaosensis* were sampled. Pupae of males and females were quantified to estimate the sex ratio. Data analysis was focused on matched mating or crosses that produced offspring in a 1 : 1 sex ratio. The goodness-of-fit was performed by a G-test [64]. The polymorphism at the sex-determining locus was estimated by Laidlaw's equation [16, 65] [$n = 2M(N + 1)/(H + 1)$], where n = sex allele number, N = sampled colonies number, H = number of colonies that segregate diploid male, and M = number of males that fertilized the queen. According to the technique described by Imai et al. [66], Francini et al. [67], in each colony with a 1 : 1 sex ratio, we carried out cytogenetic analysis of 20–30 males to confirm male diploidy and just 1-2% of the males analyzed cytogenetically were not diploid. In these colonies, the workers' behavior was observed daily and photo documented. We also randomly sampled colonies with other than a 1 : 1 sex ratio to perform cytogenetic analysis of males.

3. Results

Data of sex segregation and cytogenetic analysis confirmed diploid male production in both species (Table 1). Three of thirty-one *M. s. merrillae* colonies monitored presented offspring in a 1 : 1 sex ratio, while the same was verified in two of the thirty-two colonies of *M. i. manaosensis.* In these colonies, most males analyzed cytogenetically showed a diploid number of $2n = 18$ in *M. i. manaosensis* and $2n = 22$ in *M. s. merrillae.* We also observed diploid males in colonies with other than a 1 : 1 sex ratio. Assuming monandry (queen



(a) (b)

FIGURE 1: *Melipona seminigra merrillae* (a) showing the queen marked on the pronotum and workers showing the characteristic color of the scutellum in this subspecies; *Melipona interrupta manaosensis* (b) queen and worker characteristic colorations.

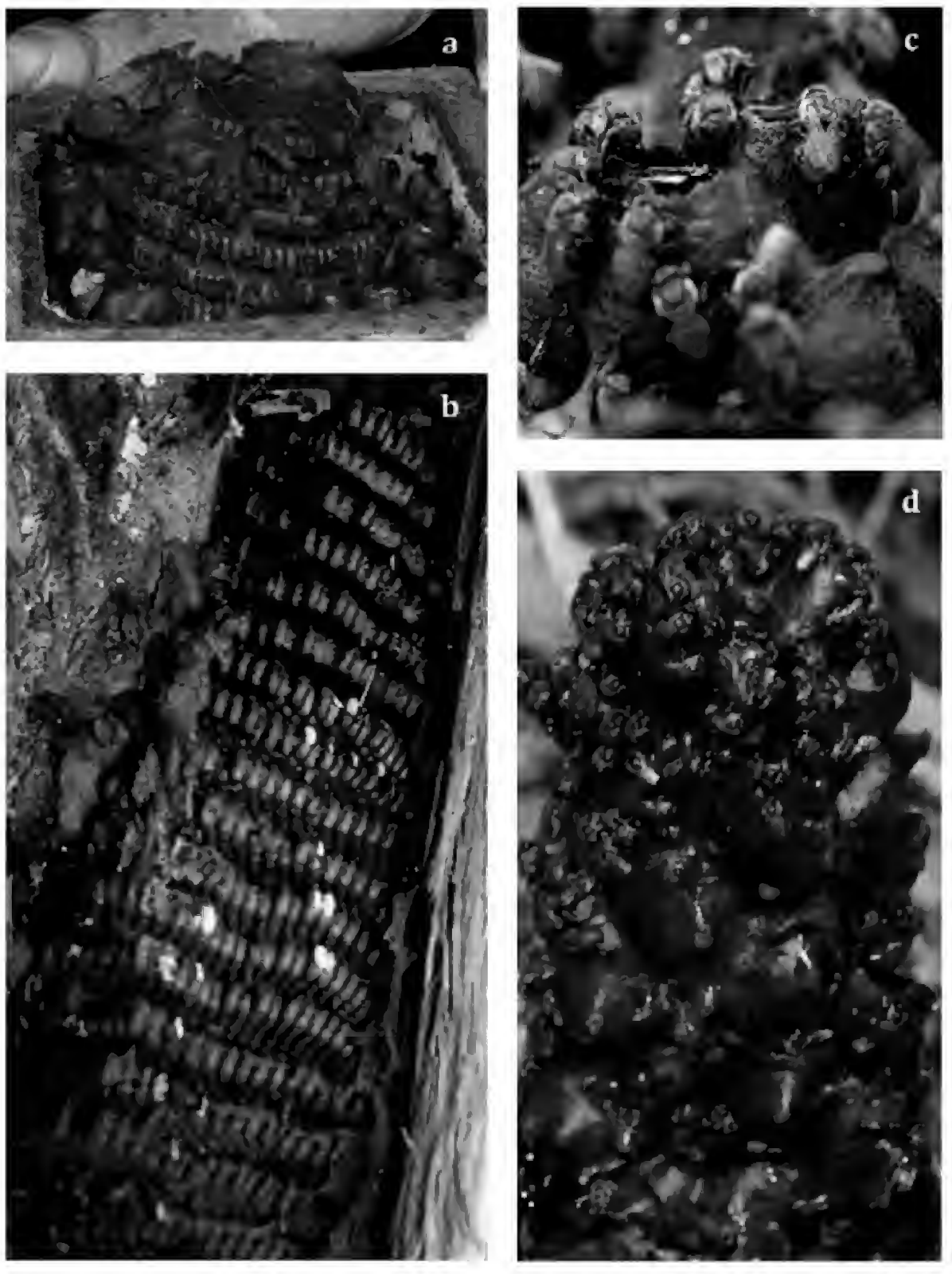


FIGURE 2: Colony conditions. (a) Colony in standard box, with large brood combs and surrounding pollen-pots; (b) colony inside tree-trunk with many brood combs, the age of developmental stage increases from upper to down, well evidenced by the darker color of combs in initial development; (c) workers of *Melipona seminigra merrillae* in honey-pots; (d) honey and pollen pots provision of the tree-trunk colony showed in (b) and many workers feeding on.

Table 1: Sex segregation of *Melipona seminigra merrillae* (colonies MSM) and *M. interrupta manaosensis* (colonies MIM).

Colonies MSM	♂	♀	Sex ratio	*G*-test	Colonies MIM	♂	♀	Sex ratio	*G*-test
01	06	89	0.06	S	01	00	74	0.00	S
02	00	74	0.00	S	02	00	36	0.00	S
03	07	55	0.11	S	03	00	63	0.00	S
04	09	78	0.10	S	04	00	31	0.00	S
05	00	60	0.00	S	05	00	53	0.00	S
06	00	63	0.00	S	06	00	69	0.00	S
07	14	55	0.20	S	07	00	81	0.00	S
08	00	69	0.00	S	08	08	32	0.20	S
09	06	82	0.07	S	09	05	78	0.06	S
10	13	21	0.38	S	⟹10	39	56	0.41	**NS**
11	09	33	0.21	S	11	00	65	0.00	S
12	07	46	0.13	S	12	00	84	0.00	S
13	00	52	0.00	S	13	01	64	0.02	S
14	27	66	0.29	S	14	00	57	0.00	S
15	00	81	0.00	S	15	01	62	0.02	S
16	87	00	1.00	S	16	13	59	0.18	S
17	00	70	0.00	S	17	11	51	0.18	S
18	35	76	0.31	S	18	00	88	0.00	S
19	04	55	0.07	S	19	03	66	0.04	S
⟹20	53	54	0.49	**NS**	20	00	78	0.00	S
21	03	80	0.04	S	21	00	46	0,00	S
22	16	43	0.27	S	22	03	57	0.05	S
23	16	84	0.16	S	23	00	53	0.00	S
24	86	13	0.87	S	24	00	58	0.00	S
25	03	56	0.05	S	25	00	64	0.00	S
26	22	61	0.26	S	26	00	51	0.00	S
⟹27	39	54	0.42	**NS**	27	00	57	0.00	S
28	77	52	0.60	S	28	01	62	0.02	S
⟹29	26	32	0.45	**NS**	29	00	48	0.00	S
30	3	97	0.03	S	30	00	63	0.00	S
31	12	48	0.20	S	31	00	79	0.00	S
					⟹ 32	21	29	0.42	**NS**

* Null hypothesis, sex ratio 1 : 1; *G*-test, critical values ($G = 3.841$; DF = 1; $P = 0.95$ and $\alpha = 0.05$); *S*: significant; NS: nonsignificant; ⟹Colonies with a 1 : 1 sex ratio.

mates once) as predicted for Meliponini queens [16, 63], we estimated 16 sex-determining alleles in *M. s. merrillae* and 22 in *M. i. manaosensis*.

In *M. i. manaosensis*, the workers' behavior in colonies with a 1 : 1 sex ratio confirmed what was predicted for the *Melipona* genus [11, 13, 16]: workers killed both the diploid males (Figure 3(a)) and the queen mother (Figure 3(b)) that produced them as soon as the diploid males emerged. However, this behavior was not observed in *M. s. merrillae*.

To verify that the queen continued to produce diploid males, we sampled the second and the third brood combs in addition to the first in colonies that had a 1 : 1 sex ratio in the first brood comb. In *M. s. merrillae* colonies with a 1 : 1 sex ratio in the first brood, we found that this ratio was not maintained in the subsequent combs. We recorded a deviation in this ratio in the second and the third brood comb, both female biased and male biased (Table 2).

4. Discussion

The frequency of diploid male for the majority of Hymenoptera studied is an indicator of genetic diversity and its loss [18]. This is a parameter that should be highlighted in stingless bees, both for the sake of conservation and for beekeeping as an economic alternative [16, 42, 68] for the Amazon people. The viability of diploid males was described previously for three *Melipona* species. In all cases, the workers killed their diploid brothers and the queen mothers that produced them [11, 13, 16], as also documented here for *M. i. manaosensis* (Figure 3). We did not observe workers of *M. s. merrillae* killing their diploid brothers or their mothers. Thus, in *M. s. merrillae* the workers behavior in colonies that produce diploid males seems to contradict that recorded previously for *Melipona*.

Data indicated that the queens of *M. s. merrillae* had mated with two or more males. The deviation from a 1 : 1

(a) (b)

FIGURE 3: *Melipona interrupta manaosensis,* the workers' behavior in colonies with a 1 : 1 sex ratio. Workers are attacking a male (a) and killing the queen (b) in the same colony at the same time.

TABLE 2: Variation of the sex ratio in colonies of *Melipona seminigra merrillae*. Queens that produced diploid males (a 1 : 1 sex ratio in the first brood comb) did not maintain this ratio in the second and third brood combs.

Colony	♂	♀	Sex ratio	*G*-test
20/D1	53	54	0.49	NS
20/D2	28	15	0.65	S
20/D3	19	99	0.16	S
27/D1	39	54	0.42	NS
27/D2	44	36	0.55	NS
27/D3	17	41	0.29	S
29/D1	26	32	0.45	NS
29/D2	33	65	0.34	S
29/D3	03	75	0.04	S

* Null hypothesis, in a 1 : 1 sex ratio; *G*-test, critical values ($G = 3.841$; DF = 1; $P = 0.95$ and $\alpha = 0.05$); *S*: significant; NS: nonsignificant; D1, first brood comb; D2, second brood comb; D3, third brood comb.

sex ratio along the different brood combs of the same queen (Table 2) seems to be evidence of polyandry [1, 4]. Additionally, we observed diploid males in colonies with other than a 1 : 1 sex ratio, which corroborated data in Table 1. Polyandry increases genetic variability, which is advantageous in a complementary sex-determination system [69]. An increase in mating frequencies leads the queen to produce diploid males at a frequency of $1/n$ of the population in the condition of panmixia [70]. Thus, polyandry can explain the variation in the sex ratio of the brood combs from the same queen, observed here in *M. s. merrillae*. Polyandry may be a good strategy evolved by *M. s. merrillae* to avoid the costs of the DMP [11, 13, 16]. This behavior is probably unique to *Melipona,* but more studies are necessary.

Despite the evidence here, the number sex-determining alleles was estimated under the assumption that queens are monandrous, according to the available information on *Melipona* [63]. Using Laidlaw's equation, estimates will be even higher with polyandry, so our estimates are likely low for *M. s. merrillae*.

Among the problems of the conservation of native bee fauna in Latin America, there is a need for basic information on taxonomy, genetics, ecology, and reproduction biology [70, 71]. The results presented herein should contribute to maintain local biodiversity associated with pollination by wild bees [68]. This work is an effort toward filling the lack of indispensable knowledge for native bee conservation in the Neotropics, especially in the Amazon Basin.

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Research Article

Contribution to the Knowledge of the Genus *Linda* Thomson, 1864 (Part I), with the Description of *Linda* (*Linda*) *subatricornis* n. sp. from China (Coleoptera, Cerambycidae, Lamiinae)

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Linda (Linda) subatricornis n. sp is described from Sichuan (holotype locality), Fujian, Shaanxi, Hebei, Ningxia of China. It is separated from the most similar species *L. atricornis* Pic by differences in genitalia and antennal insertions. Detailed descriptions, photographs of habitus and genitalia, distribution of the two sibling species and short discussion on the related species are presented.

1. Introduction

Linda Thomson, 1864 [1], includes two subgenera, *Linda* and *Dasylinda*, mostly confined to China [2]. While studying more than 150 specimens of *Linda* (*Linda*) *atricornis* Pic from different localities, we were surprised to observe two very different kinds of male genitalia. We concluded that two superficially similar species have been historically misidentified as one species. We had examined the types of *L. atricornis*, *L. gracilicornis*, *L. major* (the three known species of subgenus *Linda* with elytra and antennae all black), and most of the other species of this genus. After careful observation and dissection, we separate *L. subatricornis* n. sp. and herein describe it as new to science.

2. Materials and Methods

Types and other material studied are deposited in the following institutions or private collections.

CCH: Collection of Dr. Carolus Holzschuh, Villach, Austria.

CPS: Collection of Dr. Carlo Pesarini and Dr. Andrea Sabbadini, Milano, Italy.

HBU: Museum of Hebei University, Hebei, China.

IRSNB: Institut royal des Sciences naturelles de Belgique, Bruxelles, Belgique.

IZAS: Institute of Zoology, Chinese Academy of Sciences, Beijing, China.

MHNG: Muséum d'Histoire Naturelle de Genève, Switzerland.

MNHN: Muséum National d'Histoire Naturelle, Paris, France.

SYSU: Sun-Yatsen University, Guangzhou, China.

3. Results

3.1. Linda (Linda) atricornis Pic (Figures 1 and 2). *Linda atricornis* Pic, 1924 [3]: 19 (Jiangsu, Shanghai) (MNHN). *Linda atricornis*; Savio, 1929 [4]: 3; Gressitt, 1939: 126 [5]; 1940: 197 [6]; 1942 [7]: 10 (part); 1942: 41 [8]; 1947 [9]: 548 (part); Gressitt, 1951 [10]: 605 (part); Hua et. al, 1992 [11]: 54, 55, 170, 303; Hua, 2002 [12]: 213 (part).

Linda (Linda) atricornis; Löbl and Smetana, 2010 [2]: 293 (part).

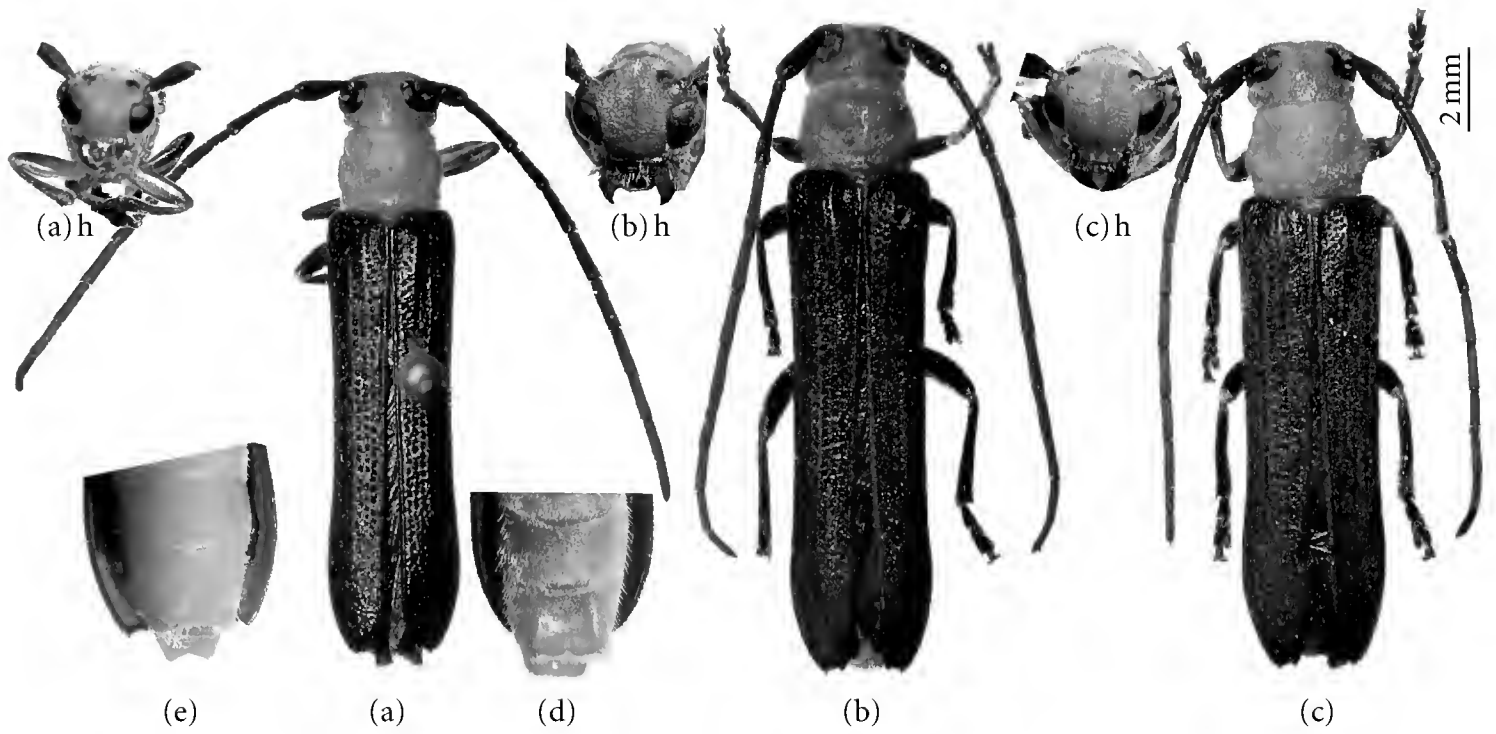

FIGURE 1: Habitus, *Linda atricornis* Pic, 1924. (a) Holotype, male, from Shanghai (Jiangsu). (b) Male, from Fujian. (c) Female, from Jiangsu. h: head, in frontal view. (d-e). Showing last visible sternite, not to scale. (d) Male. (e) Female. Scale 2 mm.

Redescription. Male (Figures 1(a) and 1(b)): length: 14.0–16.5 mm, humeral width: 3.0–3.6 mm. Female (Figure 1(c)): length: 14.2–17.5 mm, humeral width: 3.2–4.0 mm. Head (except eyes, labrum and mandibles), prothorax, scutellum, ventral surface of body, basal third of femora, extreme bases of tibiae and tarsal claws reddish testaceous; antennae, elytra, eyes, labrum, mandibles and most of legs black; pale portions covered with fine silvery pubescence and erect hairs; bases of elytra and undersurfaces of antennae with sparse erect hairs. Head densely and rugulose punctuate; vertex shallowly grooved; antennae shorter than body, about 5/6 (female) to 6/7 (male) of body length, antennomere ratio: male: 13 : 3 : 16 : 15 : 14 : 13 : 13 : 12 : 11 : 10 : 10; female: 14 : 3 : 16 : 15 : 14 : 13 : 12 : 11 : 10 : 9 : 9. Prothorax much broader than long, swollen above and behind middle of each side; scutellum declivitous, truncate. Elytron slightly emarginate apically, with sutural and outer angles slightly projected. Last visible sternite with a broad and deep groove and apex with a small nick in middle (male, Figure 1(d)) or with a thin line and apex smoothly emarginated (female, Figure 1(e)).

Male Terminalia (Figures 2(a)–2(d)). Tegmen length about 3.0 mm; lateral lobes not so stout, each about 0.5 mm long and 0.2 mm wide, mostly covered with moderate long setae, with one short but broad basal lobe furnished with short setae (in ventral view, Figure 2(d)); median lobe plus median struts slightly curved (Figure 2(b2)), a little longer than tegmen (7 : 6); the median struts slightly longer than half of the whole median lobe in length; dorsal plate slightly shorter than ventral plate; apex of ventral plate pointed (Figure 2(d)); median foramen slightly elongated; internal sac about twice as long as median lobe plus median struts, with 3 pairs of basal armature, and 2 pair of rods of endophallus; 2 longer rods each about 1.5 mm, about one-half of tegmen length, the shorter pair about 0.6 mm. The ratio of short pair to long pair always bigger than 1/3. Tergite VIII (Figure 2(a)) broader than long, apex truncated, rounded at side, with dense but short setae (hairs).

Female Genitalia (Figure 2(e)). Spermathecal capsule having a strongly sclerotized rounded apical lobe (with a very short stalk) and a not so sclerotized basal stalk, spermathecal duct not very longer than spermathecal capsule. Spermathecal gland extended from a strongly sclerotized broad ring, which attach to duct directly. Tignum shorter than abdomen. In our observation, tignum 6.5 mm for an adult with a 7.8 mm abdomen in ventral view.

Diagnosis. Femera mostly black, body not over 20 mm, these two characters easily separate it from *L. major* and *L. gracilicornis*.

Host (mixed with host of L. subatricornis). Cydonia sp. (ROSACEAE), *Juglans regia* Linnaeus (JUGLANDACEAE), *Malus* sp. (ROSACEAE), *Morus alba* Linnaeus (MORACEAE), *Populus davidiana* Dode (SALICACEAE), *Prunus armeniaca* Linnaeus (ROSACEAE), *Prunus mume* Siebold and Zuccarini (ROSACEAE), *Prunus persica* (Linnaeus) Batsch (ROSACEAE), *Prunus salicina* Lindley (ROSACEAE), *Rubus* sp. (ROSACEAE), and *Salix* sp. (SALICACEAE).

Remarks. The records from 17 provinces of China by Hua [12] or Löbl and Smetana [2] need confirmation based on specimens. The following provinces may misidentifications of *L. subatricornis*: Ningxia, Shaanxi; the northern provinces may not have this species: Inner Mongolia, Gansu, Hebei; the others may have this species but specimens are required to confirm it: Henan, Hubei, Hunan, Guizhou, Yunnan.

Distribution (Based on Specimens). China: Jiangsu, Zhejiang, Jiangxi, Fujian, Guangdong, Guangxi, Sichuan.

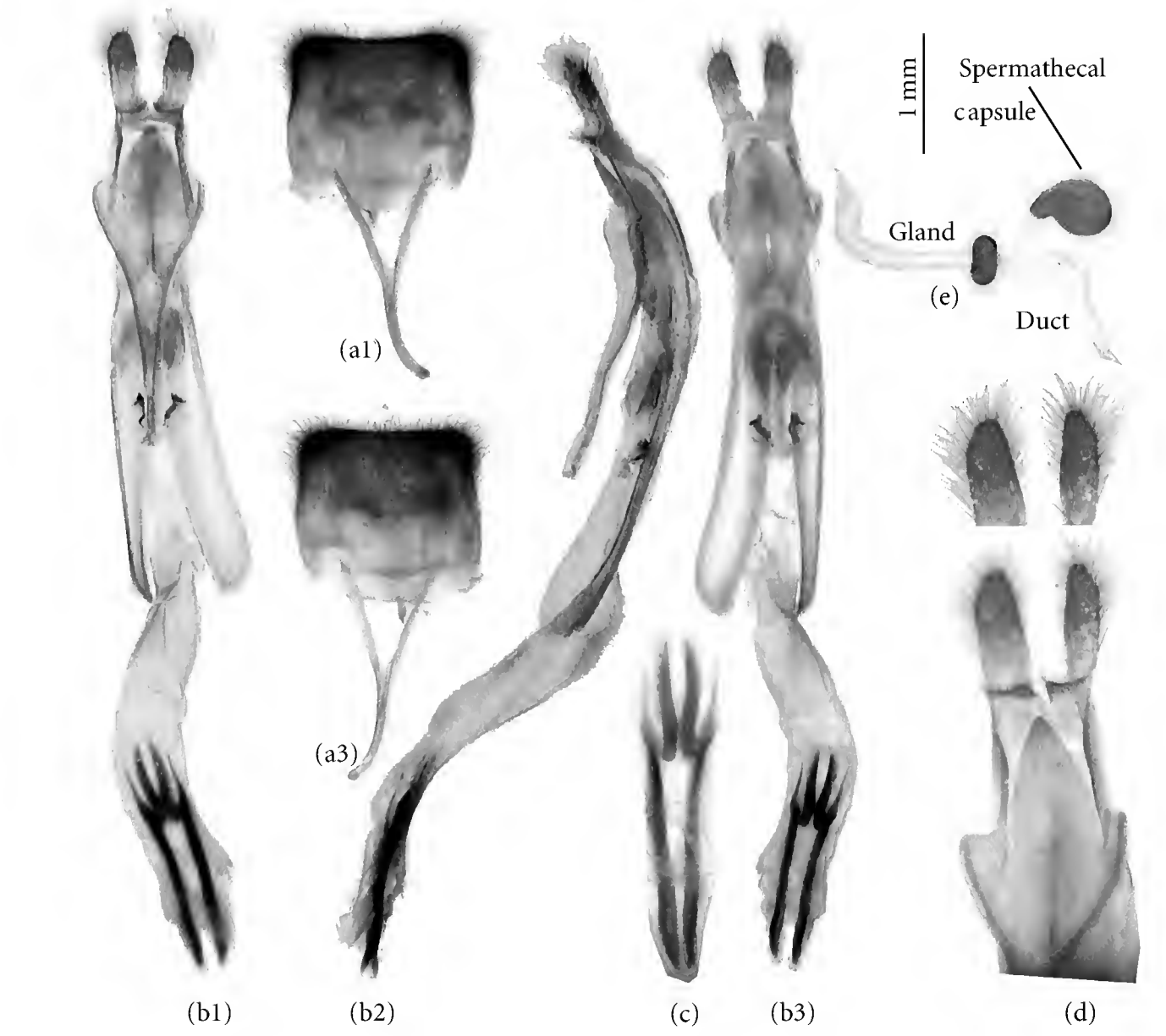

FIGURE 2: Genitalia of *Linda atricornis* Pic, 1924. (a) Tergite VIII and sternites VIII and IX. (b) Male genitalia. 1: ventral view; 2: lateral view; 3: dorsal view. Scale 1 mm. (c–e) not to scale. (c) Showing rods of endophallus. (d) Showing apex of ventral plate of median lobe and lateral lobes. (e) Spermatheca.

Type Specimens Examined. Type, male, Zi-ka-wei (MNHN, ex Coll. M. Pic).

Other Specimens Examined. Jiangsu: 1 female, Ihing, 1923. VII.16 (IZAS); 1 female, Shanghai, 1935.VII (IZAS); 1 female, Shanghai, 1939.VI.5, leg. O. Piel (IZAS).

Zhejiang: 1 female, T'ienmu Shan, 1936.VIII.1, leg. O. Piel (IZAS); 1 male 2 females, T'ienmu Shan, 1935.VIII.4 (IZAS); 1 female, Huangyan, 1955.VI.26 (IZAS); 1 female, Chekiang, Mokanshan, env. 50 k de Hangtcheou, 1925, leg. A. Pichon (MNHN); 2 males, Chusan, 1931.VI.12, leg. O. Piel (IZAS).

Jiangxi: Kiang-si, 1901, leg. C. L. Gonon (MNHN, ex Coll. R. Oberthür, 1952).

Fujian: 1 female, Chongan, Xingcun, Guadun, alt. 900–1100 m, 1963.VII.6, leg. ZHANG Youwei (IZAS); 1 female, same data but alt. 840–1160 m, 1960.VII.14, leg. ZHANG Yiran; 1 male, Chongan, Xingcun, Sangang, alt. 740 m, 1960. VII.30, leg. MA Chenglin (IZAS); 1 male, Dehua, Chengguan, alt. 510–550 m, 1960.VI.1, leg. PU Fuji (IZAS); 1 male, Dehua, Shangyong, Guifu, alt. 780–950 m, 1960.VI.18, leg. MA Chenglin (IZAS); 1 male, Fuzhou, Gushan, 1953.VI, leg. HUANG Jiabin (IZAS).

Guangxi: 4 males 2 females, Kouangsi, Region de Nanning, 1931 (MNHN, ex Coll. R. Oberthür, 1952); 9 males 4 females, same data but (IRSNB); 1 female, Prov. Kwangsi, Mts. Toyen-chan (MNHN, ex Coll. M. Pic); 1 female, Huangshahe, 1955.VIII.14 (IZAS).

Sichuan: 1 male, Pengshui, alt. 850 m, 1989.VII.11, leg. SUN Baowen (IZAS).

3.2. Linda (Linda) subatricornis n. sp. (Figures 3 and 4). *Linda gracilicornis* m. *tatsienlui* Breuning, 1954 [13]: 550 (Sichuan). (MHNG) infrasubspecies, nomen nudum.

Linda (s. str.) atricornis; Pu, 1992 [14]: 611 (misidentification).

Linda atricornis; Pic, 1935 [15]: 12 (misidentification); Gressitt, 1942 [7]: 10 (part); 1947 [9]: 548 (part); Gressitt, 1951 [10]: 605 (part); Wang and Chiang, 1988 [16]: 144 (misidentification); Hua, 2002 [12]: 213 (part).

Linda (Linda) atricornis; Löbl and Smetana, 2010 [2]: 293 [part].

Description. Male (Figures 3(a) and 3(b)), length: 13.5–16.0 mm, humeral width: 2.8–3.4 mm. Female (Figure 3(e)–3(g)), length: 15.4–18.5 mm, humeral width: 3.2–4.2 mm. Head (except eyes, antennal tubercles, labrum, and mandibles), prothorax, scutellum, ventral surface of body, basal thirds of femora, and tarsal claws reddish testaceous; antennae, antennal tubercles, elytra, eyes, labrum, mandibles, and most of legs black; pale portions covered with fine silvery pubescence and erect hairs; bases of elytra

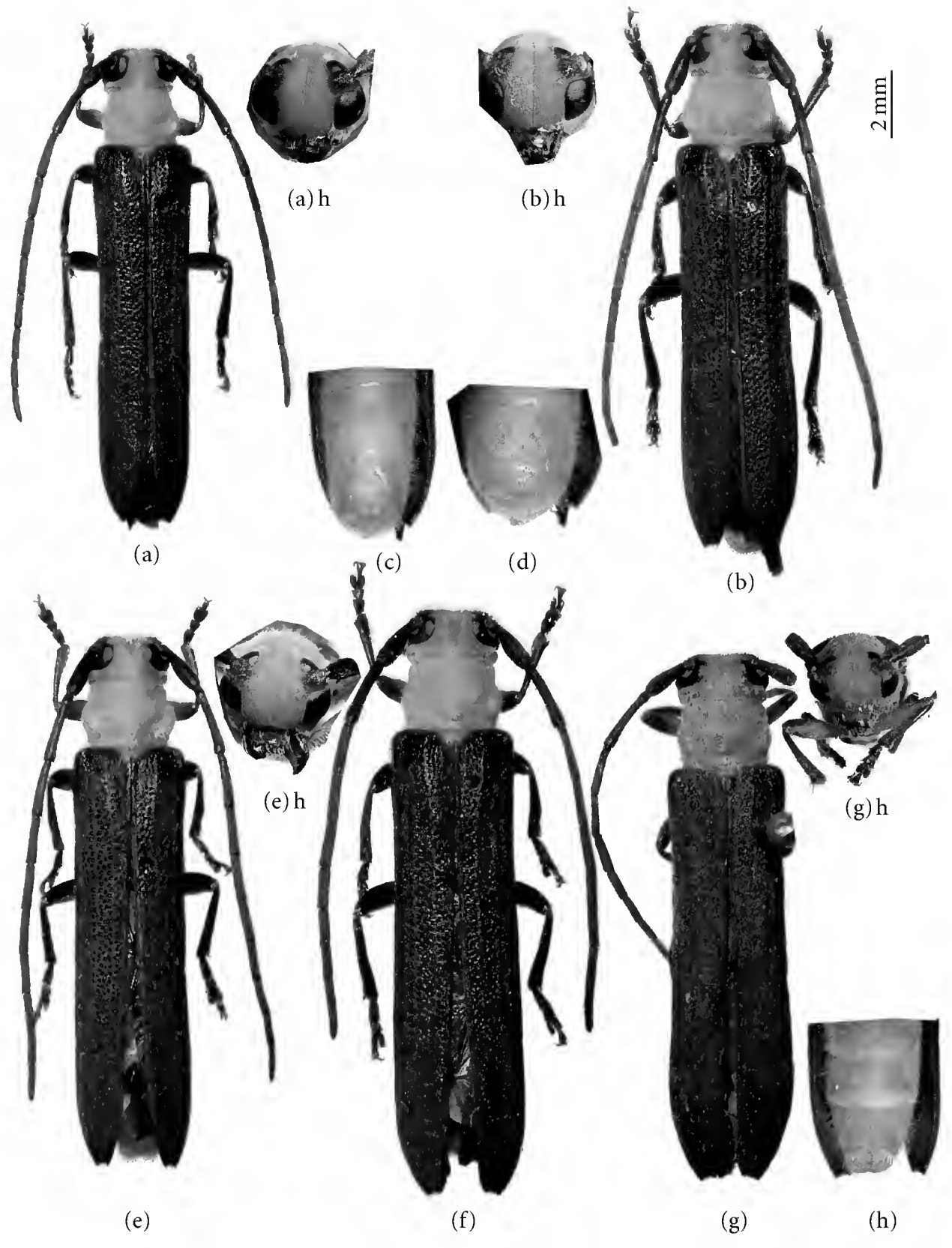

FIGURE 3: Habitus, *Linda subatricornis* n. sp. (a) Holotype, male, from Sichuan. (b) Paratype, male, from Beijing. (e–f) Paratype, female, from Sichuan. (g) Holotype of *Linda* (*Linda*) *gracilicornis* m. *tatsienlui* Breuning, 1954, female, from Sichuan. Scale 2 mm. h: head, in frontal view. (c-d) and (h) showing last visible sternite, not to scale. (c-d) Male. (h) Female.

and undersurfaces of antennae with sparse erect hairs. Head densely and rugulose punctuate; vertex shallowly grooved; antennae shorter than body, about 4/5 (female) to 9/10 (male) of body length, antennomere ratio: male: 13 : 2 : 16 : 14 : 13 : 13 : 12 : 11 : 10 : 9 : 10; female: 15 : 3 : 19 : 16 : 15 : 14 : 13 : 12 : 11 : 10 : 11. Prothorax much broader than long, swollen (three) above and behind middle of each side; scutellum declivitous, truncate. Elytron slightly emarginate apically, with sutural and outer angles slightly projected. Last visible sternite with a moderate broad and deep groove and apex with a small groove in middle (male, Figure 3(c) and 3(d)) or with a thin line and apex smoothly emarginated (female, Figure 3(h)).

Male Terminalia (Figures 4(a)–4(f)). Tegmen length about 3.0 mm; lateral lobes stout, each about 0.5 mm long and 0.25 mm wide, mostly covered with moderate long setae, with one short but broad basal lobe furnished with short setae (in ventral view, Figure 4(d)); median lobe plus median struts slightly curved (Figure 4(b2)), a little longer than tegmen (6 : 5); the median struts slightly longer than half of the whole median lobe in length; dorsal plate slightly shorter than ventral plate; apex of ventral plate narrowly rounded (Figure 4(c)); median foramen elongated; internal sac less than twice of median lobe plus median struts in length, with 3 pairs of basal armature, and 2 pairs of rods of endophallus; 2 longer rods each about 1.9 mm, longer than one-half of tegmen, the shorter pair about 0.6 mm. The ratio of short pair to long pair always smaller than 1/3. Tergite VIII (Figure 4(a)) broader than long, apex truncated, rounded at side, with dense but short setae (hairs).

Female Genitalia (Figures 4(g)–4(j)). Spermathecal capsule having a strongly sclerotized rounded apical lobe (with

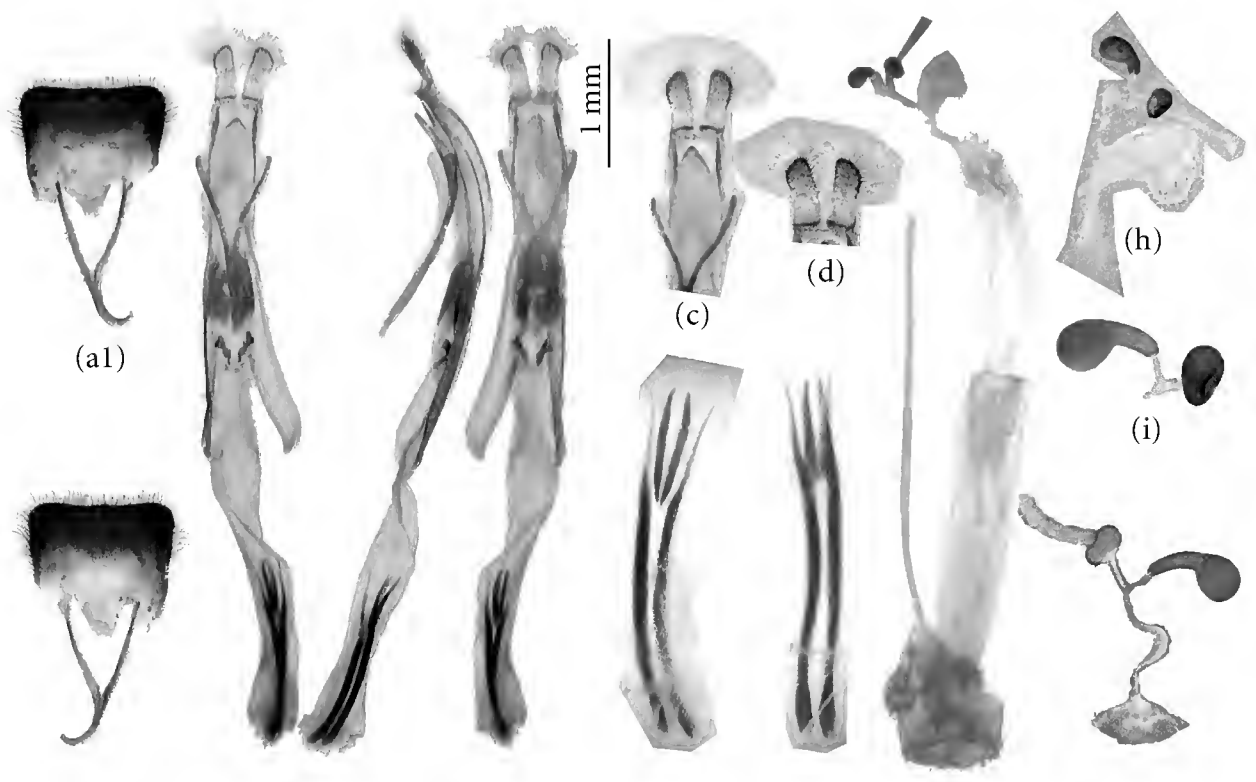

FIGURE 4: Genitalia of *Linda subatricornis* n. sp. (a) Tergite VIII and sternites VIII and IX. (b) Male genitalia. 1: ventral view; 2: lateral view; 3: dorsal view. Scale 1 mm. (c–j) Not to scale. (c-d) Showing apex of ventral plate of median lobe and lateral lobes. (e-f) Showing rods of endophallus. (e) From Sichuan. (f) From Beijing. (g–j) Female genitalia. (g-h) From Sichuan. (i) From Sichuan. (j) From Beijing.

a short to long stalk) and a not so sclerotized basal stalk, spermathecal duct not very longer than spermathecal capsule. Spermathecal gland extended from a strongly sclerotized broad ring, which attach to duct directly. Tignum shorter than abdomen. In our observation, tignum 6.8 mm for an adult with a 9.0 mm abdomen in ventral view.

Diagnosis. Differs from *L. atricornis* by antennal insertions black, extreme bases of tibiae black, groove of last visible sternite of male not so broad, last antennomere longer than tenth antennomere, rods of endophallus slender and the ratio of short pair to long pair smaller than 1/3, lateral lobes stouter, and so forth.

Differs from *L. major* and *L. gracilicornis* by antennal insertions black, femera mostly black, and body not over 20 mm. Differs from all the other species of subgenus *Linda* by antennae and elytra all black.

Etymology. Named after misidentification as *L. atricornis* in the collections.

Remarks. The female genitalia is difficult to separate species. In this species, the stalk attached to the strongly sclerotized rounded apical lobe is quite variable in length (Figure 4(h)–4(j)).

L. (*L.*) *gracilicornis* m. *tatsienlui* Breuning, 1954 [13] is a nomen nudum of this species, while *L.* (*L.*) *gracilicornis* m. *rufofemorata* Breuning, 1954 [13] should be a nomen nudum of *Linda femorata* (Chevrolat, 1852).

Host (mixed with host of L. atricornis). *Cydonia* sp. (ROSACEAE), *Juglans regia* Linnaeus (JUGLANDACEAE), *Malus* sp. (ROSACEAE), *Morus alba* Linnaeus (MORACEAE), *Populus davidiana* Dode (SALICACEAE), *Prunus armeniaca* Linnaeus (ROSACEAE), *Prunus mume* Siebold and Zuccarini (ROSACEAE), *Prunus persica* (Linnaeus) Batsch (ROSACEAE), *Prunus salicina* Lindley (ROSACEAE), *Rubus* sp. (ROSACEAE), *Salix* sp. (SALICACEAE).

Distribution. Sichuan, Fujian, Shaanxi, Hebei, Ningxia.

Type specimens examined. *Holotype,* male, Sichuan, Luding, Moxi, alt. 1600 m, 1983.VI.20, leg. CHAI Huaicheng (IZAS).

Paratypes. Sichuan: 13 males 9 females, Luding, Moxi, alt. 1500 m, 1983.VI.20, leg. ZHANG Xuezhong (IZAS); 1 male 4 females, same data but alt. 1600–1650 m, 1983.VI.18-19, leg. WANG Shuyong; 1 female, same data but 1982.IX.14, leg. WANG Shuyong; 10 males 7 females, Luding, Xinxing, alt. 1800–2100 m, 1983.VI.13–19, leg. WANG Shuyong, ZHANG Xuezhong, CHEN Yuanqing (IZAS); 1 male, Luding county, Moxi env., 1994.V.22–VI.10, leg. V. Beneš (CCH); 1 male, Abazhou, Nanping, Jiuzhaigou, alt. 2000 m, 1991.VI.8–13, leg. C. Holzschuh (CCH); 9 males, Emeishan, Baoguosi, 1957.V.5, leg. HUANG Keren (IZAS); 3 males, same data but 1957.V.12; 6 males 2 females, Emeishan, Baoguosi, alt. 550–750 m, 1957.V.3–VI.5, leg. HUANG Keren, ZHU Fuxing, LU Youcai (IZAS); 1 male, Emeishan, alt. 700 m, 1957.VI.1, leg. ZHU Fuxing (IZAS); 1 male, Emeishan, Qingyinge, alt. 800–1000 m, 1957.VI.11, leg. LU Youcai (IZAS); 4 females, Emeishan, 1955.VI.13–14, leg. HUANG Keren, JIN Gentao (IZAS); 1 female, Emeishan, alt. 1100–1800 m, 1955.VI.23, leg. GE Zhonglin (IZAS); 1 female, Mt. Emei, alt. 1050 m, 1990.VII.18, leg. L. & M. Bocák (CCH); 2 males, Wenchuan, Yingxiu, alt. 900 m, 1983.VIII.3, leg. WANG Shuyong (IZAS); 1 male, Jintang, 1943.V.9, leg. K. O. V. Lieu (IZAS); 1 female, Guanxian, Qingchengshan, alt. 700–1600 m, 1963.V.4, leg. ZHANG Xuezhong (IZAS); 1 female, Fengjiexian, 1980.VI.30, leg. QIAN Yuanzhi (IZAS);

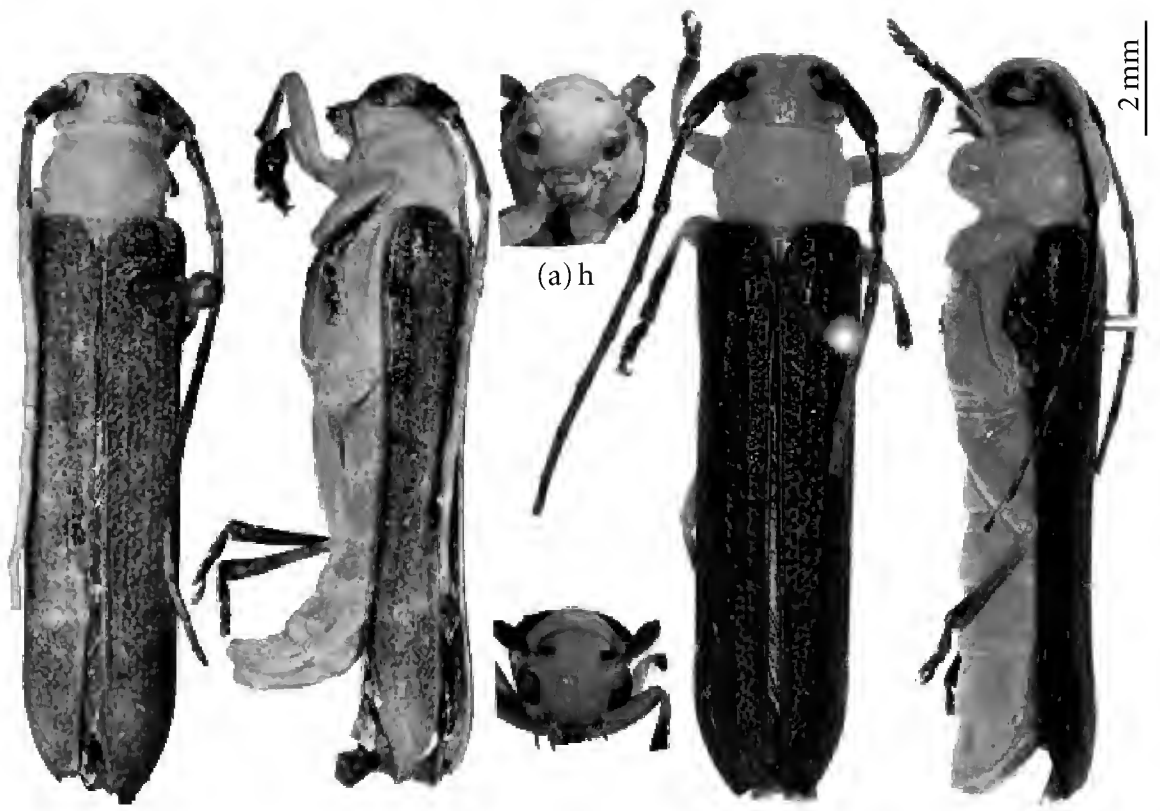

FIGURE 5: Habitus, holotype. (a) *Linda gracilicornis* Pic, 1907, male, from Yunnan. (b) *Linda major* Gressitt, 1942, female, from Anhui. 1. dorsal view. 2. lateral view. h: head, in frontal view. Scale 2 mm.

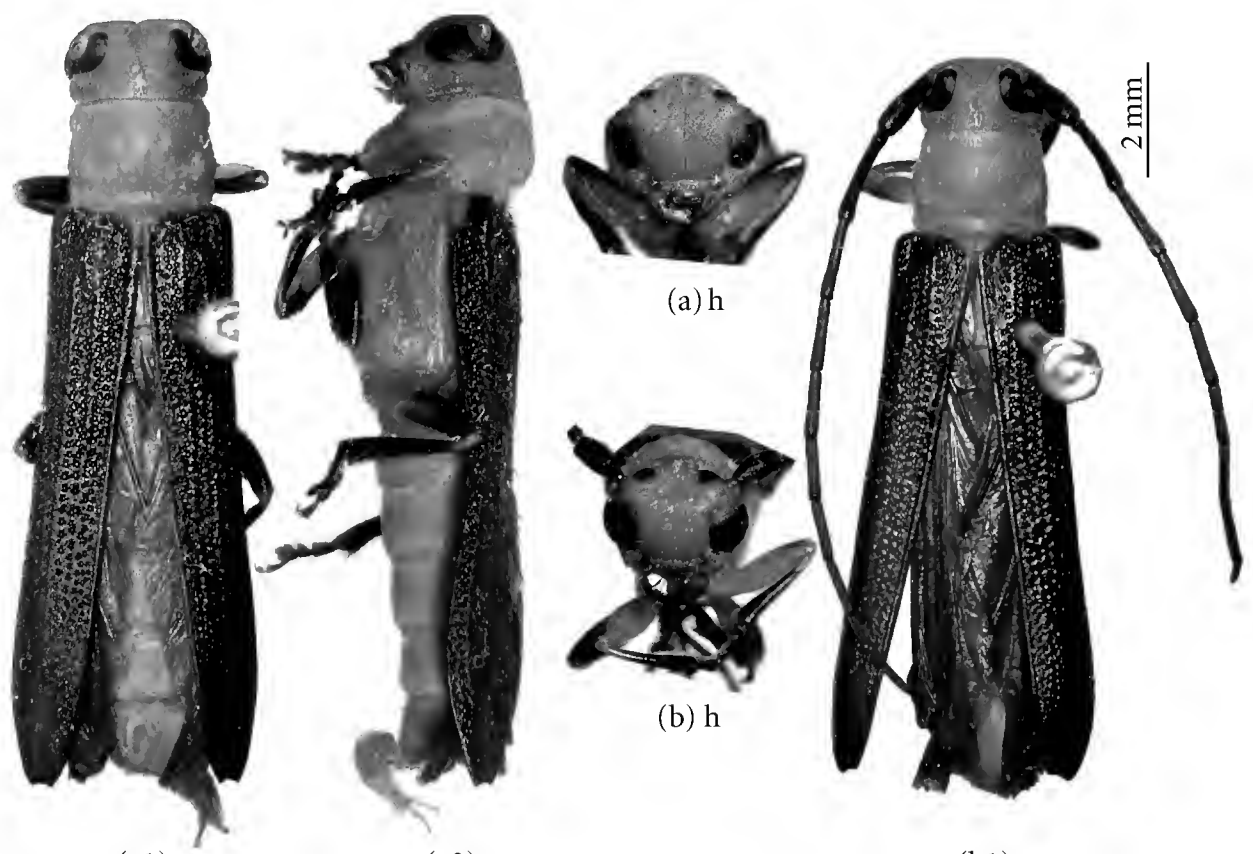

FIGURE 6: Habitus, *Oberea holatripennis* Breuning, 1982. (a) Holotype, female, from Beijing. (b) "Paratype," male, from Beijing, may be *Linda atricornis* Pic, 1924. 1: dorsal view; 2: lateral view; h: head, in frontal view. Scale 2 mm.

1 female, Yuechi, 1981.VIII.7, leg. LUO Dongming (IZAS); 1 female, Kangding, alt. 2500 m, 1983.VI.26, leg. WANG Shuyong (IZAS); 1 male, Kangding, 10 km North, alt. 2600 m, 1992.VII.9, leg. G. C. Bozano (CPS); 1 female, Xingou, 180 km S.W of Chengdou, alt. 1600 m, 1991.VII.16, leg. E. Giacomazzo (CPS); 2 females, Su-Tchuen, Siào-Lou, 1897 (MNHN, ex Coll. R. Oberthür, 1952); 2 females, Siào-Lou, 1901/1904, leg. Chasseurs du P. Dejéan (MNHN, ex Coll. R. Oberthür, 1952); 2 females, Siao-Lou-Lou-Chan, 1897, leg. Chasseurs Thibétains (MNHN, ex Coll. R. Oberthür, 1952); 1 male 1 female (holotype of *Linda gracilicornis* m. *tatsienlui* Breuning, 1954), Szetschuan, Tatsienlu (MHNG, ex Coll. S. Bruning, ex Coll. Reitter); 3 males 4 females, Su-Tchuen, 1903, leg. Chasseurs Indignes (MNHN, ex Coll. R. Oberthür, 1952).

Fujian: 1 male, Foochow (MHNG).

Shaanxi: 1 male, Qinlingshan, 6 km East of Xunyangba, alt. 1000–1300 m, 2000.V.23–VI.13, leg. C. Holzschuh (CCH); 1 male, Danfeng, NE env., alt. 900–1500 m, 1995.V.28–29, leg. L. & R. Businský (CCH); 1 male, Shaanxi (IZAS).

Hebei: 2 males, Beijing, Sanpu, 1964.VII.9, leg. LIAO Subai (IZAS); 1 female, Beijing, Sanpu, alt. 550 m, 1972.VII.4, leg. JIANG Shengqiao (IZAS); 1 male, Beijing,

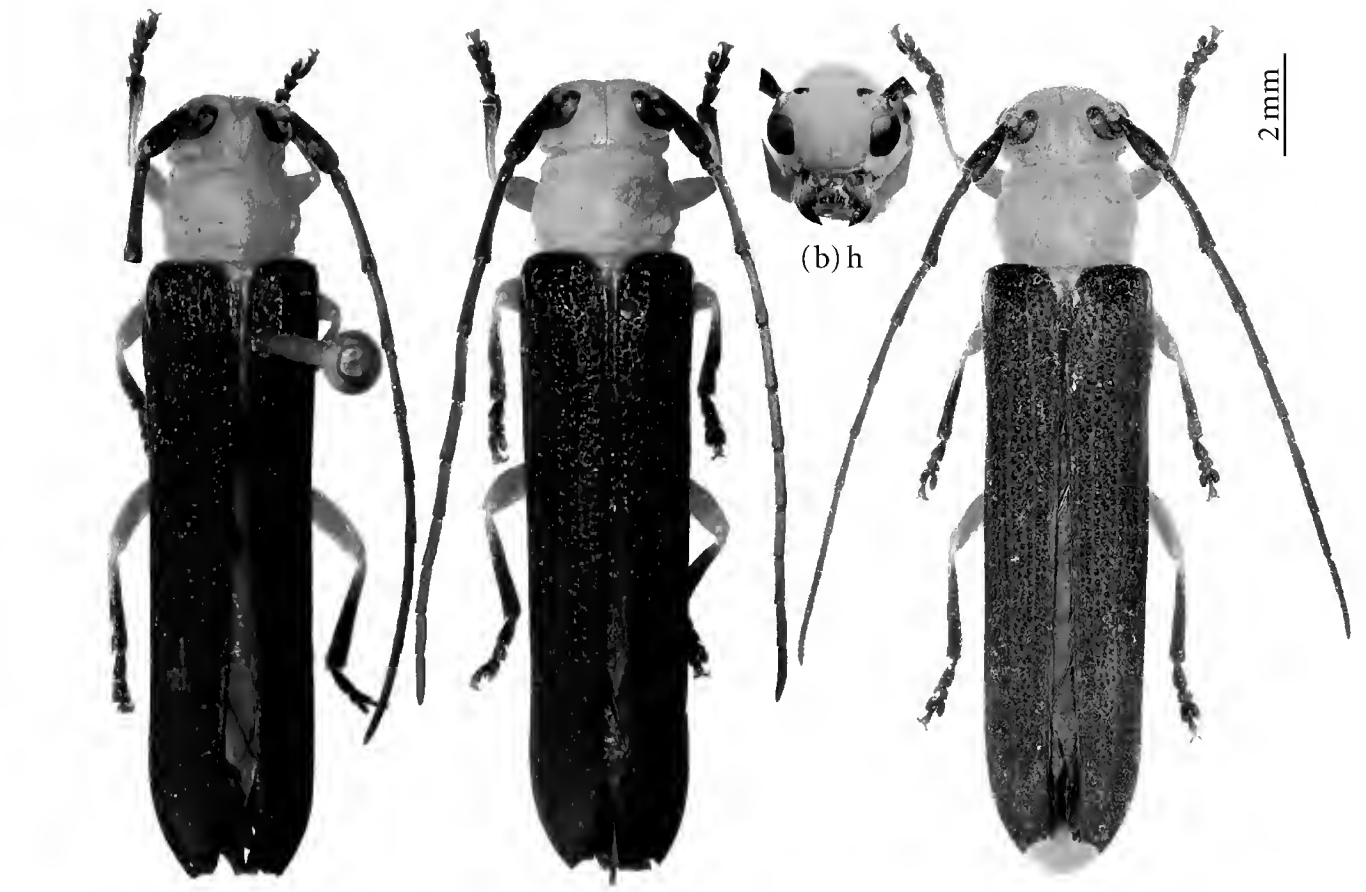

FIGURE 7: Habitus. (a-b) *Linda* cf. *gracilicornis*. (a) Male, from Sichuan. (b) Female, from Guangxi. h: head, in frontal view. (c) *Linda* cf. *major*, female, from Zhejiang. Scale 2 mm.

Shangfangshan, alt. 400 m, 1961.VII.17, leg. WANG Shuyong (IZAS); 1 female, Beijing, Shangfangshan, 1979.VII.25, leg. JIANG Shengqiao (IZAS).

Ningxia: 1 male, Jingyuan, Mt. Liupanshan, 1995.VI.14, leg. LIN924 group (HBU); 1 female, Jingyuan, 1981.VI.8 (IZAS); 1 male, Guyuan, 1981.VI.17 (IZAS).

4. Discussion

Including the new species described above, there are four species of subgenus *Linda* with elytra and antennae all black. They are *Linda atricornis*, *L. subatricornis*, *L. gracilicornis*, and *L. major*. *L. gracilicornis* Pic, 1907[17], was described based on one male from Yunnan. The holotype (Figure 5(a1), 5(a2), and 5(a)h deposited in MNHN) is in bad condition, with mud covering the punctures. It is very difficult to conclude if *L. major* Gressitt, 1942 [18] (Figure 5(b1), 5(b2), and 5(b)h deposited in SYSU) is a synonym of *L. gracilicornis* or a separate species. In the keys by Gressitt [7, 9, 10], *L. gracilicornis* "Elytra irregularly punctured; body slender; antennae relatively slender and as long as body," while *L. major* "Elytra subregularly punctured, body more or less stout; antennae relatively thick and shorter than body; elytra with round punctures; femora entirely testaceous and length over 20 mm," descriptions which did not well match with the types. Before enough material are available for further study, we consider them as two different species and *L. major* differs from *L. gracilicornis* by pronotum quite smooth, without accidented tubercles, elytral punctures denser, and more irregular. One male from Sichuan (Figure 7(a)) and one female from Guangxi (Figure 7(b) and 7(b)h) are identified as *L.* cf. *gracilicornis*, while one female from Zhejiang (Figure 7(c)) as *L.* cf. *major* according to above consideration. We wait for more material especially specimens from the type localities to make a better conclusion.

Based on the holotype (MNHN, ex Coll. J. Thomson, 1952, ex Musaeo ARM. DAVID, 1900), *Oberea holatripennis* Breuning, 1982 [19], from Beijing (Figure 6(a1), 6(a2), 6(a)h) is similar to *L. atricornis* but can be separated by the denser and irregular elytral punctures, and the pronotum with three visible swollen. The "paratype" (Figure 6(b1) and 6(b)h, determined by Breuning, deposited in MHNG, ex Musaeo Arm. David, 1900) is possibly a male of *L. atricornis*. More material and genitalia dissections are needed for further study.

Acknowledgments

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Research Article

Life History of the Camelthorn Gall Leafhopper, *Scenergates viridis* (Vilbaste) (Hemiptera, Cicadellidae)

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The world's only member of Hemiptera Auchenorrhyncha known to form true galls, the leafhopper *Scenergates viridis* (Vilbaste) (Cicadellidae), transforms leaves of camelthorn (*Alhagi maurorum* Medikus, Fabaceae) into pod-like chambers, up to 35 mm long, inside which individual leafhoppers develop, mate, and lay eggs. At the study site 40 km SE of Bukhara (Uzbekistan), two generations develop annually. First-instar nymphs cause young leaves to fold along the midrib. The subsequent development takes place inside the tightly closed growing gall, plugged at both ends with a mixture of leafhopper excrement, brochosomes, and crushed exuviae. These plugs act as mechanical barriers and sticky traps for intruders. The inner surface of the gall, lined with brochosomes and wax platelets, is hydrophobic. Adult males emerge from their galls and squeeze into female galls. Fertilized females insert an average of 146 eggs under the gall's inner epidermis and remain inside, possibly protecting the brood, until they die. The walls of the galls containing eggs are approximately three times thicker than regular leaves. The galls are subject to predation by Gelechiidae caterpillars; the eggs of the leafhopper are parasitized by two species of Trichogrammatidae and one Mymaridae (Hymenoptera), and its larvae by one species of Pipunculidae (Diptera).

1. Introduction

1.1. Background. Gall formation is arguably the most sophisticated strategy of plant parasitism. Among sap-sucking insects of the order Hemiptera, it is found mostly in the suborder Sternorrhyncha, particularly among aphids [1], jumping plant lice [2], and scale insects [3], and is rare among Heteroptera [4, 5]. Although malformations of host plants caused by feeding of some Auchenorrhyncha have also been observed [6, 7], so far, only one of the 42,000+ [8] known species of that suborder was found to induce true galls, which provide the parasite with food and shelter. Over a half century ago Ivan Mitjaev discovered that the leafhopper *Scenergates viridis* (Vilbaste) (Cicadellidae) develops inside closed pod-like leaf galls on camelthorn (*Alhagi*, Fabaceae) (Figures 1(a)–1(d)). His brief but highly informative report, two pages in Russian with a single sketch drawing [9], has remained mostly forgotten.

1.2. Scenergates viridis: Taxonomy, Distribution, and Previous Research on the Biology. The camelthorn gall leafhopper is the sole member of the genus *Scenergates* Emeljanov within the subfamily Deltocephalinae, comprising over 6,200 [10] phloem-feeding leafhopper species which inhabit virtually every terrestrial habitat and include some economically important and therefore better studied vectors of plant pathogens. All the known species except one are free-living; both nymphs and adults are capable of jumping, and the majority is capable of flight.

S. viridis was described almost simultaneously but independently by two authors, who even used the same species epithet, referring to the greenish color, in the Latin names they devised. Vilbaste [11] described it based on two males and several females from Golodnaya Steppe, Uzbekistan, and placed it in the newly created monotypic genus *Platytettix*. Emeljanov [12] described it based on ten males and nine females collected in Khiva, Uzbekistan, and the unknown

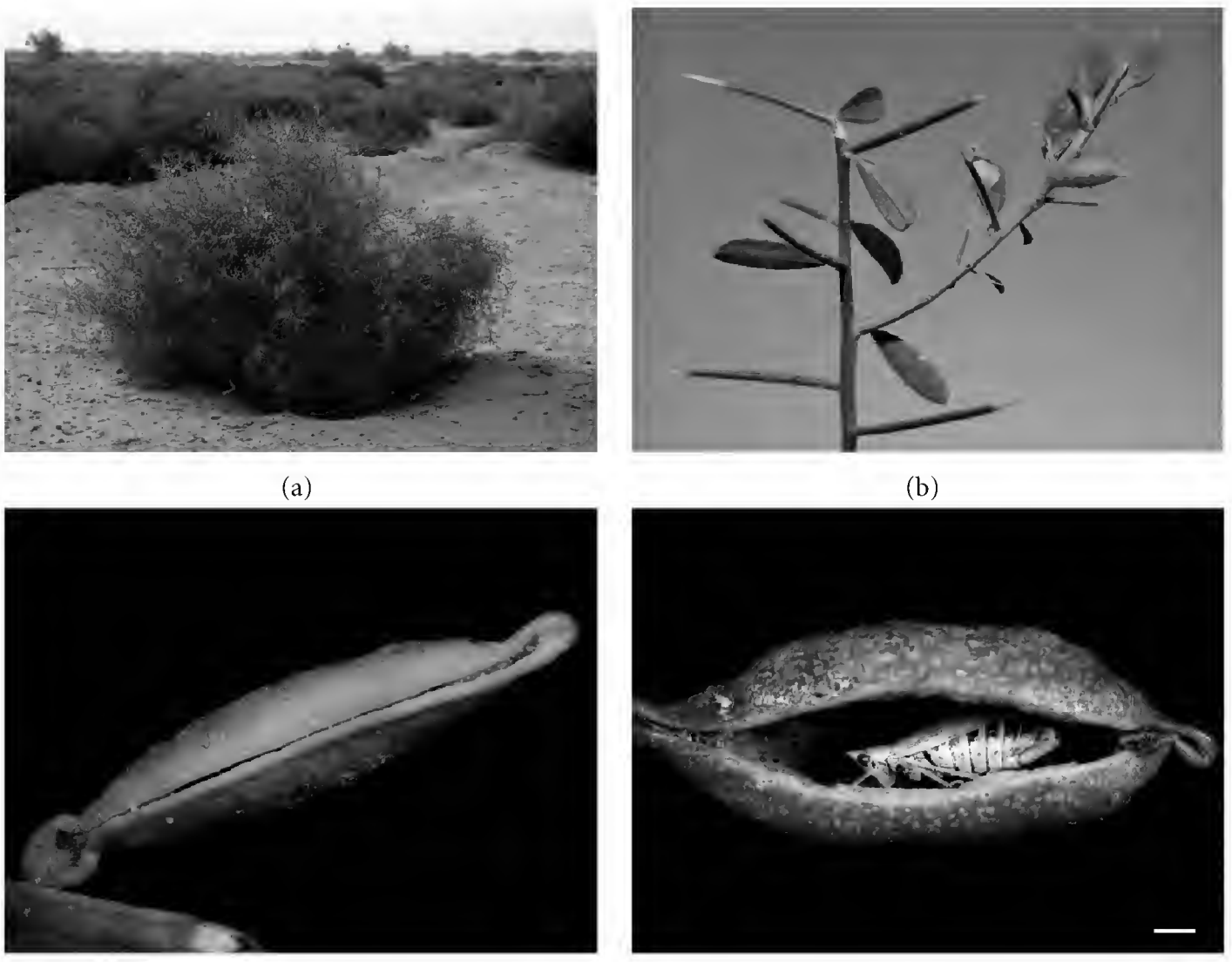


FIGURE 1: (a) Camelthorn (*Alhagi maurorum*) on the territory of the Ecocenter "Dzheiran" in Uzbekistan; note fresh green growth on the left and dead branches from the previous year on the right side of the shrub. (b) A camelthorn branch with thorns, ungalled leaves, and galls of *Scenergates viridis*. (c) An intact gall of *Scenergates viridis*. (d) An artificially opened gall with a female of *Scenergates viridis* inside. Scale bar: 1 mm (d).

locality "Nuriobay," and placed it in the new monotypic genus *Papyrina*. The priority name *Platytettix* turned out to be preoccupied and was later replaced by the name *Scenergates* [13].

Mitjaev [9] observed *S. viridis* near the village of Baltakul' in southern Kazakhstan. He reported that young nymphs fed on the adaxial surfaces of *Alhagi* leaves, causing their folding along the midrib into pod-like galls with individual leafhoppers developing inside, and that the exuviae were crushed and pressed together with dried leafhopper excrement, the latter forming white masses at both ends of the gall chamber. He also observed adult males on the outside of female galls, apparently waiting for copulation. Some additional data on the species were included by Dubovskii and Sulaimanov in their check-list of Auchenorrhyncha of the so-called Karshi Steppe, a semidesert plain approximately centered on the town of Karshi in southern Uzbekistan [14]. Of the 100 galls examined by the authors, 78 contained immatures or adults and also, remarkably, some eggs of *S. viridis*; 15 galls contained puparia of unidentified dipteran parasitoids, and seven contained lepidopteran larvae. Dubovskii and Sulaimanov [14] summarized the available records of this leafhopper, known mostly from Uzbekistan and a few localities in southern Kazakhstan and eastern Turkmenistan (Figure 2).

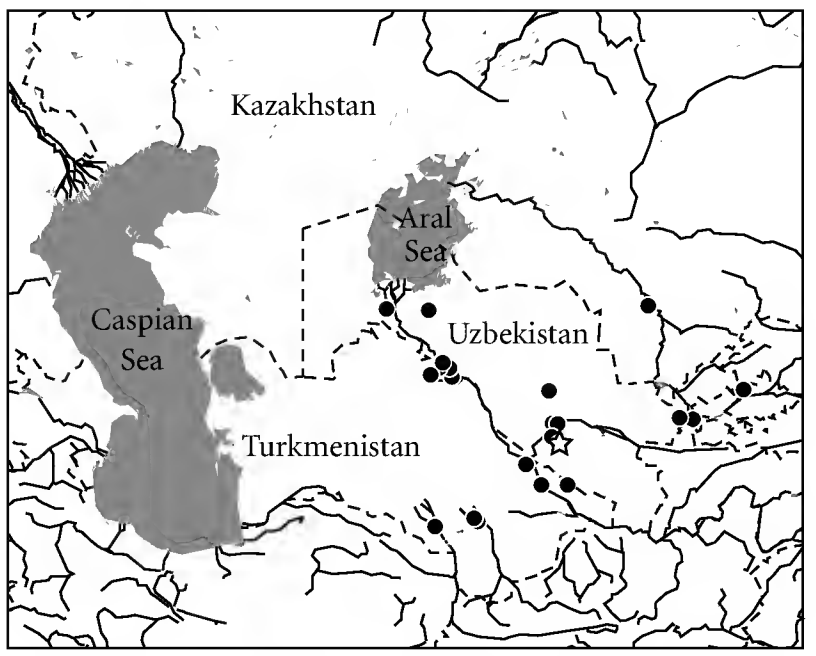


FIGURE 2: The distribution of *Scenergates viridis* based on records summarized by Dubovskii and Sulaimanov [15]. The star shows the location of the Ecocenter "Dzheiran."

1.3. Host Plant. The genus *Alhagi* (Fabaceae) includes perennial shrubs with prickly thorns formed by rudimentary lateral shoots (Figures 1(a) and 1(b)), endemic to arid areas of the Old World and invasive in the western United States. The shrubs are usually less than one meter high, but their roots can reach depths exceeding 15 meters below the ground surface, allowing the plant to stay green and vigorous throughout the summer drought. Camelthorn propagates mostly as clones connected by long underground stolons. In Uzbekistan, the aboveground part of the shrub dies off

by late November except for a few basal centimeters of the main stem; these bear resting buds that produce new shoots in late March-early April [15]. According to Yakovlev [16], the five species of *Alhagi* previously recognized within the territory of the former Soviet Union belong to the same variable species, *Alhagi maurorum* Medikus, which occurs from North Africa and the Mediterranean throughout the Middle East to western China and northern India (see also [17]).

1.4. Goals and Scope of the Study. The observations of Mitjaev [9] and Dubovskii and Sulaimanov [14] have not completely elucidated the unique biology of the camelthorn gall leafhopper. Where mating takes place, how the eggs are laid, how the offspring leave the galls, how many generations develop annually, and at which stage the leafhopper overwinters all remain unknown. Therefore, the first goal of our field work in Uzbekistan was the elucidation of the complete life cycle of the leafhopper. The second goal was to find out how typical leafhopper traits are modified in this species in connection with its galling life style. In particular, disposal of dangerous sticky liquid waste is a challenge for phloem-feeding gallers [18]. In order to avoid entrapment in their liquid waste, all free-living Auchenorrhyncha forcefully eject droplets of liquid excrement; leafhoppers are additionally protected by a hydrophobic coat of brochosomes (reviewed in [19]). Therefore, among other traits, we wanted to find out how *S. viridis* maintains a safe environment inside the galls.

2. Study Site and Timing

The research was conducted at the Ecocenter "Dzheiran" (39.579°N, 64.723°E) near the town of Kagan in Bukhara Province, Uzbekistan, during two visits, September 4–13, 2010, and August 3–17, 2011. Additionally, at our request, 10 overwintering galls were collected at the same locality by Anastasia Shilina on December 18, 2010.

The site is located in the southwestern reaches of the Kyzylkum desert (Figure 2) and falls within the area studied by Dubovskii and Sulaimanov [14]. It is characterized by a strongly continental, hot, and arid climate with a mean annual precipitation of 110 mm and rains occurring from mid-October to mid-May, and mean January and July temperatures of −1°C and +31°C, respectively (data from the study by Balasheva, Sabinina, and Semenov as quoted in [20]). The site is an alluvial plain with sparse semi-desert vegetation, including camelthorn, which grows mostly on flats near the Amu-Bukhar Canal (Figure 1(a)) and along roads. According to Yakovlev [16], the area is within the range of *Alhagi maurorum* ssp. *canescens* (Regel) Yakovlev, but our plants lacked the pubescent calyx characteristic of that subspecies and must therefore be classified in the nominal *A. m.* ssp. *maurorum*.

3. Material and Methods

Hundreds of galls were observed in the field and examined intact under a stereomicroscope in the laboratory. Freshly cut short branches with galls, inserted in moist sand, remained fresh for one to two days. Leafhopper behavior was documented using a JVC Everio camcorder with an attached macro lens. Photographs were taken with a Canon EOS camera equipped with a Canon MP-E 65 mm macro lens.

For a more detailed survey of their contents, the galls were preserved in 70% ethanol. In addition to counting the number of intact and parasitized eggs, we examined nymphal exuviae or their remains. For this purpose the masses of dried excrement (see below) were dissected in droplets of water. The number of instars was determined based on the number of stylet bundles, which remain intact even when the rest of the exuviae have been disintegrated; remains of the last-instar female exuviae were recognized based on the presence of nymphal ovipositor sheaths.

Ethanol-preserved galls were critical-point dried and examined in a Tescan Vega XMU scanning electron microscope (Tescan, Brno, Czech Republic). In order to examine wax secretions of the gall epidermis, intact galls stored in a refrigerator at +5°C for 17 days were frozen in liquid nitrogen and examined in their frozen state in a Hitachi S-4800 cryo-scanning electron microscope (Hitachi High-Technologies Corp., Tokyo, Japan) equipped with a Gatan ALTO 2500 cryo-preparation system (Gatan Inc., Abingdon, UK).

To characterize the wettability of the gall surfaces, contact angles of small water droplets placed on the inner and outer surfaces of two galls were measured using an OCAH 200 high-speed contact angle measuring system (DataPhysics Instruments GmbH, Filderstadt, Germany). Seven measurements were taken at different points on each examined surface. Prior to measurements the galls had been stored in a refrigerator at +5°C for 17 days. The thickness of the wall or lamina was measured under a stereomicroscope on 44 galls and 18 leaves, respectively, preserved for histological examination in a 9.0 : 0.5 : 0.5 mixture of 70% ethanol, glacial acetic acid, and neutral-buffered formalin.

4. Results

4.1. Life Cycle. The life cycle of *S. viridis*, inferred from our observations, is shown in Figure 3. First-instar nymphs cause camelthorn leaves to fold into pod-like chambers, each containing a single leafhopper. The subsequent development, including five molts, takes place inside the tightly closed growing gall. Adult females remain inside their galls, while adult males emerge from their galls and penetrate into closed female galls. The male leaves after mating, while the fertilized female stays inside and inserts eggs under the inner epidermis of the gall. First-instar nymphs eventually disperse from the galls.

Although our own observations were restricted to August and September, the entire annual cycle of *S. viridis* can be reconstructed with reasonable confidence (Figure 4). Two generations develop annually (Figure 5). According to Gushchin [15], camelthorn begins sprouting young leaves in late March-early April. Soon afterwards, first-instar nymphs must emerge from overwintered galls and initiate new galls. We refer to this as the *overwintering generation*. Mitjaev [9] observed nymphal galls from the second half of May,

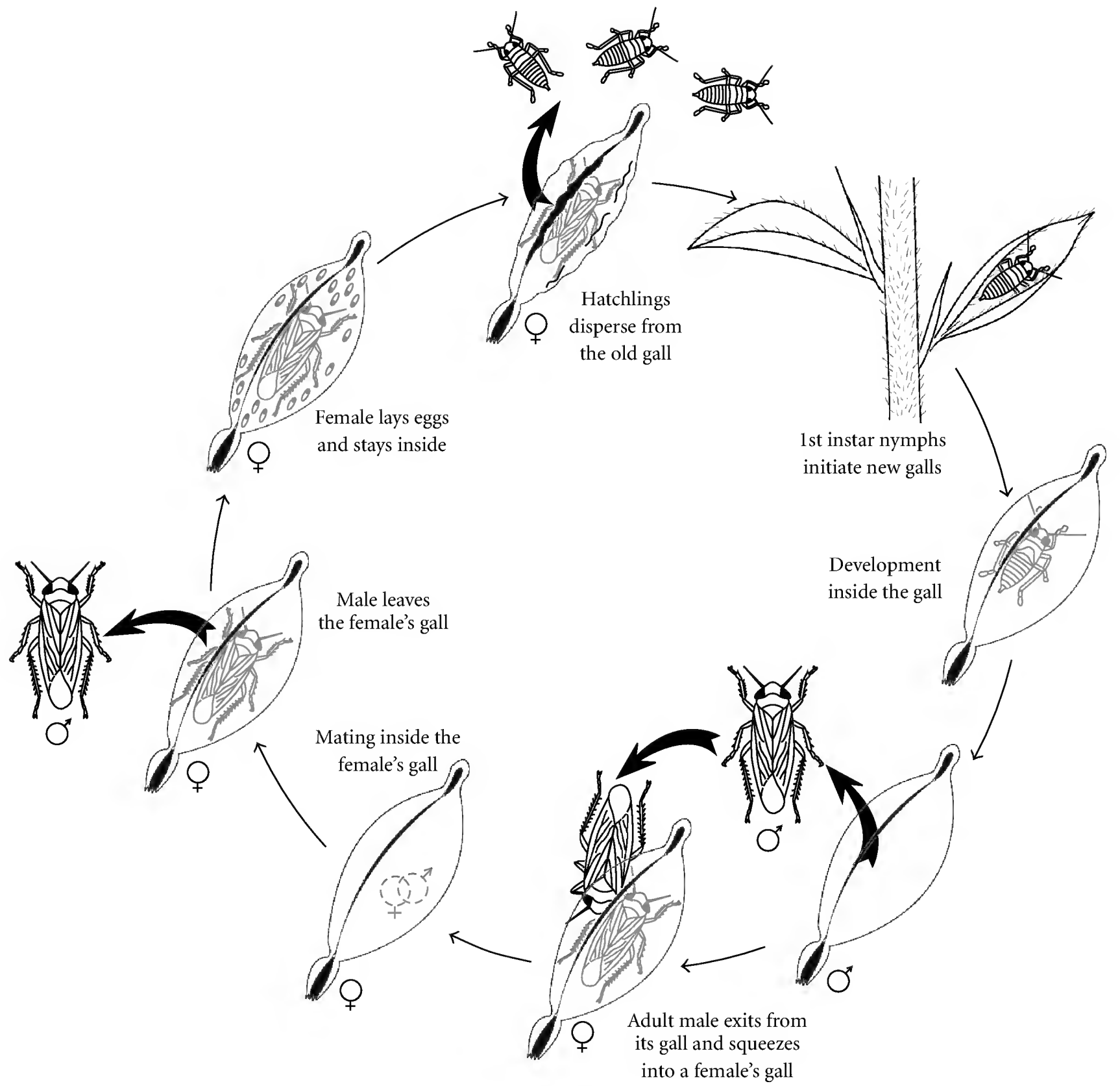


Figure 3: The life cycle of *Scenergates viridis*.

but already by mid-June most galls contained adults. These adults must mate and lay eggs approximately from mid-June to mid-July. During the first half of August 2011, numerous older looking galls contained live adult females of the overwintering generation next to their fully developed eggs and/or hatchlings, referred to as the *summer generation* (Figure 6(a)). This means that females of the overwintering generation remain alive inside the galls throughout the embryonic development of their offspring. Remarkably, 34 out of 38 such examined females (89.5%) had apical parts of their hindlegs injured or missing (Figure 6(b)). During the same period we observed fresher galls, which belonged to the summer generation and contained every developmental stage of the leafhopper from the first instars (very numerous) to adults (very few). By mid-August these adults had already begun mating and laying eggs of the next overwintering generation. Therefore, the two generations overlap to such an extent that some females of one overwintering generation survive until the first eggs of the next overwintering generation are laid (Figure 4).

In early September 2010, a few galls still contained summer-generation nymphs and virgin adults, while the majority contained summer-generation females laying eggs (Figure 1(d)). Only 16 out of 50 such females (32%) had apical parts of their hindlegs injured or missing. The dry galls collected in December 2010 contained dead females next to their live overwintering eggs (Figures 7(a) and 7(b)). The aboveground parts of camelthorn shrubs die off by late November [15]; drying up of phloem sap around that time limits the potential life span of postoviposition females (Figure 4).

It appears that adult females do not typically leave their galls. Our attempts during both trips to collect *S. viridis* by beating camelthorn with a sweep net yielded no specimens of either sex. However, in August 2011 a postoviposition female was spotted walking freely; apparently it had left its

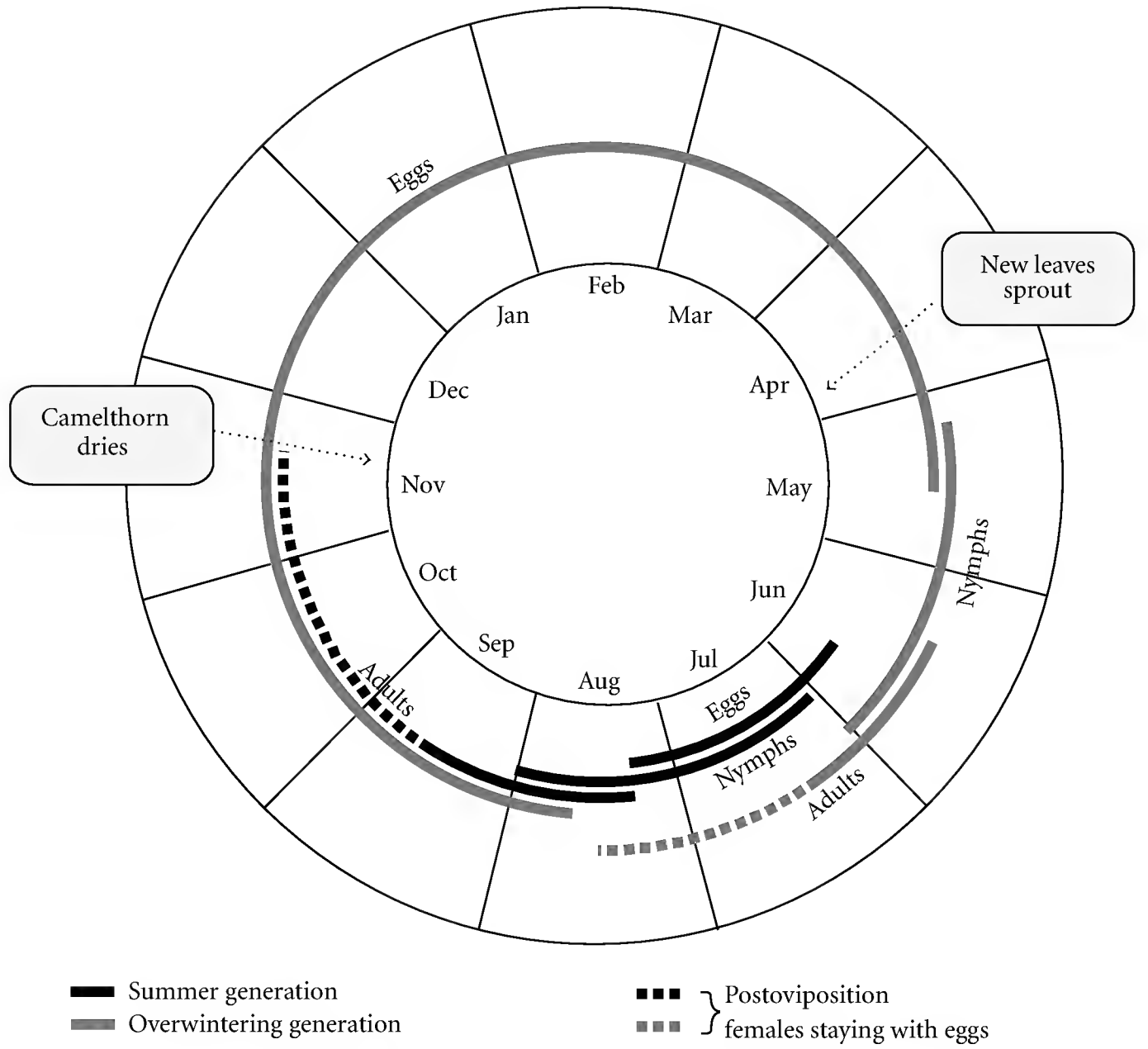


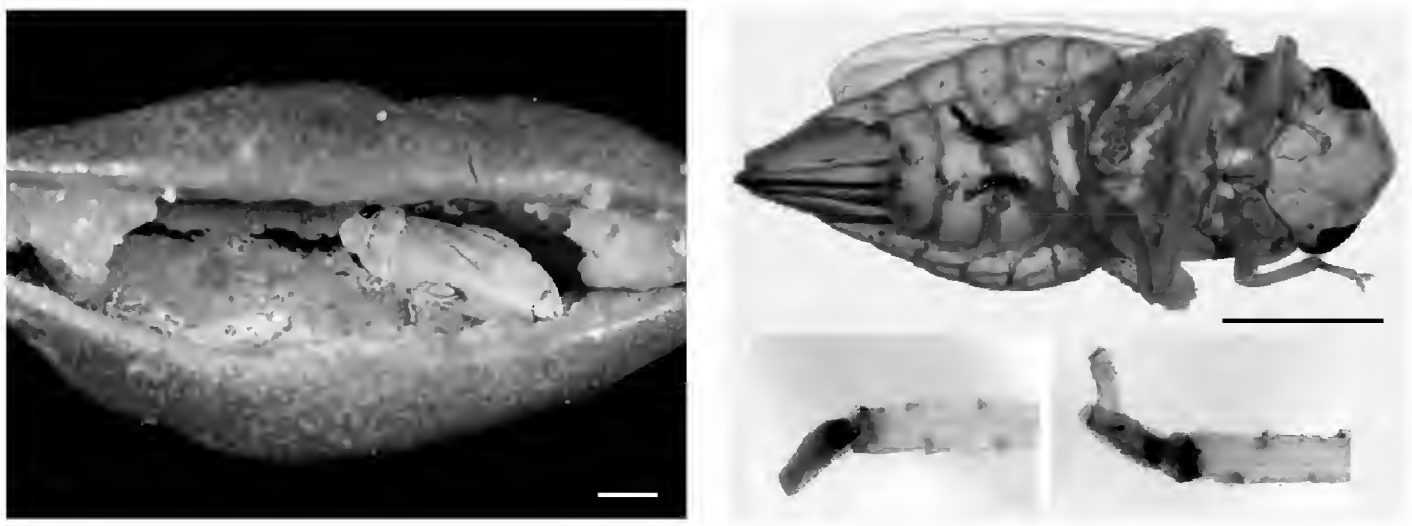
FIGURE 4: The annual cycle of *Scenergates viridis*.

FIGURE 5: A camelthorn branch with galls induced by the overwintering and summer generations of *Scenergates viridis* on the main stem and lateral shoot, respectively. Note that the older galls are slightly open.

gall through the same crack as its offspring (see below). This may explain how the females studied by Vilbaste [11] and Emeljanov [12] were collected; in neither case were the examined specimens labeled as originating from galls.

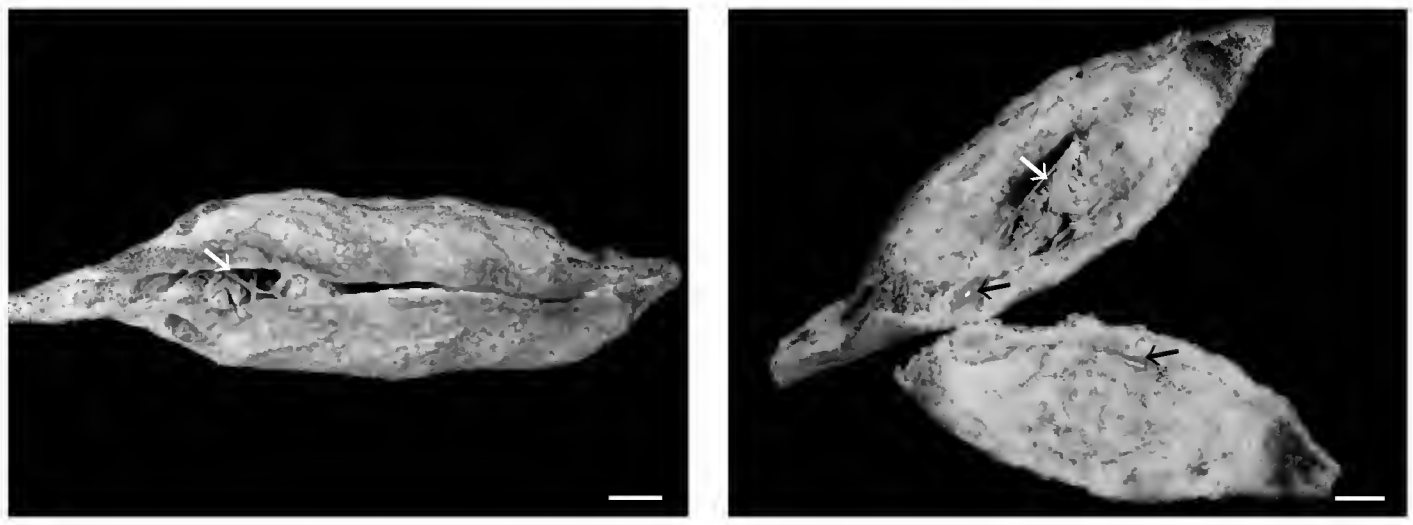
4.2. Gall Initiation. Despite the abundance of first-instar nymphs, both hatching inside mother galls and having already formed galls of their own, we failed to observe initiation of new galls. In the field we never found exposed nymphs or any incompletely formed, nascent galls. Attempts to place hatchlings onto young leaves at room-temperature conditions (+20–25°C) proved unsuccessful except in one case. After a period of feeding most nymphs escaped, but one produced a nascent gall when it was left unobserved for a few hours (Figures 8(a) and 8(b)). In the field the galls containing first-instar nymphs were 5–7 mm long; on one occasion two nymphs were discovered inside one gall.

4.3. Gall Structure. The number of galls per shrub varied between none and ca. 1,000. In the latter case virtually every leaf was transformed into a gall. The galls had the appearance of fusiform pods, round in cross-section or slightly flattened laterally, with a short funnel-shaped basal petiole and, typically, a similar apical lobe (Figures 1(c), 9(a)–9(d)). The orifice of each funnel was plugged from the inside by masses of dried excrement (see below). Except for these terminal openings, the valves of inhabited galls were tightly shut (Figures 1(c), 9(b)). In the galls containing



(a) (b)

FIGURE 6: (a) A gall of *Scenergates viridis* split open to expose the female (overwintering generation) next to its offspring, first-instar nymphs (summer generation). (b) A postoviposition female with damaged apical parts of the hindlegs (insets). Scale bars: 1 mm ((a)-(b)).



(a) (b)

FIGURE 7: A dry overwintering gall of *Scenergates viridis,* collected in December. (a) Intact. (b) Split open. Note the dead female (white arrows) and live eggs (black arrows). Scale bars: 1 mm ((a)-(b)).

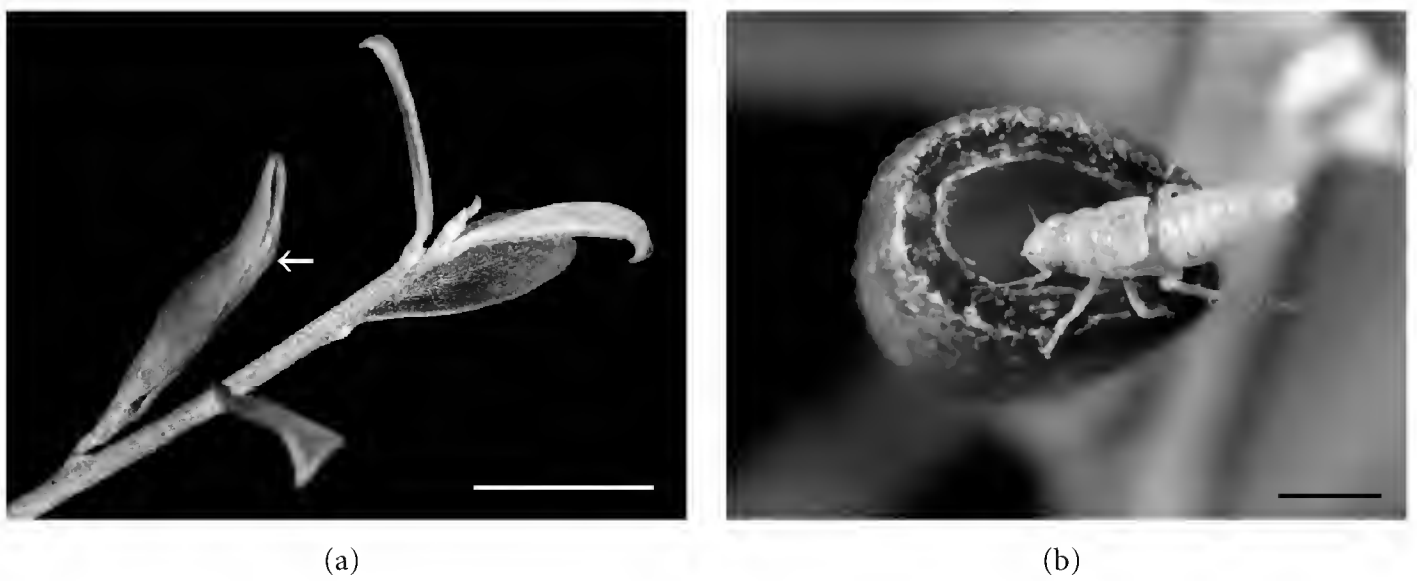
summer-generation eggs ready to hatch, the valves were slightly open (Figure 9(c)), while the galls abandoned by adult males and those of nymphs killed by pipunculids were widely open (Figure 9(d)).

The galls remain green and grow as leafhopper nymphs grow inside them. The length of the galls containing adult males we observed was 10.1 ± 2.10 mm (mean $\pm$ SD, $n = 24$), and those containing adult females 14.0 ± 3.10 mm ($n = 189$). The largest gall observed was 35 mm long. The average length of a mature egg-containing gall is close to the average length of leaves on the same shrub. In contrast, the mean maximum wall thickness of such galls, 1.6 mm, is three times the thickness of mature leaves, 0.5 mm (Figures 10(a) and 10(b)). The wall thickness of the last-instar immature male galls only slightly exceeds that of regular leaves, but the galls of the last-instar immature females and preoviposition adult females have conspicuously thicker walls (Figure 10(b)).

The gall's inner surface corresponds to the adaxial and its outer surface to the abaxial sides of the original leaf. Young galls are variably pubescent on both sides. In older galls the outer surface is usually bare and glossy (Figure 11(a)), while the inner surface retains appressed hairs, ca. 150 μm in length (Figure 11(b)). On a smaller scale, the inner epidermis is coated with wax platelets and brochosomes (Figures 11(c) and 11(d)). The latter can be found already in the youngest galls but appear to be more abundant in the egg-containing female galls. Wax platelets and brochosomes are responsible for the pruinose appearance of the gall's inner surface (Figure 11(e)). The inner surface is more hydrophobic than the outer one (Figure 11(f)). The static contact angles of small water droplets placed onto the outer and inner surfaces were $110.2 \pm 18.94^\circ$ (mean $\pm$ SD, $n = 14$) and $150.1 \pm 9.89^\circ$ ($n = 14$), respectively.

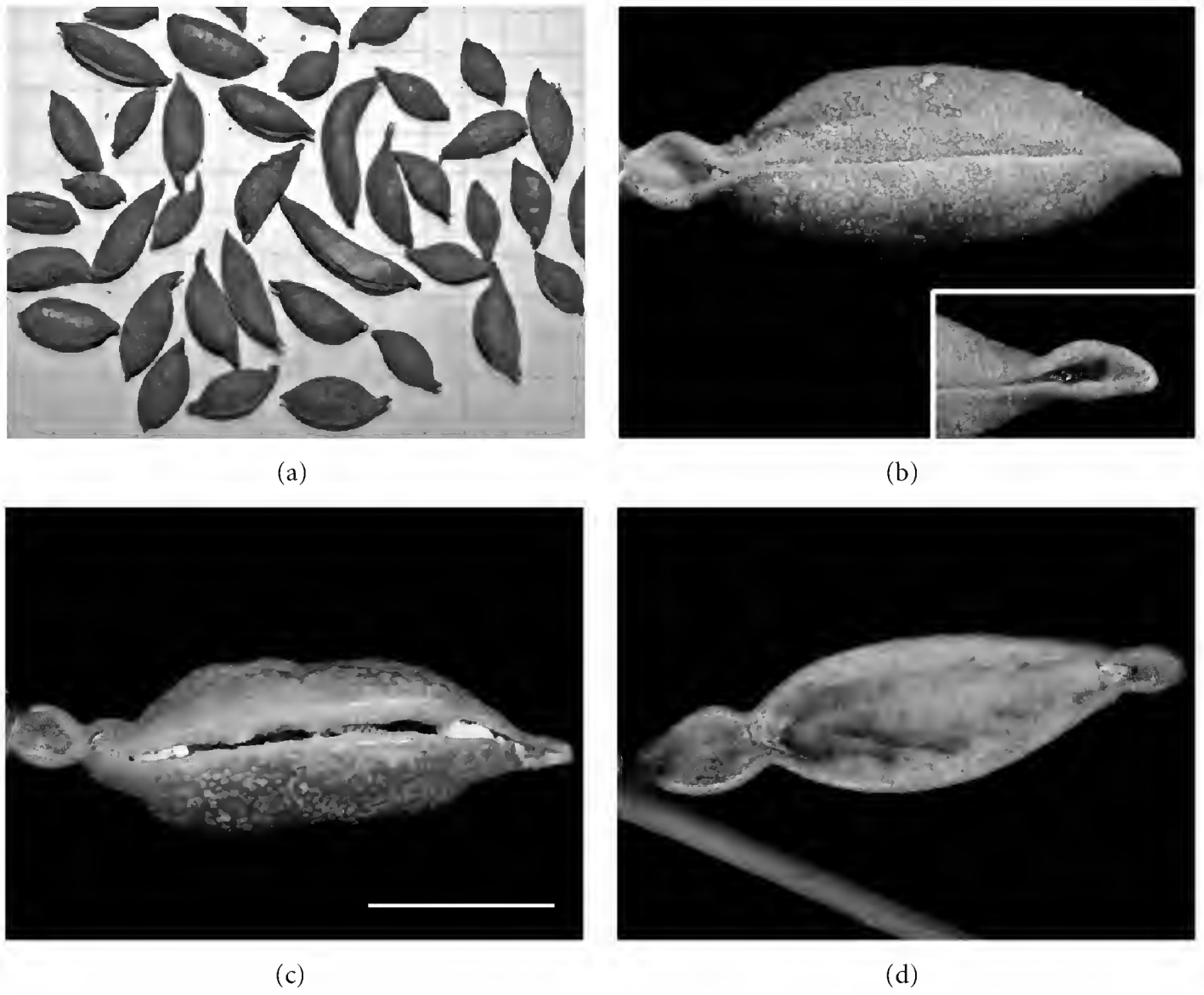
4.4. Disposal of Excrement and Exuviae. The gall interior is remarkably neat and clean (Figure 12(a)). The basal and apical ends of the gall contain amorphous white or light yellow masses consisting of dried leafhopper excrement (honeydew), brochosomes, and crushed exuviae (Figures 12(a)–12(d)). These masses plug the terminal orifices of the gall cavity and therefore are referred to here as *excrement plugs*. Occasionally they also contain dead parasitoids or other small intruders (Figure 12(e)). The plugs do not melt when touched with a red-hot needle, indicating that they do not contain wax. When galls are placed in sealed plastic bags, the plugs quickly get mouldy.

Mitjaev [9] observed that the exuviae were crushed and pressed together with excrement but that the last 5th-instar



(a) (b)

FIGURE 8: A nascent gall of *Scenergates viridis*, a few hours old, rolled in the laboratory. (a) The branch with the gall (arrow). (b) The same gall cross-sectioned, with the first-instar nymph exposed. Scale bars: 5 mm (a), 1 mm (b).



(a) (b)

(c) (d)

FIGURE 9: General structure of galls of *Scenergates viridis*. (a) Galls on 5 mm grid paper. (b) A closed gall containing a female and developing eggs; such galls often have funnel-shaped apices (inset). (c) A slightly open gall containing a female and ready-to-hatch eggs or first-instar hatchlings. (d) An open male gall after the male has exited. Note remnants of excrement plugs near both ends of the gall. Scale bar: 5 mm (c).

exuviae remained intact. We have found this to be true only for males, their intact last-instar exuviae lying freely in the gall cavity (recorded in 23 galls, Figure 12(f)). Females at least partly crush the exuviae of all nymphal instars (recorded in 30 galls). The exuviae are often reduced to small, hardly recognizable pieces (Figure 12(c)).

4.5. Mating. Mitjaev [9] observed adult males on the outsides of closed female galls and suggested they were waiting for females to come out. In August 2011 we observed such "waiting" males (Figures 13(a)–13(c)) eventually squeeze into the galls (Figures 13(d) and 14(a)–14(e)). First the male pushed one side of its body in between the valves with a lateral thrust and somewhat raised the posterior body, so that one anterolateral head margin became wedged in the slit. Then, in small increments, the rest of the body was pushed inside. During this process the male became noticeably compressed so that a part of its mesonotum normally

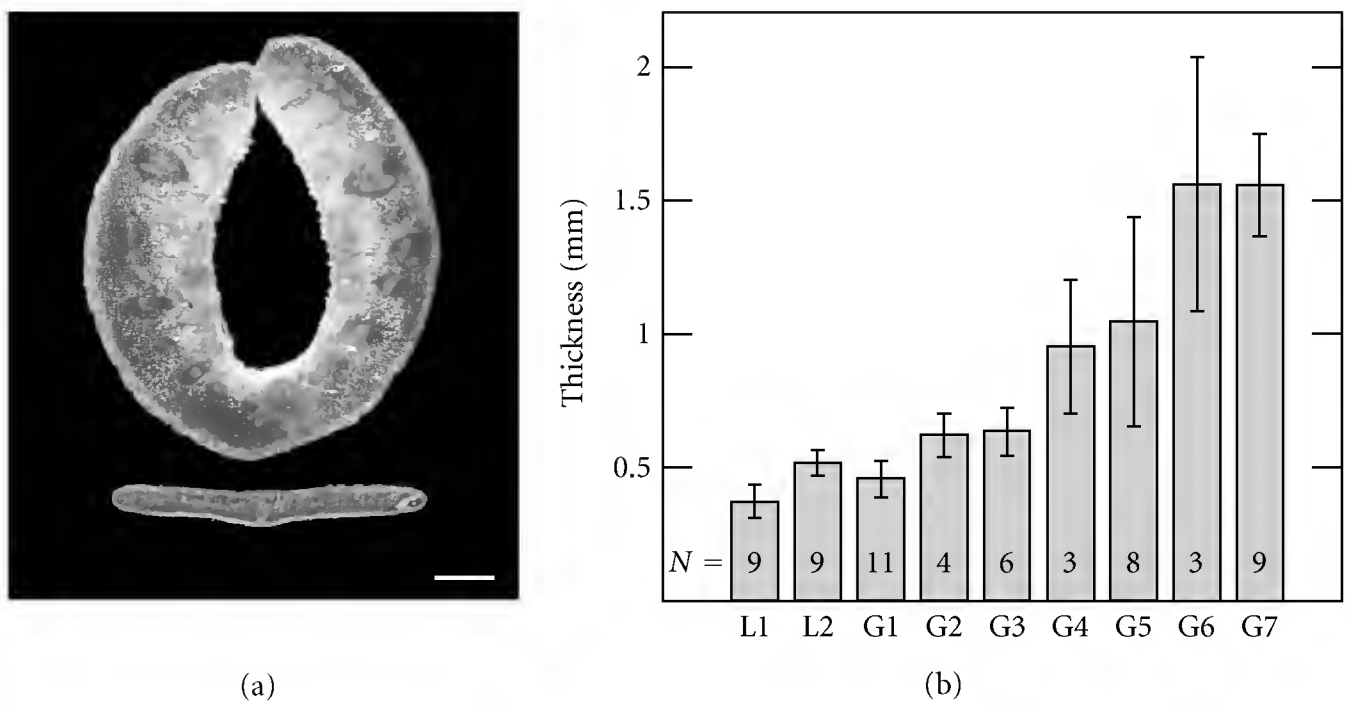


FIGURE 10: Changes of the leaf lamina thickness during the development of galls of *Scenergates viridis*. (a) Cross-sections of an egg-containing gall (top) and an ungalled camelthorn leaf of the same length (bottom). (b) The maximum thickness of camelthorn leaves (L1-L2) and *Scenergates viridis* gall walls (G1–G7). Numbers on the bars indicate sample sizes; error bars indicate the standard deviation. L1, young leaves; L2, mature leaves; G1, galls with 2nd- or 3rd-instar nymphs, sex not identified; G2, galls with 4th-instar nymphs, sex not identified; G3, galls with 5th-instar male nymphs; G4, galls with 5th-instar female nymphs; G5, galls with virgin females; G6, galls with postoviposition females and freshly laid eggs (overwintering generation); G7, galls with postoviposition females and fully developed eggs (summer generation). Scale bar: 1 mm (a).

concealed under the pronotum got exposed (Figures 13(d) and 14(b)). This strenuous process appeared to be assisted by vigorous pushing movements of all leg pairs, even though both hindlegs and at least one midleg were mostly just dangling in the air. After some time, the male left the female gall.

Altogether we observed two males penetrating into female galls and one male penetrating, obviously mistakenly, into a gall containing a last-instar immature male. In two instances, the process was recorded on video (Figures 14(a)–14(e)). In one case the penetration took 2.2 minutes and the exit 15 seconds; in the second case the penetration took 8.5 minutes and the exit 25 seconds. Additionally, we recorded an unsuccessful attempt of penetration; when the male was not able to insert more than its head margin into the slit, it eventually abandoned the task. All three penetrations observed in the field took place in full sun during the hottest hours of the day, between noon and 2 pm, when temperatures in the shade reached 37–39°C. In two cases we were able to confirm mating inside the gall upon penetration of the male. Three out of 170 examined ethanol-preserved galls containing adult females also contained a male; in two such galls the male was partly destroyed.

4.6. Oviposition. The eggs were inserted under the epidermis of the inner side of the gall nearly parallel to the surface (Figures 15(a)–15(c)), their anterior poles slightly protruding from the scars (Figure 15(d)). In the proximal half of the gall, the posterior poles of the eggs were directed towards, and in the apical half away, from the gall's base (Figure 15(c)), indicating that during oviposition the female turns 180°. Both the gall cavity surface (Figure 15(b)) and the protruding egg poles (Figures 15(e)-15(f)) were densely powdered with brochosomes.

The number of eggs per gall of the overwintering generation, counted in August 2011, was 146 ± 53.2 (mean ± SD, $n = 50$). Since the females do not leave their galls, this figure is an estimate of their entire lifetime reproductive output. In early September 2010, when summer-generation females were still actively laying eggs, the number of eggs per gall was just 11.3 ± 13.3 (mean ± SD, $n = 138$).

4.7. Hatching and Nymphs. As in other Auchenorrhyncha [21, 22], the hatching phase is a pronymph, which molts into a first-instar nymph as soon as it is out of the egg (Figures 16(a) and 16(b)); the cast-off pronymphal exuvia often remains stuck to the empty chorion (Figure 16(c)). The hatchlings exit the gall through a narrow crack between the partially open valves (Figures 7(a) and 9(c)). However, we observed several galls that failed to open, with dead summer-generation hatchlings trapped inside (Figure 16(f)). The first-instar nymphs display two color morphs, one almost unpigmented and the other dark-patterned (Figure 16(d)). Although sexual dimorphism is not commonly observed among young instars of leafhoppers, these can be females and males, respectively. Coloration of the older nymphal instars is sexually dimorphic, males being darker than females (Figure 16(e)).

4.8. Locomotion. Nymphs and adults of both sexes were capable of jumping. The maximum recorded horizontal distance jumped was 8 cm for a first-instar nymph and 20 cm for an adult female released from its gall. Both males (Figures 13(a)–13(d)) and females (Figures 15(a) and 16(f)) were

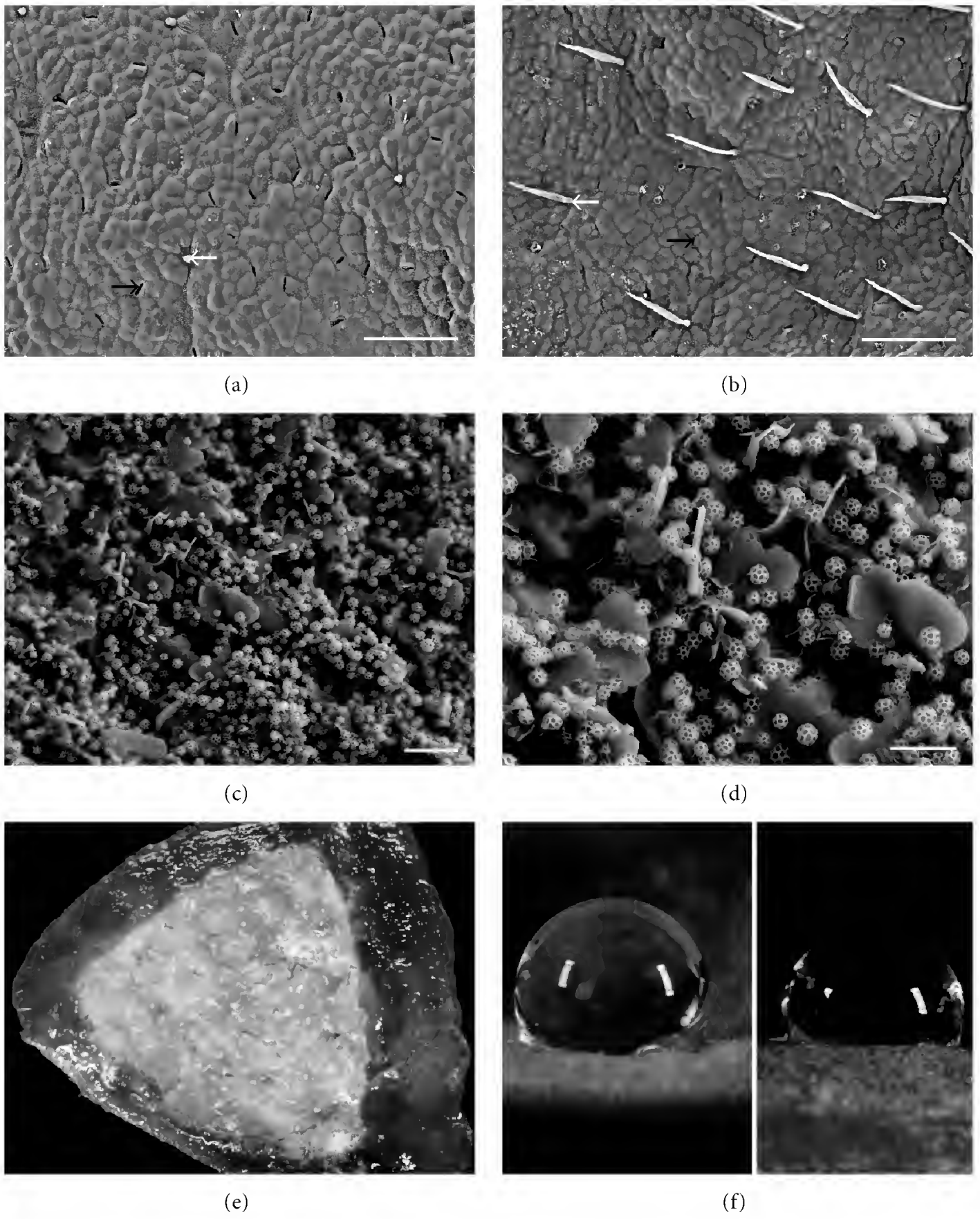


FIGURE 11: Microstructure and water repellence of the surface of galls of *Scenergates viridis*. (a) The outer surface of an ethanol-preserved gall, critical-point dried, and observed in a regular SEM: the white arrow points to the base of a broken hair, the black arrow points to a stoma. (b) Same, the inner surface: the white arrow points to a hair, the black arrow points to a stoma. (c) The inner surface of a frozen gall observed in a Cryo-SEM, showing brochosomes and wax platelets. (d) A closeup of the same. (e) A fragment of a valve of an egg-containing gall, showing its inner epidermis coated with the whitish pruinose layer of brochosomes and wax platelets. (f) Droplets of water resting on the inner (left) and outer (right) surfaces of a gall. Scale bars: 200 μm ((a)-(b)), 3 μm (c), 2 μm (d).

macropterous, with normally developed fore and hind wings, but flight was not observed.

4.9. Natural Enemies

4.9.1. Lepidoptera. Numerous galls are destroyed by caterpillars of *Filatima* sp. (Gelechiidae). The younger caterpillars mine the gall valves, while the older ones devour the fleshy inner layer of the gall together with leafhopper eggs and the mother leafhopper without damaging the outer epidermis (Figures 17(a)–17(d)). Once the interior of the gall is destroyed, the caterpillar chews an exit hole and leaves; such damaged galls remain closed (Figure 17(d): inset). We never found pupae of this moth in the field but were able to rear two adult females in captivity (Figure 17(d)); an additional female moth was collected directly from a camelthorn. In the absence of males, the moths could not be identified beyond the genus level (Alexei Bidzilya, personal communication).

4.9.2. Diptera. Some partially or completely open galls contained puparia of *Tomosvaryella argyrata* De Meyer (Pipunculidae) glued to their inner surface, often next to

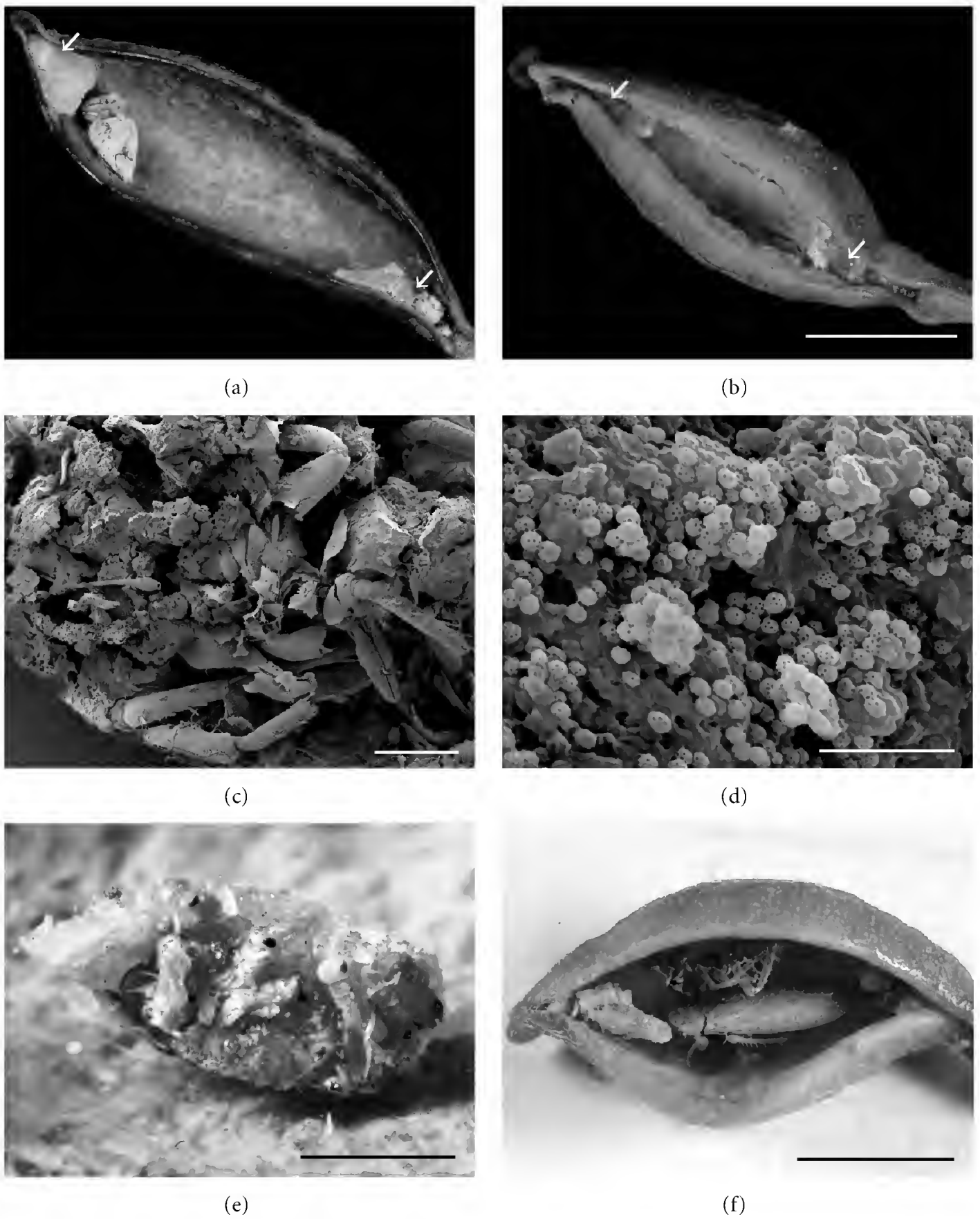


FIGURE 12: Disposal of excrement and exuvia in the galls of *Scenergates viridis*. ((a), (b)) Two adult galls opened artificially to expose their contents. Arrows point to the excrement plugs. The gall shown in (a) contains an etherized female. (c) A fragment of an excrement plug with remnants of crushed exuviae (pieces of legs, proboscis, and antenna are discernible). (d) A fragment of an excrement plug showing brochosomes. (e) A chunk of an excrement plug from the gall shown in (a); note ten trapped female *Aphelinoidea* sp. wasps. (d) A male gall artificially opened to expose the adult male and its intact last-instar exuvia; note also massive excrement plugs at both ends of the gall. Scale bars: 5 mm (b), 200 μm (c), 5 μm (d), 1 mm (e), 2.5 mm (f).

sucked-out remains of a last-instar *S. viridis* nymph (Figures 18(a) and 18(b)). Nymphs with pipunculid larvae in the abdomen were also observed (Figure 18(c)). One male and three female adult flies were reared in the laboratory (Figure 18(d)).

4.9.3. Hymenoptera: Trichogrammatidae. Among egg parasitoids of *S. viridis*, an undescribed *Aphelinoidea* sp. (Hymenoptera, Trichogrammatidae), 0.5–0.8 mm in length, kills the largest proportion of eggs. In August 2011 among 50 sampled galls containing a total of 7,152 fully developed summer-generation eggs, the mean percentage of parasitized eggs per gall was 43.0% (range, 0.0–98.2%). From such galls we reared 181 female and 171 male *Aphelinoidea* sp. The anterior poles of parasitized eggs become dark (Figure 19(a)) and their posterior poles become filled with an orange substance, apparently the meconium excreted by the wasp larva prior to pupation. The emerging wasp chews an exit hole next to the egg's anterior pole on the inner surface of the gall wall (Figure 19(b)). A number of emerged wasps were found inside the galls, and one instance of mating was observed there. During the same period, in August

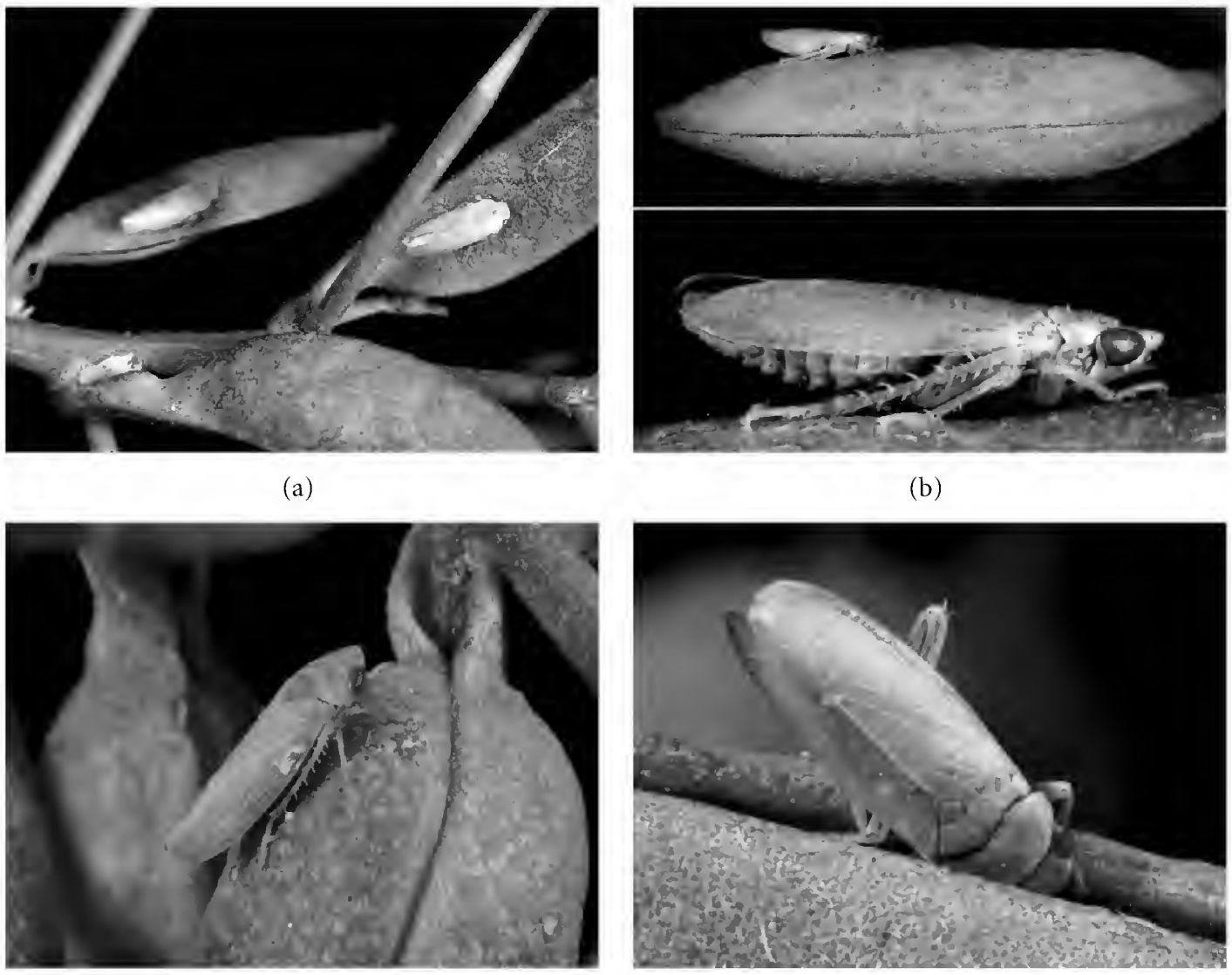


FIGURE 13: Premating behavior of males of *Scenergates viridis*. ((a)–(c)) Males "waiting" on the outside of female galls. (d) A male squeezing into a female's gall.

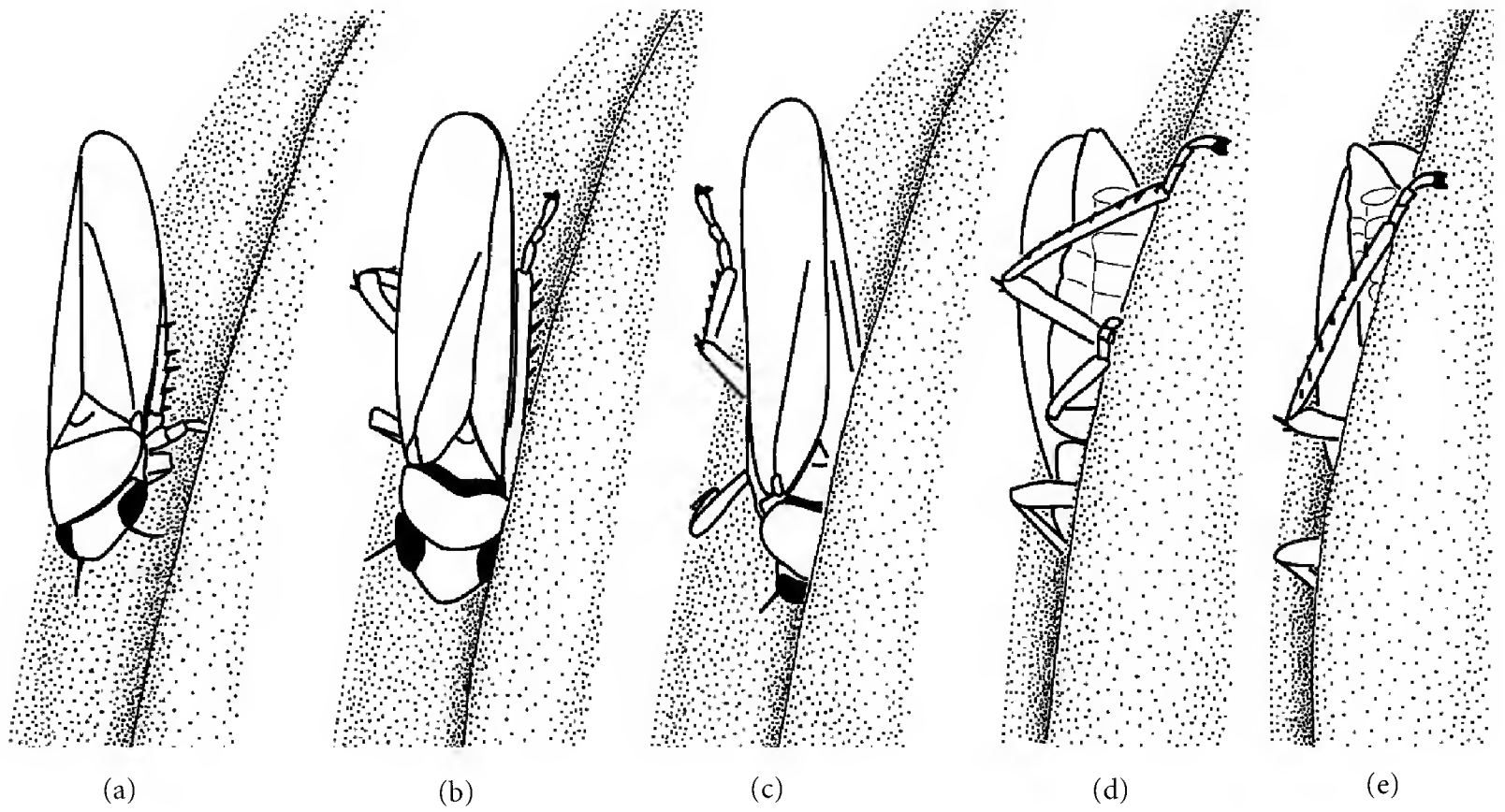


FIGURE 14: A male *Scenergates viridis* squeezing into a female's gall. ((a)–(e)) Successive stages, based on a video recording. The stages (d) and (e) were observed from the ventral side.

2011, we observed female *Aphelinoidea* sp. running on the outsides of the galls containing females just beginning to lay eggs of the overwintering generation, apparently looking for a way in. Some such galls contained dead *Aphelinoidea* sp. females stuck in the excrement plugs (Figure 12(e)). In September 2010, 21 of 88 examined galls with overwintering-generation eggs contained between 1 and 10 (average, 2.2) dead *Aphelinoidea* sp. females (but no males) stuck in the excrement plugs. From nine dry overwintering galls collected in December 2010 (Figure 7(a)), five male and nine female

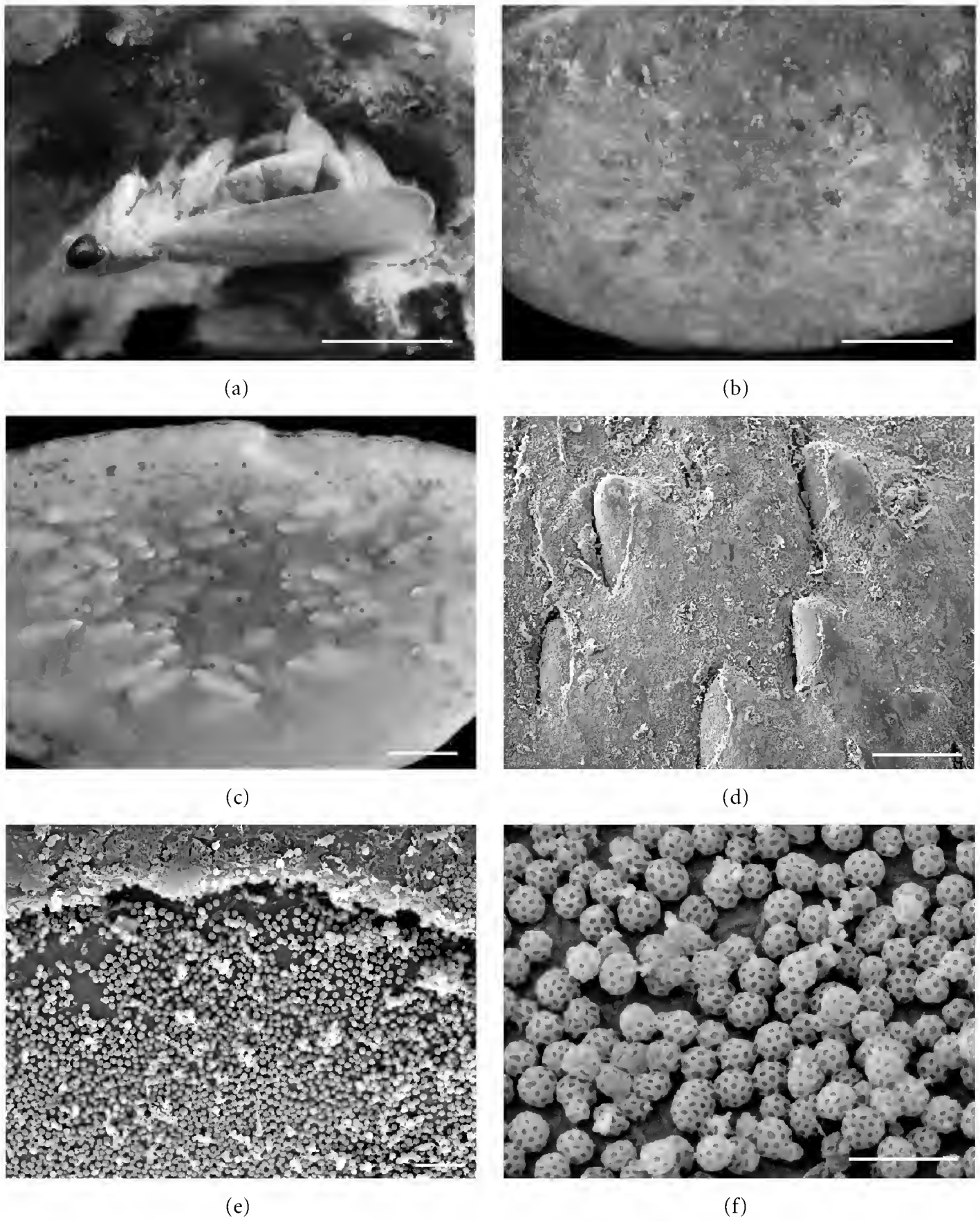


FIGURE 15: Oviposition and eggs of *Scenergates viridis*. (a) Remnants of a female that died in the course of egg laying with her ovipositor inserted under the gall's inner epidermis. (b) The inner surface of an intact gall valve with yellow eggs partially visible under the plant epidermis; note the conspicuous whitish pruinosity (brochosomes) on the latter. (c) The inner surface of a gall valve soaked in ethanol, with eggs visible underneath the epidermis; note that only some of the embryos have already developed their eyes. (d) The inner surface of a gall valve showing anterior poles of the eggs protruding from oviposition slits. (e) Closeup of an egg tip (lower part of the photo) protruding from under the epidermis (upper part of the photo). (f) Brochosomes on the egg surface. Scale bars: 1 mm ((a)–(c)), 200 μm (d), 5 μm (e), 2 μm (f).

Aphelinoidea sp. were reared next May. The first of these wasps emerged a month later than the first leafhopper hatchlings, by that time most leafhopper nymphs had already hatched out.

The second species of Trichogrammatidae, *Paracentrobia* sp., apparently also emerges from the gall's inner surface. Only eight females and four males were reared from galls containing summer-generation eggs in August 2011. No details on the appearance of the eggs parasitized by this species or the rate of parasitization are available.

4.9.4. Hymenoptera: Mymaridae. Unlike *Aphelinoidea* sp., an undescribed *Gonatocerus* sp. (Hymenoptera, Mymaridae), 0.8–1.1 mm in length, parasitizes eggs of *S. viridis* through the gall's outer epidermis. Consequently, adult wasps emerge by chewing exit holes in the gall's outer surface (Figures 19(c) and 19(d)). As in *Aphelinoidea* sp., the larvae of *Gonatocerus* sp. excrete a bright orange substance from their rear end, but since the two parasitoids have opposite orientations within the host eggs, those containing *Gonatocerus* sp. are easily recognizable (Figure 19(a)). In August 2011, among 50

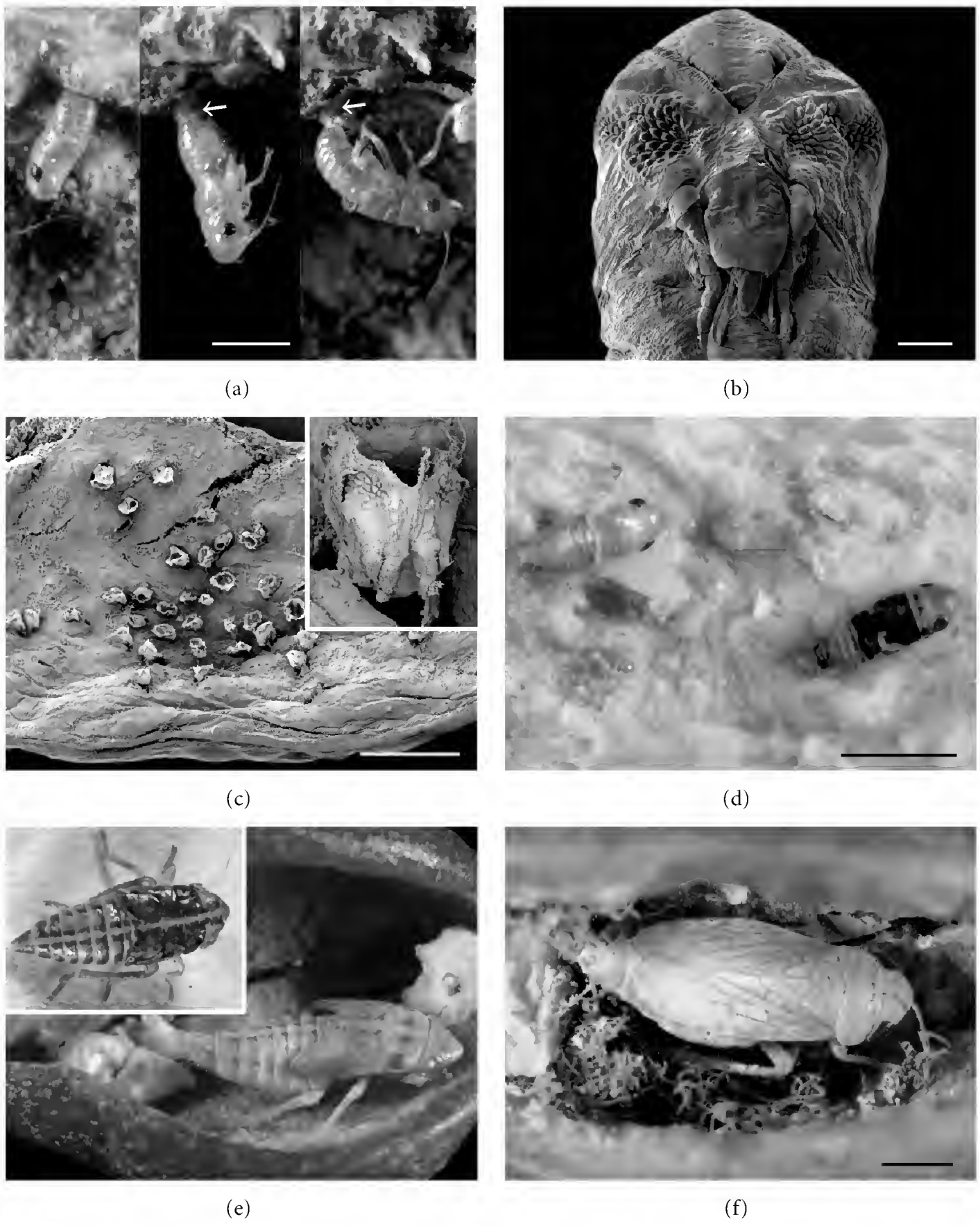


FIGURE 16: Eclosion and immatures of *Scenergates viridis*. (a) Three stages of hatching: arrow points to the pronymphal exuvia. (b) The anterior end of a pronymph in ventral view, with the first-instar nymph's head emerging from the V-shaped rupture at the apex. (c) The inner surface of a dry overwintered gall after the nymphs have hatched out, with numerous pronymphal exuviae attached to eclosion slits; one exuvia is magnified (inset). (d) Two differently colored first-instar nymphs from the same brood. (e) Female (below) and male (inset) last-instar nymphs; the nymphal gall was opened artificially. (f) A gall which failed to open, here opened artificially to expose the dead female atop the mass of dead hatchlings. Scale bars: 0.5 mm ((a), (d)), 50 μm (b), 1 mm (c), 1 mm (f).

ethanol-preserved galls containing fully developed summer-generation eggs, the mean percentage of parasitized eggs per gall was 1.5% (range, 0–7.7%). Fifty-two females and 33 males were reared from such galls. During the same period we recorded on video two female *Gonatocerus* sp. probing the outer surface of a gall with their ovipositors.

4.10. Other Associates. In one area we observed a group of camelthorn shrubs with galls containing summer-generation immatures of *S. viridis* and covered externally with unidentified apterous aphids tended by the *Tapinoma karavaievi* Emery ants (Hymenoptera, Formicidae) (Figures 20(a)–20(c)). A few open galls were both covered and filled with aphids (Figure 20(d)). Remarkably, except for a few individuals that were dispersing, other parts of the plants, including normal leaves, were free of aphids.

Besides trichogrammatids, the gall excrement plugs often contained trapped unidentified mites and adult and immature thrips from the families Thripidae and Phloeothripidae (Thysanoptera). Since these were observed both in galls that contained and those that did not contain eggs of *S. viridis*, they were probably inquilines feeding on the gall tissue rather than the leafhopper eggs.

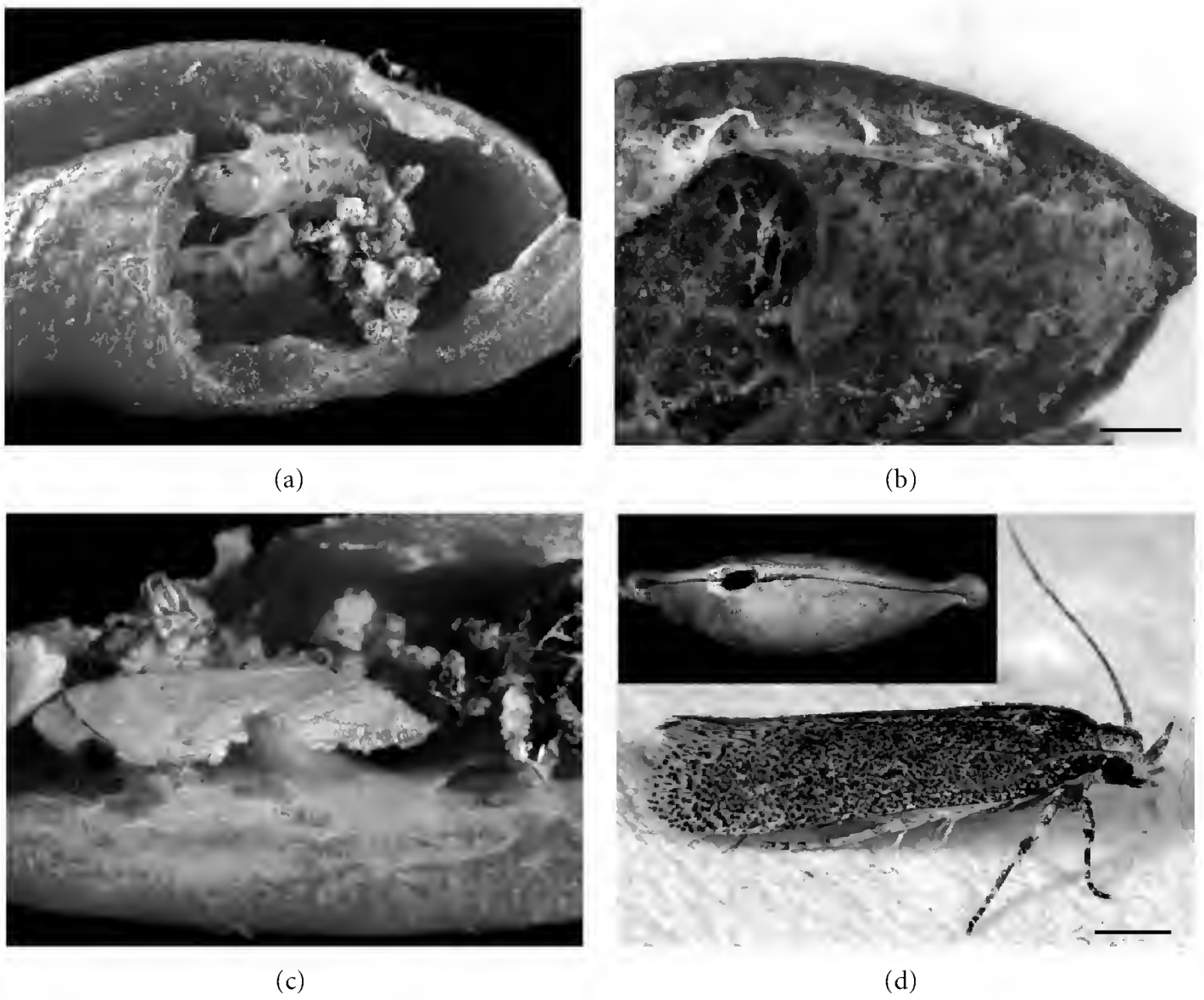


FIGURE 17: *Filatima* sp. moth (Gelechiidae), a predator of the galls of *Scenergates viridis*. (a) A gall broken open to expose the caterpillar; note the caterpillar's frass and silk netting. (b) The inner surface of a gall valve, partially eaten (in the lower left area) by a caterpillar. (c) Remnants of a partially eaten *S. viridis* next to caterpillar frass; note also the silk netting. (d) A reared female moth and a gall with a caterpillar's exit hole (inset). Scale bars: 1 mm ((b), (d)).

5. Discussion

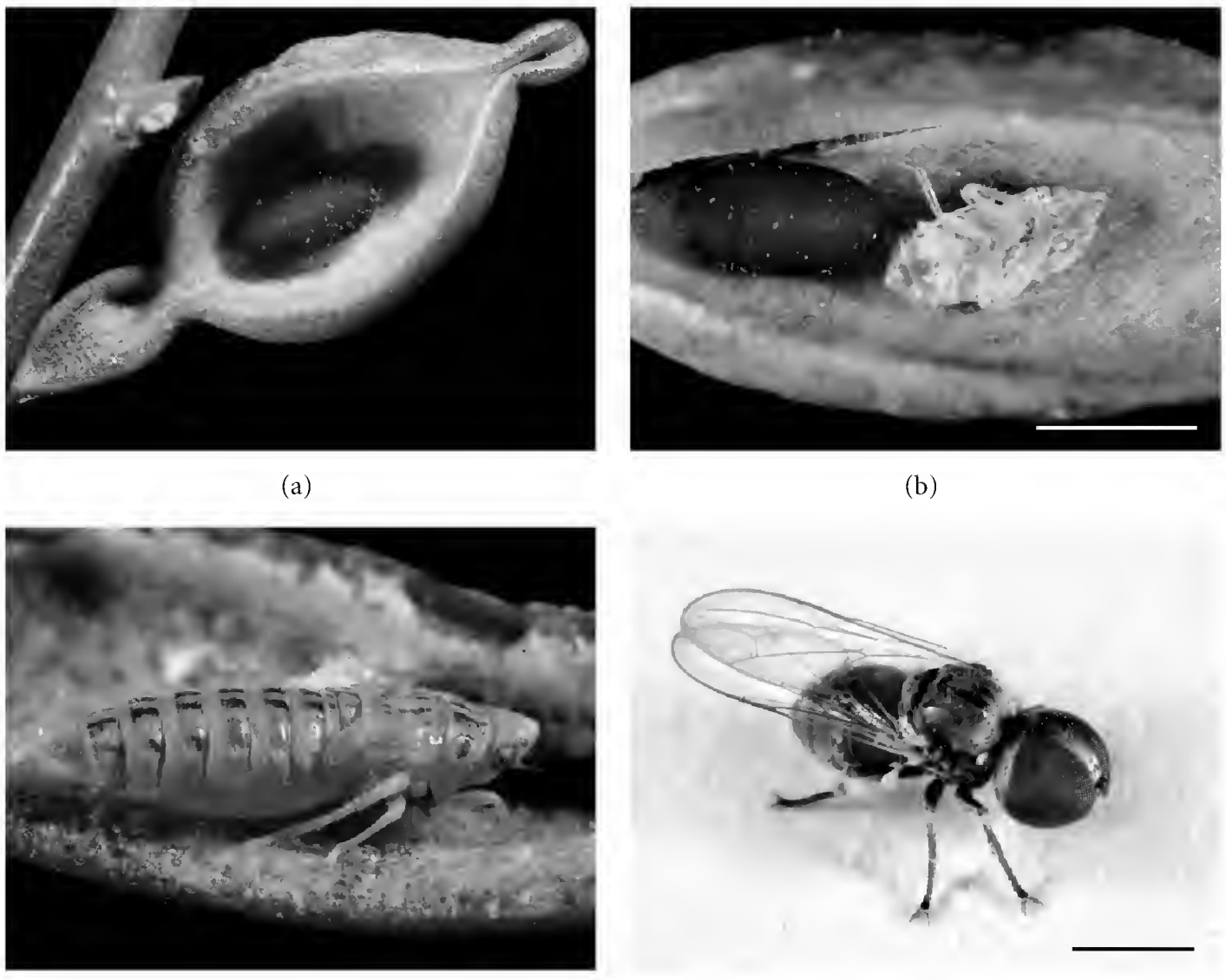
5.1. Key Galling Traits of Scenergates. The camelthorn gall leafhopper is an accomplished gall maker and displays a number of related adaptations (reviewed below). This is particularly striking given the lack of any known galling prowess in other Auchenorrhyncha. It is worth noting, however, that these adaptations coexist in *S. viridis* with the general appearance, morphology, and behaviors typical of free-living leafhoppers, such as jumping and the presence of wings in both sexes.

5.1.1. Control over Leaf Growth and Movement. The nymphs of *S. viridis* induce profound modifications of *Alhagi* leaves. The following three aspects of their control over leaf growth and movement may each potentially involve a separate mechanism.

(1) Triggering of the Initial Folding of the Leaf. The fact that we were unable to find incompletely formed galls or exposed nymphs suggests that initial folding of the young leaf is too rapid to involve growth of the tissue. Such folding may instead result entirely from the changed turgor pressure within the leaf, as in nastic movements. Nascent camelthorn leaves are longitudinally folded, and as they gradually unfold, they pass through a boat-shaped stage resembling a partially open pod. Hypothetically, some galls may be initiated at this stage, thus limiting the parasite's task to merely reclosing the pod. However, it appears that many young leaves passing through this stage are too small for the nymph to squeeze in.

(2) Modifying Further Growth of the Leaf. The galls are similar to normal leaves in length but their surface area is significantly larger, indicating increased lateral growth of the leaf lamina. Moreover, the gall valves are thicker than normal leaves, the egg-containing female galls being particularly succulent (Figure 10(a)). Measurements demonstrated that the walls of female galls begin swelling prior to oviposition, with the difference in wall thickness between male and female galls becoming noticeable during the last nymphal instar (Figure 10(b)). In similarly shaped leaf galls induced by Psyllidae, swelling of the walls results mostly from cell proliferation and to a lesser degree from an increase in cell volume [23].

The increased thickness of the female galls of *S. viridis* is likely an adaptation to their special role in reproduction. A thick wall may protect the eggs from desiccation, particularly during winter months when the galls dry out. It may additionally act as a barrier against *Gonatocerus* sp. wasps, which reach leafhopper eggs from outside the gall. Given that the wasps' ovipositors range in length between



(a) (b) (c) (d)

FIGURE 18: *Tomosvaryella argyrata* (Pipunculidae), a parasitoid of *Scenergates viridis*. (a) A puparium glued to the inner surface of a gall; note that the gall has fully opened. (b) A puparium next to sucked-out remains of a last-instar immature *S. viridis*. (c) A last-instar female nymph of *S. viridis* with a pipunculid larva inside its swollen abdomen (compare with an unparasitized nymph in Figure 16(e)). (d) An adult *T. argyrata* reared from *S. viridis*. Scale bars: 2 mm (b), 1 mm (d).

approximately 0.55 and 0.65 mm (*S. Triapitsyn*, personal communication) and that leafhopper eggs lie close to the wall's inner surface, the average maximum wall thickness of 1.6 mm (Figure 10(b): G6-7) puts the eggs well out of the wasps' reach. Although the gall walls are not uniformly thick, the swelling may contribute to the considerably lower rate of parasitization by *Gonatocerus* sp. than that by *Aphelinoidea* sp. Lastly, thick walls may be essential for accommodating a large number of leafhopper eggs without killing the gall.

(3) Maintenance of the Closed State of the Gall. While opening of dry overwintering galls is a trivial result of their shriveling (Figure 7(a)), opening of summer-generation galls when the progeny is ready to hatch out (Figure 9(c)) requires a mechanism. We hypothesize that the closed state (Figure 9(b)) is maintained by the leafhopper's feeding inside the gall, accompanied by injecting the plant with certain chemicals contained in the saliva, while cessation of feeding leads to the opening of the gall. This hypothesis explains the fact that male galls become broadly open after the males leave them (Figure 9(d)) and so do the galls of nymphs killed by pipunculids (Figures 18(a) and 18(b)). At the same time, the galls damaged by caterpillars stay closed (Figure 17(d): inset), which may be a consequence of the fleshy inner part of their valves being destroyed (Figure 17(b)).

5.1.2. Safe Waste Disposal and Protection against Intruders

(1) Use of Brochosomes as Lining of the Gall Chamber. Brochosomes form hydrophobic coats on the integuments of Cicadellidae [19]. Adults and, in many subfamilies including Deltocephalinae, immatures use their legs to actively spread brochosomes over their body and appendages. The hypothetical primary role of the brochosomal coats is repellence of the liquid excrement produced by leafhoppers. Coating of plant surfaces with brochosomes is unknown among free-living leafhoppers, except in one group of genera in which females powder the plant epidermis above their eggs with specialized brochosomes, which are produced exclusively by gravid females [24] and apparently provide protection against egg parasitoids [25]. We could not observe *S. viridis* coating the gall chamber surface with brochosomes, but this is most likely done by scraping the secretion off the body with the legs during grooming. The galls of young nymphs already displayed numerous brochosomes on their inner surface. Together with wax platelets produced by the plant epidermis, such lining (Figures 11(c) and 11(d)) is likely to contribute to the higher hydrophobicity and "nonstickiness" of that surface (Figure 11(f)). These properties are essential to prevent contamination of the gall with the sticky honeydew produced by the phloem-feeding leafhopper. The interiors of the galls are indeed strikingly clean (Figure 12(a)). The

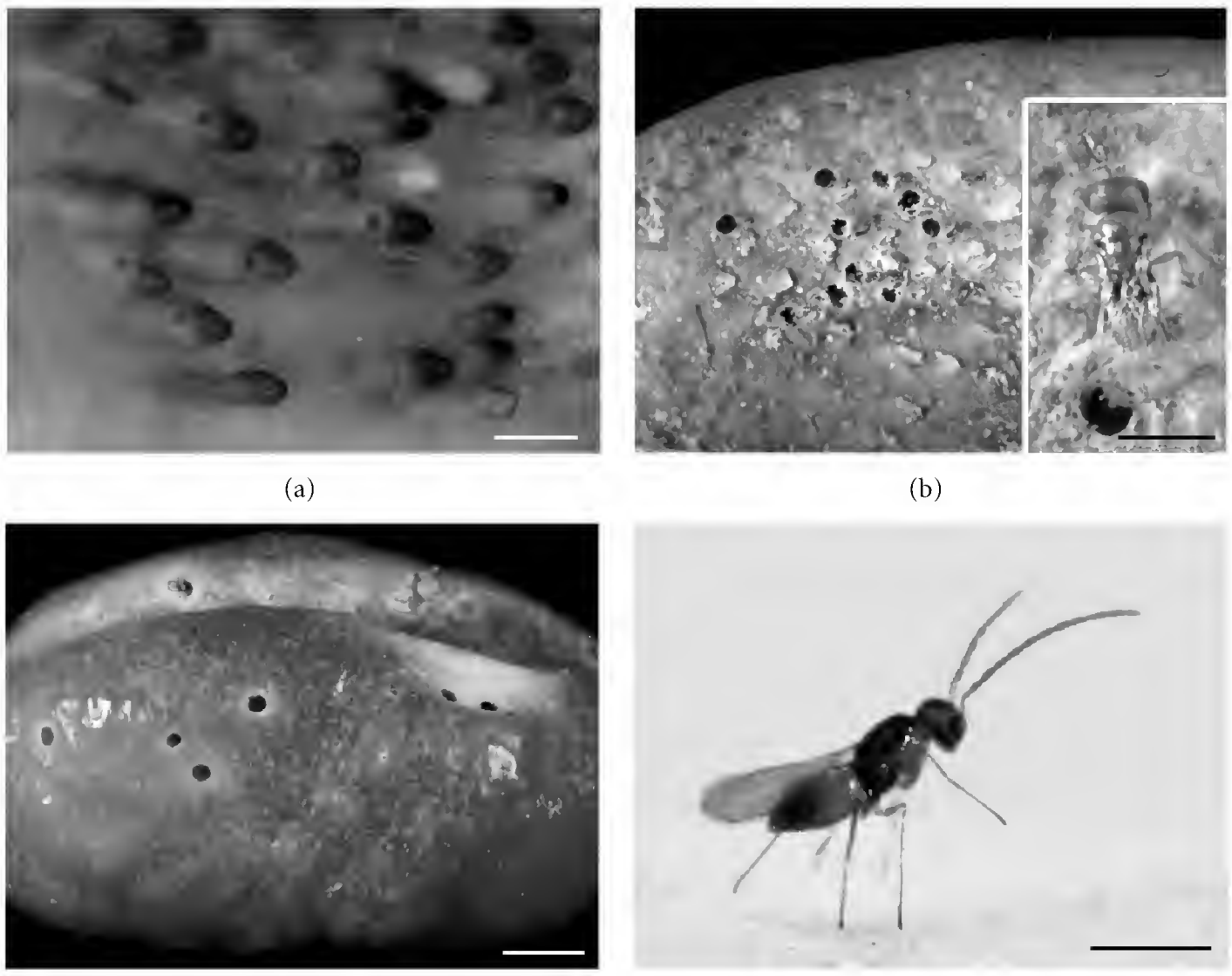


FIGURE 19: Egg parasitoids of *Scenergates viridis*. (a) The inner surface of a gall soaked in ethanol, with eggs of *S. viridis* visible underneath the epidermis; those with dark apices have been parasitized by *Aphelinoidea* sp. (Trichogrammatidae), and the egg with an orange apex has been parasitized by *Gonatocerus* sp. (Mymaridae). (b) *Aphelinoidea* sp. Exit holes on the inner surface of a gall and an adult female next to the hole (inset). (c) Exit holes of *Gonatocerus* sp. on the outer surface of a gall; note that one hole is on the commissural area of a valve. (d) An adult male *Gonatocerus* sp. reared from galls of *S. viridis*. Scale bars: 0.5 mm ((a), (d)), 1 mm ((b), (c)).

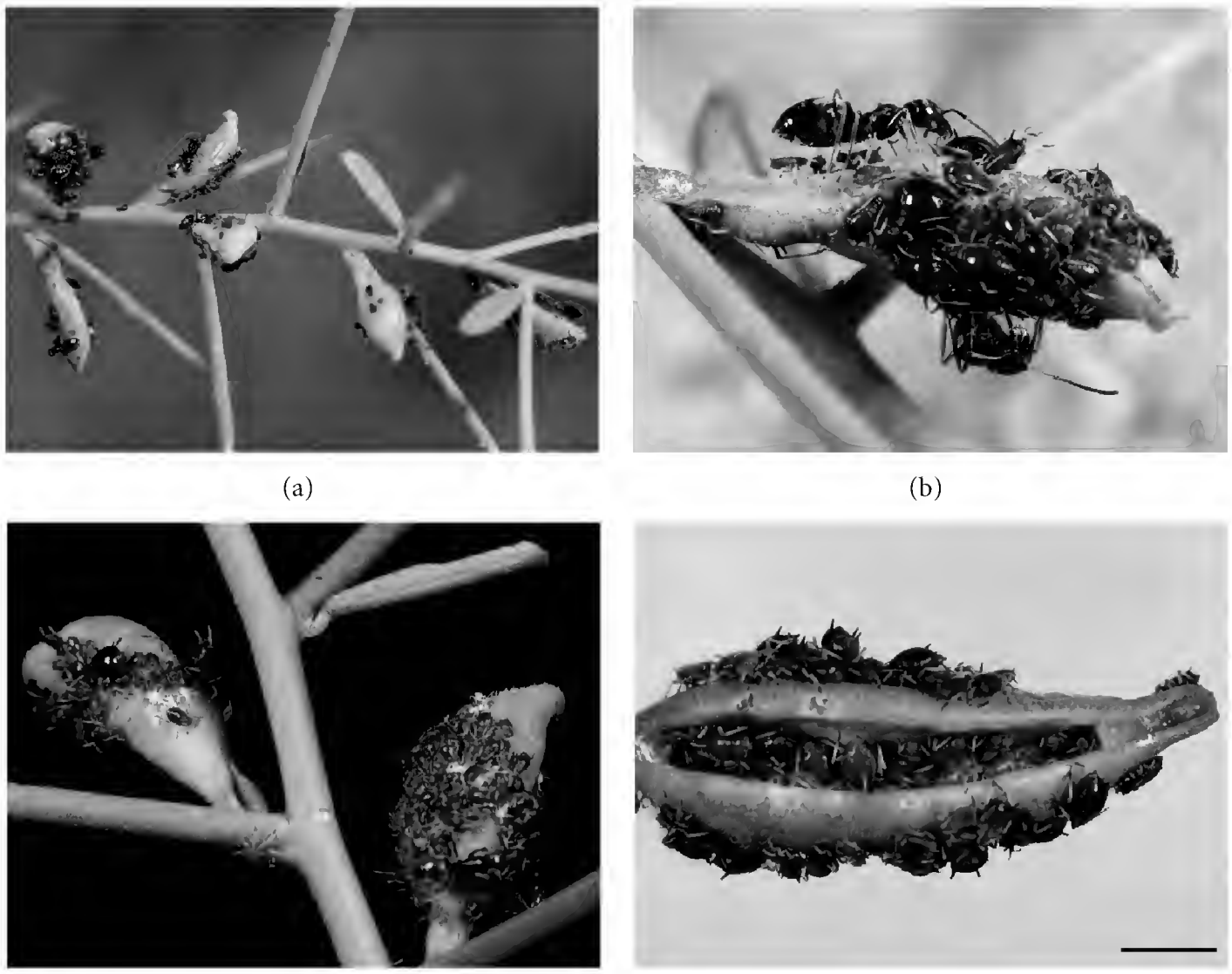
female galls containing eggs appear most heavily coated with brochosomes (Figures 15(b), 15(e) and 15(f)); the latter may to some extent deter trichogrammatid egg parasitoids.

(2) "Excrement Plugs." The excrement plugs appear to be an ingenious way to simultaneously dispose of the dangerous liquid waste within the narrow space of the gall and utilize it to protect the gall from intruders. They act as mechanical barriers which block larger intruders from entering the chamber through the orifices at the basal and apical ends of the gall. Because the leafhopper moistens the plugs by excreting new honeydew, they may also act as sticky traps for the smallest intruders such as thrips, mites, and trichogrammatid wasps, all of which we found trapped in the plugs (Figure 12(e)). It is likely that the leafhoppers, particularly postoviposition females, can use their hindlegs to actively push the intruder into the sticky plug.

It is not clear how exactly the plugs are formed. Most probably, prior to excreting the next droplet of honeydew, the leafhopper moves to one end of the chamber and deposits it there. Crushed exuviae also become incorporated into the plugs (Figure 12(c)). The crushing behavior, unknown in any free-living auchenorrhynchans, apparently serves to maintain the gall chamber clean and unobstructed. Crushing was not observed directly, but is most likely performed with the powerful hindlegs. It is remarkable that males, which leave the galls after they reach adulthood, "do not bother" or do not have time to dispose of their last-instar exuvia (Figure 12(f)) while the females, confined to the galls, process their last-instar exuvia like those from the younger instars. Plugging of gall orifices with exuviae is known in galling scale insects [3].

Our observation that the excrement plugs become mouldy in moist conditions suggests that extreme aridity of climate is a prerequisite for the galling behavior of this leafhopper. Thus, if gall formation occurs among other species of Auchenorrhyncha, such cases are more likely to be found in arid habitats.

5.1.3. Exiting and Entering Galls. The ability to squeeze through narrow slits, as observed in males of *S. viridis* (Figures 13(d) and 14), is another derived behavior not known among free-living leafhoppers. The process is obviously facilitated by the flattened body shape and particularly by the anteriorly flattened head (Figure 13(b)). Both traits occur in many free-living leafhopper species, apparently serving as camouflaging features. Penetration between closed gall valves is a strenuous task. It is remarkable in this respect that the few observed penetrations took place when the outdoor temperature reached its maximum. It is possible that males



(a) (b) (c) (d)

FIGURE 20: Unidentified aphids feeding on the surface of galls of *Scenergates viridis*. ((a)–(c)) Closed galls covered with aphids tended by *Tapinoma karavaievi* ants (Hymenoptera, Formicidae). (d) An open gall with aphids both outside and inside. Scale bars: 2 mm (d).

standing on the outside of female galls (Figures 13(a)–13(c)) wait for the rising temperature to decrease turgor pressure in the gall valves or perhaps just warm up their muscles for the intrusion. Exiting galls is easier and faster because the valves offer less resistance when pushed from the inside.

5.1.4. Maternal Guarding. Among Auchenorrhyncha, maternal guarding behavior is known only in the treehopper families Membracidae and Aetalionidae. In these two families, females stand by and protect their single egg batches from predators and parasitoids [26]. Other species produce multiple egg batches or scattered eggs and leave them unattended. Female *S. viridis* stay with their eggs inside the gall, and in the case of the overwintering generation, even until their offspring hatch (Figure 6(a)). Indirect evidence indicates that such females continue feeding and, at a decreased rate, laying eggs. It is most likely that they also actively protect the gall from intruders, including predators and parasitoids of eggs. Presumably, the females can kick and push these intruders out of the gall or into the excrement plugs with their hindlegs. The damaged hindlegs of postoviposition females (Figure 6(b)) may result from such activity.

5.2. Natural Enemies and Associates. The abundance of galls containing all life stages of *S. viridis* at the study site enabled us to identify major natural enemies and associates of this leafhopper within the narrow time limits of the study.

The galls are subject to predation by *Filatima* sp. moth larvae. According to Alexei Bidzilya (personal communication), this Holarctic genus includes up to 15 described and some undescribed Palearctic species that occur mostly in steppes and semideserts. Many Gelechiidae are leaf miners or gall inducers [27], while some facultatively feed on galls induced by other insects [28]. Whether *Filatima* sp. is a specialist predator of the galls of *S. viridis* remains to be found out. The caterpillars found by Dubovskii and Sulaimanov [14] in seven of the 100 examined galls probably belonged to this species.

The complex of parasitoids of *S. viridis*, including trichogrammatid and mymarid egg parasitoids and pipunculid parasitoids of larvae, is typical of Cicadellidae. The chalcidoid wasp genera *Gonatocerus, Aphelinoidea,* and *Paracentrobia* and the pipunculid fly genus *Tomosvaryella* all contain known parasitoids of free-living Cicadellidae [29, 30].

Among the parasitoids of *S. viridis* only *T. argyrata* has hitherto been described taxonomically, based on a series of males collected in Israel but with no information on the hosts (Christian Kehlmaier, personal communication). The type locality of *T. argyrata* is, therefore, outside the known distribution range of *S. viridis* (Figure 2) but within the range of *Alhagi maurorum*. It is possible that the range of *S. viridis* is wider than is currently known. Since pipunculids inject their eggs directly into immature auchenorrhynchans [31], the most likely targets in the case of *S. viridis* are the first-instar nymphs during the period between leaving their

mother's gall and forming their own gall. We suggest that this time frame is short. Thus, the attack tactics of the fly are an intriguing subject for future investigation. The eventual opening up of the galls of pipunculized nymphs (Figures 18(a) and 18(b)) appears to be crucial for the fly's exit.

The reared new species of trichogrammatid and mymarid wasps will be taxonomically described elsewhere. These species are likely to be adapted to the galling life style of their host. In particular, *Aphelinoidea* sp. are capable of locating and penetrating egg-containing galls, and *Gonatocerus* sp. can locate both such galls and the eggs lying deeply underneath the epidermis. One potential drawback of host specialization for egg parasitoids could be the absence of host eggs during a certain part of the year. It is worth noting in this respect the broad overlap between two generations of *S. viridis* during the late summer (Figure 4) which may facilitate continuity in the life cycle of the parasitoids.

It is known that the phloem sap of some aphid-induced galls contains more nutrients than ungalled plant parts [32]. The observed concentration of unidentified aphids on camelthorn galls (Figure 20) may indicate an increased nutritional quality of the phloem sap produced in these leafhopper-induced galls. A similar case, also observed in Middle Asian deserts, has been reported previously: the aphid *Brachyunguis (Xerophilaphis) saxaulica* (Aphididae) feeds both on and under the scales of cone-shaped bud galls induced on *Haloxylon* by the jumping plant lice of the genus *Caillardia* (Psyllidae) [33]. This aphid is so intimately associated with the psyllid galls—it rarely occurs on ungalled parts of the plant—that it has been mistaken for their inducer. The fact that the aphid often feeds on the exposed gall surface has been interpreted as an indication that it not merely uses the gall as a shelter, but that the phloem sap produced in the gall is particularly nutritious [33]. The same authors reported that *B. saxaulica* is tended by several ant species, including *T. karavajevi*.

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Research Article

Integrative Taxonomy, Phylogeny, and New Species of the Weevil Genus *Onyxacalles* Stüben (Coleoptera: Curculionidae: Cryptorhynchinae)

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A molecular phylogeny of the western Palearctic weevil genus *Onyxacalles* Stüben, 1999 is presented, combining two mitochondrial genes (COI and 16S) in a Bayesian analysis. Based on molecular data, *Onyxacalles pyrenaeus* Boheman, 1844 is transferred into the genus *Kyklioacalles* Stüben 1999 (*K. fausti* group) and—in an integrative taxonomy framework—the interaction between morphology and molecular analysis is illustrated. The species of *Onyxacalles* s. str. are assigned to three new species groups, *O. henoni*, *O. luigionii*, and *O. portusveneris* groups. The distribution of the related species in the Mediterranean area is illustrated with values of COI and 16S p-distances. Three new species are described and distinguished from their related species: *Onyxacalles nuraghi* Stüben sp.n. from Italy (Sardinia), *Onyxacalles torre* Stüben and Astrin sp. n. from France (Corsica) and *Onyxacalles vilae* Stüben sp. n. from Croatia (Velebit Mts.). A catalogue of all 20 species of *Onyxacalles* is given, and a key is finally presented combined with image stacking of the habitus and aedeagus for all species.

1. Introduction

Together with a number of other genera, the genus *Onyxacalles* Stüben, 1999 (Curculionidae: Cryptorhynchinae) was separated by Stüben [1] from the formerly excessively broadly circumscribed genus *Acalles* Schoenherr, 1825 as a group with initially 8 species. Since then, many new species of *Onyxacalles* have been described, mainly from Spain and North Africa. These discoveries were supported by the morphological finding that the three species from the Canary Islands belong to this genus [1, 2], a thesis that gained support from recent molecular analysis and has contributed to the new subgenus *Araneacalles* Stüben and Astrin [3]. This closed a "biogeographical gap" (between the Pyrenees and northwestern Africa) as a direct consequence of target-oriented collecting activities and descriptions of many new *Onyxacalles* species over the past decade [4–9]. Thus, including the new species presented in this work, the genus now comprises 20 valid species.

Most species of *Onyxacalles* are found in the west Mediterranean area and on the Macaronesian Islands. Only one species, *Onyxacalles croaticus* (H. Brisout, 1897) [10], reaches Eastern Europe (Carpathians); another species, *O. amasyaensis* Wolf, 2001, was described from Turkey, but could be a synonym of *Onyxacalles denominandus* A. and F. Solari, 1907. This species richness in the west Mediterranean is well founded in the ecological preferences of *Onyxcalles*.

As "nocturnal goblins of the last primeval forests" [7], the species of *Onyxacalles* are not common in the often disturbed landscapes of the Iberian Peninsula and North Africa [11, 12]. These conditions provide good maps of relictual vegetation and information about the habitats, allowing us to trace these nocturnal Cryptorhynchinae in the dark and humid relicts of natural forests under big

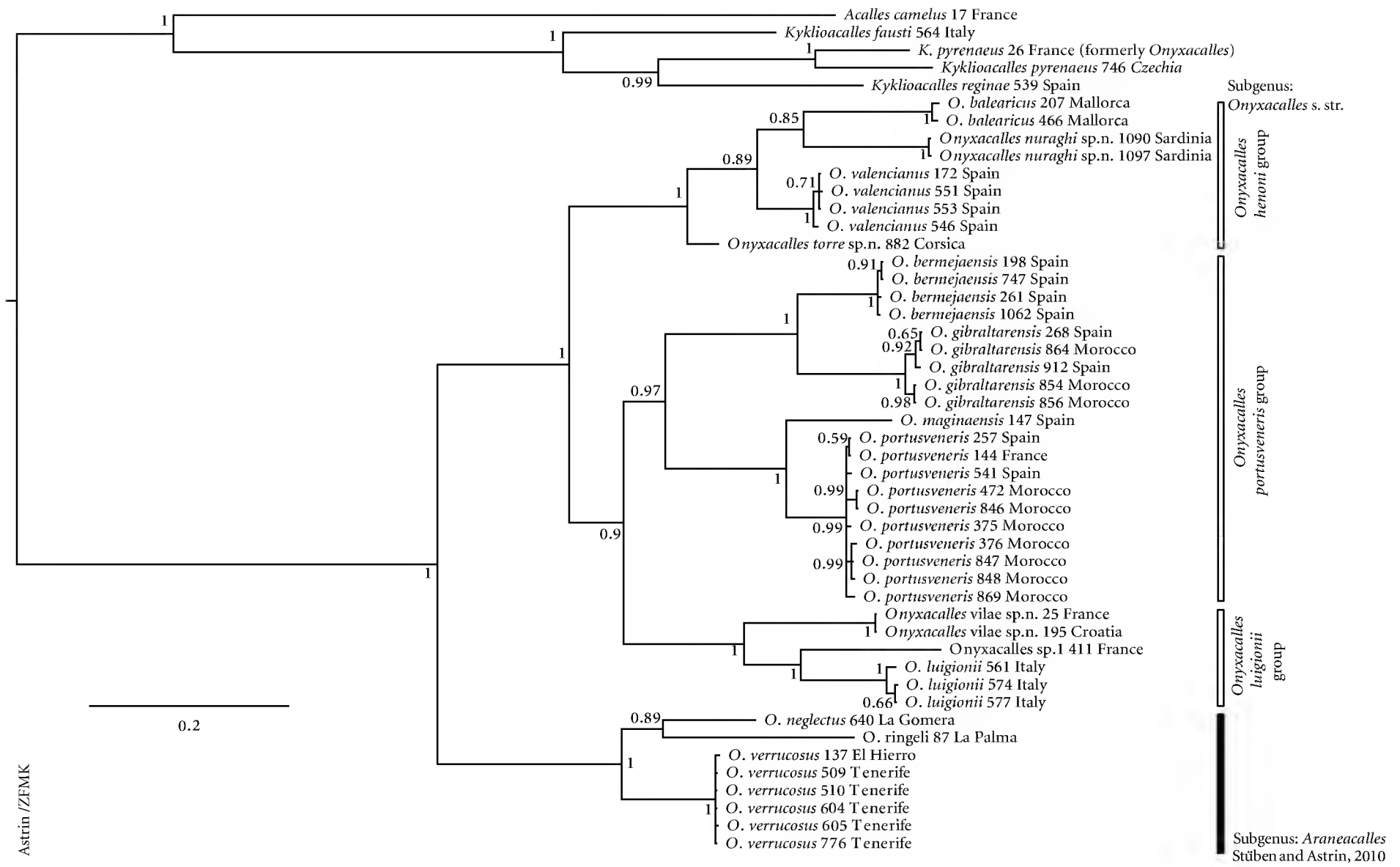

Figure 1: Bayesian consensus tree (50% majority rule) for COI and 16S.

oak trees and behind a dense jungle of *Smilax aspera* L. Therefore, sifting by day is not the ideal method of catching *Onyxacalles*. With their long legs, *Onyxacalles* species climb trees by night and were fogged by us from the canopy of the *laurisilva* on the Canary Islands of Tenerife and La Gomera [13, 14]. The flightless *Onyxacalles* are highly specialised woodlander inhabitant, and because of their restricted dispersal (above all on the European continent), they are an ideal bioindicator of original forest, highlighting that protection and sustainable development should concern us as entomologists and conservationists.

Moreover, with the study of the cryptic and similar *Onyxacalles* species, the taxonomist, especially the morphologist, enters a minefield. Due to intraspecific variability, the few external characteristics that are suitable for a differential diagnosis do not guarantee a reliable (re)identification of species: if the tufts of bristles exist or not, if the elytra are short oval or elongated, or if the midgroove of pronotum is more or less distinct—all these are important pointers, but are not conclusive. All *Onyxacalles* species have the hooked apex of the aedeagus in common, as the genus name implies (cf. Figures 9(a)–9(l)). However, a complex internal sac of the aedeagus (endophallus) does not exist—as it is typical for *Kyklioacalles*, *Dichromacalles,* or most genera from Macaronesia. Here, we are quickly stretched to our limits of a descriptive morphology.

With the "**M**olecular **W**eevil **I**dentification" Project (see below), we tread a path towards an integrative taxonomy [15–21]. Molecular taxonomy is not only an addition or an accessory "immunisation" to confirm morphological results. The integration of the two approaches must be done with the intention of *falsifying* and, if so, it can lead to a new way of viewing morphology: *interspecific* characteristics are discovered that are not regarded as belonging to *intraspecific* variability, or—more often—substituting the "human eyes," the way we look at things. Instead of "anthropocentric conspicuities" (e.g., forms, colours, sizes), we focus on constitutive characteristics (e.g., apomorphies and homologies) that were previously overlooked in our diagnostic keys. An example is the latest history of the science behind the original species *Acalles pyrenaeus* Boheman, 1844 (see below).

Another telling example is the present classification of *Onyxacalles* s. str. into two species groups: the *O. luigionii*- and *O. pyrenaeus*-groups, based on the bristles of the elytral intervals (cf. [1, 8]). This hypothesis is no longer tenable, and this has nothing to do with superficial diagnosis of affinities or inaccurate observations. First, molecular analysis of related species reveals new informal species groups (see Figure 1) and makes evidence available to the morphologist, who then looks for new external characteristics. These are inconspicuous paradigm changes with a high impact [22], because the "puzzles," in this case the *species*, which were previously pressurised within the framework of "normal science" (and more and more frequently caused difficulties) continue, but the emphasis of the characteristics under the new molecular phylogenetic paradigma has changed (see "Catalogue of *Onyxacalles*"), and new characteristics are discovered (see "Key to the species of *Onyxacalles*").

The assumption that the morphologist should have seen a characteristic must be abandoned in favour of the question: would the morphologist be able to "*see*" it?

2. Catalogue of *Onyxacalles*

Species included in the molecular analysis are printed in bold (l.t. = type locality).
Genus: Onyxacalles Stüben, 1999a.
177 type species *Acalles luigionii* A. and F. Solari, 1907.
Subgenus: *Onyxacalles* s. str.
Onyxacalles henoni Group

balearicus Stüben, 2005: 115, Spain: Majorca (l.t.)

croaticus H. Brisout de Barneville, 1867: 62 (*Acalles*), Croatia (l.t.), Austria, Czech Republic, Germany, Poland, Slovakia, Slovenia

hannibali Germann, 2004: 118, Tunisia (l.t.)

henoni Bedel, 1888: 36 (*Acalles*), Algeria: Mt. Edough (l.t.).

nuraghi Stüben **sp. n.**, Italy: Sardinia (l.t.).

torre Stüben and Astrin **sp. n.**, France: Corsica (l.t.).

valencianus Germann, 2005: 104, Western Spain (l.t.).

Onyxacalles portusveneris Group

bermejaensis Stüben, 2001: 145, Spain (l.t.).

gibraltarensis Stüben, 2002: 206, Morocco (l.t.), Spain.

maginaensis Stüben, 2004: 120, Southern Spain (l.t.).

portusveneris Mayet, 1903: 74 (*Acalles*), France (l.t.), Spain, Morocco

seguraensis Stüben, 2003a: 201, Spain.

Onyxacalles luigionii Group

luigionii A. and F. Solari, 1907: 521 (*Acalles*), Central (l.t.) and Southern Italy.

vilae Stüben **sp. n.**, Croatia (l.t.), France: Isère (perhaps Austria and Slovenia).

cf. luigionii, France: Alpes Maritimes.

Incertae Sedis

denominandus A. and F. Solari, 1907: 523 (*Acalles*), Turkey (l.t.).

porcheti Hoffmann, 1935: 162 (*Acalles*), France: Pyrenees (probably a synonym of *A. luigionii,* see also Stüben 2007: 149).

amasyaensis Wolf, 2001: 150, Turkey (l.t.). (probably a synonym of *Acalles denominandus*)

Subgenus: *Araneacalles* Stüben and Astrin, 2010: 78 type species *Acalles verrucosus* Wollaston, 1863.

neglectus Kulbe, 1999: 193, Canary Islands: La Gomera (l.t.), El Hierro.

ringeli Kulbe 1999: 196, Canary Islands: La Palma (l.t.).

verrucosus Wollaston, 1863: 219 (*Acalles*), Canary Islands: Tenerife (l.t.), El Hierro.

3. Materials and Methods

The molecular analysis is based on 45 (43 after transfer of *O. pyrenaeus*, see below) individuals in 15 species or putative species of *Onyxacalles* and on 5 outgroup species (Cryptorhynchinae from 4 other genera; only the two closer genera are shown in the tree for better visualisation, while the 2 more distant ones were removed; see Table 1). Most sequences have been published in Astrin et al. (2012). Collecting and vouchering information as well as GenBank accession numbers are given in Table 1. Voucher specimens and extracted genomic DNA are deposited at the Biobank of the ZFMK (Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany).

DNA extraction was carried out on samples preserved in ethanol or on dried material, using Macherey-Nagel Nucleo Spin Tissue kits (Dueren, Germany) or BioSprint 96 kits (Qiagen, Hilden, Germany). We extracted DNA from either 2-3 legs, head and prothorax, or sometimes also the whole weevil, depending on size and conservation of the sample. PCR reaction mixes (50 μL) contained 125 nmol $MgCl_2$, 5 μL 10x PCR-buffer, 25 pmol of forward and reverse primer each, 5 pmol dNTPs, 1.75 units of *Taq* polymerase, and 5 μL total undiluted DNA template. The lab chemicals were purchased from Sigma-Aldrich (Steinheim, Germany). We used the Qiagen (Hilden, Germany) Multiplex PCR kit in cases where the regular protocol failed. PCR primers were taken from Astrin and Stüben (2008; COI is based on the Folmer et al. [23] region; 16S is based on the Crandall and Fitzpatrick (1996) region). Primer sequences were as follows: LCOI490-JJ (COI forward, fw) 5′-CHACWAAYCATAAAGATATYG-G-3′, HCO2198-JJ (COI reverse, rev) 5′-AWACTTCVG-GRTGVCCAAARAATCA-3′; 16S-ar-JJ (16S fw, erroneously as "rev" in [24]) 5′-CRCCTGTTTATTAAAAACAT-3′, 16S-1472-JJ (16S rev) 5′-AGATAGAAACCRACCTGG-3′. Thermal cycling was performed on blocks of the type GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). PCR program for 16S: first cycle set (15 repeats): 35 s denaturation at 94°C, 35 s annealing at 55°C (−1°C per cycle) and 60 s extension at 72°C. Second cycle set (25 repeats): 35 s denaturation at 94°C, 35 s annealing at 40°C, and 50 s extension at 72°C. PCR program for COI: same as for 16S, but annealing temperatures at 70°C and 55°C, with a decrease of 2°C per cycle in the first cycle set. Double-stranded sequencing was carried out by a sequencing facility (Macrogen, South Korea, and Netherlands) using the same primers as in PCR.

DNA sequence alignment was performed manually (COI) or using the MUSCLE ver. 3.6 programme [28] (16S), run with default parameters. Sequence length was 554 bp for 16S (aligned; longest sequence: 544 bp; shortest: 533 bp) and 658 bp for COI, for concatenated sequence data 1212 bp. The 16S alignment comprised 29 positions with

Table 1: Collecting data, vouchers, and GenBank accession numbers for the material analysed in this study. All specimens determined by P. E. Stüben, 2010 and 2011. Vouchers (DNA, morphology) are kept at the ZFMK Biobank. Most sequences have been published (or are reviewed) in Astrin et al. 2012 [21]. GenBank accession numbers of new sequences in this study start with "JN...". Taxonomic changes with regard to this publication are printed in brackets (old name).

Taxon	Collecting data	DNA voucher	COI 16S
Acalles camelus (Fabricius 1792)	France: Isère, 2 km SE Lans en Vercors, Montagne de Lans; N45°06′45″ E05°36′21″, 1352 m; Abies, Fagus, Fraxinus, 2005, Stüben	ZFMK-DNA-JJ0017, ZFMK-TIS-cI0026cam	EU286282 EU286447
Acallorneuma doderoi A. and F. Solari 1908	Italy: Sicilia (PA), 6 km SW Godrano, Bosco Ficuzza, Mte. Rocca Busambra; N37°51′38″ E13°23′24″, 1200 m; Quercus, Fraxinus, 2002, Stüben	ZFMK-DNA-JJ0065, ZFMK-TIS-cS0082dod	EU286292 EU286457
Cryptorhynchus lapathi (Linné 1758)	Germany: Bienen bei Rees, Altrheinarm, 2004, Scharf	ZFMK-DNA-JJ0214, ZFMK-TIS-cD0354lap	EU286360 EU286523
Kyklioacalles fausti (Meyer 1896) [25]	Italy: Campania, Cilento, 6 km SE Vallo d. Lucania, M. Sacro o Gelbison; N40°12′41″ E15°19′42″, 1544 m; Fagus, 2008, Stüben	ZFMK-DNA-JJ0564, ZFMK-TIS-cI625fau	GU213776 GU213772
Kyklioacalles pyrenaeus (Boheman 1844) [26] (gen. Onyxacalles)	France: Isère, 14 km N Grenoble, Massif de la Chartreuse, NW Col de Porte; N45°18′40″ E05°45′17″, 1649 m; Abies, Fagus, Fraxinus, 2005, Stüben	ZFMK-DNA-JJ0026, ZFMK-TIS-cI0035pyr	GU988172 GU987762
Kyklioacalles pyrenaeus [26] (gen. *Onyxacalles*)	Czech Republic: W Bohemia (KT), Balkovy, Doubrava Hill (6545), 2008, Kresl	ZFMK-DNA-JJ0764, ZFMK-TIS-cCz798pyr	GU981555 GU981506
Kyklioacalles reginae Stüben 2003	Spain: Teruel, S. Javalambre, Fuente la Risca near Arcos de las Salinas; N39°59′56″ W01°01′21″, 1121 m; Amelanchier ovalis, Acer monspessulanum, Erinacea anthyllis, Ulex, 2008, Stüben	ZFMK-DNA-JJ0539, ZFMK-TIS-cE600reg	GU981544 GU981495
Onyxacalles balearicus Stüben 2005	Spain: Mallorca, 3 km SE Lluc, Sra. de Tramuntana, Sa Maleta; N39°48′47″ E02°53′23″, 571 m; Quercus ilex, 2004, Stüben	ZFMK-DNA-JJ0207, ZFMK-TIS-cE0168bal	EU286357 EU286521
Onyxacalles balearicus Stüben 2005	Spain: Mallorca, 11 km NE Lluc, Sra. de Tramuntana; N39°52′03″ E02°58′20″, 107 m; PT, Smilax aspera, Quercus ilex, 2004, Stüben	ZFMK-DNA-JJ0466, ZFMK-TIS-cE0294bal	GU988348 ---------
Onyxacalles bermejaensis Stüben 2001	Spain: Andalucía, 11 km S Ronda, Sierra de las Nieves; N36°39′51″ W05°05′01″, 1047 m; Quercus ilex, 2005, Stüben	ZFMK-DNA-JJ0198, ZFMK-TIS-cE0167ber	EU286350 EU286514
Onyxacalles bermejaensis Stüben 2001	Spain: Málaga, 9 km SE Ubrique, Sierra de Líbar; N36°36′52″ W05°23′16″, 663 m; Quercus ilex, Ceratonia, 2007, Stüben	ZFMK-DNA-JJ0261, ZFMK-TIS-cE0194ber	GU988244 GU987827
Onyxacalles bermejaensis Stüben 2001	Spain: Málaga, NW Marbella, Sierra de las Nieves; N36°39′52″ W05°04′57″, 1043 m; Quercus ilex, Echinodera spinosa, 2009, Stüben	ZFMK-DNA-JJ0747, ZFMK-TIS-cE778ber	GU988506 GU988066
Onyxacalles bermejaensis Stüben 2001	Spain: Prov. Málaga, Algatocín, near Opayar; N36°34′39″ W05°18′13″, 576 m, Quercus sp., 17.8.2010, Stüben	ZFMK-DNA-JJ1062, ZFMK-TIS-cES1062	JN121398 ---------
Onyxacalles gibraltarensis Stüben 2002	Spain: Cádiz, 10 km SW Algeciras, El Bujeo; N36°04′10″ W05°31′48″, 257 m; Quercus suber, 2007, Stüben	ZFMK-DNA-JJ0268, ZFMK-TIS-cE0206gib	GU988249 GU987832
Onyxacalles gibraltarensis Stüben 2002	Morocco: Rif, SW Oued-Laou, river, O. Laou; N35°17′47″ W05°13′38″, 210 m; Quercus suber, Smilax, Arbutus, 2009, Stüben	ZFMK-DNA-JJ0854, ZFMK-TIS-cE897gib	GU988577 GU988137
Onyxacalles gibraltarensis Stüben 2002	Morocco: W Sebta, vir. Biutz; N35°53′04″ W05°24′08″, 337 m; Quercus suber, Smilax, Arbutus, 2009, Stüben	ZFMK-DNA-JJ0856, ZFMK-TIS-cE899gib	GU988578 GU988138
Onyxacalles gibraltarensis Stüben 2002	Morocco: S Ksar-es-Seghir; N35°45′16″ W05°30′49″, 278 m; Pistacia, Quercus suber, 2009, Stüben	ZFMK-DNA-JJ0864, ZFMK-TIS-cE907gib	GU988584 GU988144
Onyxacalles gibraltarensis Stüben 2002	Spain: Cádiz, Los Barrios, Alcornocales N.P., between Facinos, Río Las Cañas and Mantera Torero; Olea europaea, 2009, Torres	ZFMK-DNA-JJ0912, ZFMK-TIS-cE949gib	GU988608 ---------
Onyxacalles luigionii (A. & F. Solari 1907)	Italy: Campania, Cilento, 6 km SE Vallo d. Lucania, M. Sacro o Gelbison; N40°12′41″ E15°19′42″, 1544 m; Fagus, 2008, Stüben	ZFMK-DNA-JJ0561, ZFMK-TIS-cI622lui	GU988407 GU987967

Table 1: Continued.

Taxon	Collecting data	DNA voucher	COI 16S
Onyxacalles luigionii (A. & F. Solari 1907)	Italy: Campania, Monti Picentini, 9 km N Acerno, Piano Laceno; N40°48′58″ E15°07′35″, 1210 m; Fagus, 2008, Stüben	ZFMK-DNA-JJ0574, ZFMK-TIS-cI635lui	GU988417 GU987977
Onyxacalles luigionii (A. & F. Solari 1907)	Italy: Basilicata, Monte Pollino, 9 km SE Rotonda, Rif. de Gasperi; N39°54′37″ E16°07′15″, 1486 m; Fagus, 2008, Stüben	ZFMK-DNA-JJ0577, ZFMK-TIS-cI638lui	GU988418 GU987979
Onyxacalles maginaensis Stüben 2004	Spain: Andalucía, 28 km E Jaén, Sierra Magina; N37°43′21″ W03°29′11″, 1600 m; Quercus ilex, 2005, Stüben	ZFMK-DNA-JJ0147, ZFMK-TIS-cE0169mag	EU286327 EU286491
Onyxacalles maginaensis Stüben 2004	Spain: Almería, 11 km NW Laujar de Andarax, Sierra Nevada, Bayárcal; N37°02′27″ W03°00′12″, 1291 m; Quercus ilex, 2007, Stüben	ZFMK-DNA-JJ0257, ZFMK-TIS-cE0187mag	submitted to GenBank
Onyxacalles maginaensis Stüben 2004	Spain: Teruel, S. Javalambre, Fuente la Risca near Arcos de las Salinas; N39°59′56″ W01°01′21″, 1121 m; Amelanchier ovalis, Acer monspessulanum, Erinacea anthyllis, Ulex, 2008, Stüben	ZFMK-DNA-JJ0541, ZFMK-TIS-cE602mag	GU988390 GU987950
Onyxacalles neglectus Kulbe 1999	Spain: Canary Islands, La Gomera, S Hermigua, El Cedro, Las Mimbreras; N28°07′27″ W17°13′26″, 901 m; laurisilva, 2008, Astrin and Stüben	ZFMK-DNA-JJ0640, ZFMK-TIS-cE713neg	FJ716525 GU988014
Onyxacalles nuraghi sp.n.	Italy: W-Sardinia, E Macomer: above Lei; N40°19′54″ E08°53′49″, 1020 m; Quercus, Acer monspessulanum, 4.10.2010, Stüben	ZFMK-DNA-JJ1090, ZFMK-TIS-cIT1090	JN642097 JN121399
Onyxacalles nuraghi sp.n.	Italy: W-Sardinia, E Macomer: above Lei; N40°19′17″ E08°53′52″, 586 m; Quercus ilex, 7.10.2010, Stüben	ZFMK-DNA-JJ1097, ZFMK-TIS-cIT1097	JN642098 JN121300
Onyxacalles portusveneris (Mayet 1903) [27]	France: Gard, 15 km NE Nimes, Pont du Gard, Collias; N43°57′03″ E04°28′59″, 68 m; Quercus ilex, 2006, Stüben	ZFMK-DNA-JJ0144, ZFMK-TIS-cF0166por	EU286326 EU286490
Onyxacalles portusveneris (Mayet 1903) [27]	Morocco: Rif Mts., 10 km W Ketama = Issague; N34°57′40″ W04°40′51″, 1600 m; Prunus lusitanica, 2001, Stüben	ZFMK-DNA-JJ0375, ZFMK-TIS-cM480por	GU988311 ---------
Onyxacalles portusveneris (Mayet 1903) [27]	Morocco: M-Atlas, 10 km S Ain-Leuh; N33°13′48″ W05°20′50″, 1700 m; Quercus ilex, Rubus, Cedrus, 2002, Stüben	ZFMK-DNA-JJ0376, ZFMK-TIS-cM481por	GU988312 ---------
Onyxacalles portusveneris (Mayet 1903) [27]	Morocco: High Atlas, E Marrakech, N Taddert, (near Tazouguerte); N31°28′07″ W07°24′59″, 1727 m; Quercus, 2009, Stüben	ZFMK-DNA-JJ0846, ZFMK-TIS-cE889port	GU988573 GU988133
Onyxacalles portusveneris (Mayet 1903) [27]	Morocco: Middle Atlas, S Azrou, Äin Leuh; N33°16′50″ W05°20′18″, 1582 m; Quercus ilex, Euphorbia, 2009, Stüben	ZFMK-DNA-JJ0847, ZFMK-TIS-cE890port	GU988574 GU988134
Onyxacalles portusveneris (Mayet 1903) [27]	Morocco: Middle Atlas, S Azrou, S Äin Leuh; N33°14′57″ W05°21′04″, 1715 m; Quercus ilex, 2009, Stüben	ZFMK-DNA-JJ0848, ZFMK-TIS-cE891port	GU988575 GU988135
Onyxacalles portusveneris (Mayet 1903) [27]	Morocco: Rif, 10 km W Ketama; N34°57′40″ W04°40′51″, 1600 m; Cedrus, Prunus, 2009, Stüben	ZFMK-DNA-JJ0869, ZFMK-TIS-cE912port	GU988587 GU988147
Onyxacalles portusveneris (Mayet 1903) [27] (*Onyxacalles* sp.)	Morocco: High Atlas, 59 km SE Marrakech; N31°28′19″ W07°24′22″, 1500 m; Quercus ilex, Quercus suber, 2002, Stüben	ZFMK-DNA-JJ0472, ZFMK-TIS-cM482mag	GU988350 GU987922
Onyxacalles ringeli Kulbe 1999	Spain: Canary Islands, La Palma, Cumbre Nueva, 4,5 km SE El Paso, El Pilar; N28°37′37″ W17°49′45″, 1432 m; laurisilva, 2006, Stüben	ZFMK-DNA-JJ0087, ZFMK-TIS-cC0171rin	EU286300 EU286465
Onyxacalles sp. 1 (*O. luigionii*)	France: Alpes-Maritimes, 3 km W Sospel, Col de Braus; N43°52′34″ E07°24′17″, 1051 m; Quercus pubescens, Ostrya carpinifolia, broom, 2007, Stüben	ZFMK-DNA-JJ0411, ZFMK-TIS-cF440lui	GU988325 GU987897
Onyxacalles torre sp. n. (*O. henoni*)	France: Corsica, Col de Vizzavona, 22 km S Corte; N42°06′45″ E09°06′49″, 1100 m; Fagus, 2001, Stüben	ZFMK-DNA-JJ0882, ZFMK-TIS-cF479hen	GU988592 ---------

Table 1: Continued.

Taxon	Collecting data	DNA voucher	COI 16S
Onyxacalles valencianus Germann 2005	Spain: Alicante, 7 km SW Alcoi, Sierra de Menechaor, Santurio de la Font Roja; N38°39′34″ W00°32′29″,1296 m; Quercus ilex, 2007, Stüben	ZFMK-DNA-JJ0172, ZFMK-TIS-cE0180val	EU286331 EU286495
Onyxacalles valencianus Germann 2005	Spain: Castellón, Morella, Barranco de la Bota; N40°33′12″ W00°00′27″, 814 m; Quercus ilex, Hedera helix, 2008, Stüben	ZFMK-DNA-JJ0546, ZFMK-TIS-cE607val	GU988393 GU987953
Onyxacalles valencianus Germann 2005	Spain: Barcelona, above dry river bed, near Vallirana; N41°22′36″ E01°55′02″, 245 m; Quercus ilex, Ficus carica, Smilax aspera, 2008, Stüben	ZFMK-DNA-JJ0551, ZFMK-TIS-cE612val	GU988398 GU987958
Onyxacalles valencianus Germann 2005	spain: Barcelona, S. Montseny, Tordera valley, near St. Marçal; N41°48′01″ E02°25′15″, 1060 m, 2008, Stüben	ZFMK-DNA-JJ0553, ZFMK-TIS-cE614val	GU988400 GU987960
Onyxacalles verrucosus (Wollaston 1863)	Spain: Canary Islands, El Hierro, 7 km W La Frontera, Pista Derrabado; N27°44′29″ W18°03′24″, 895 m; Laurus azorica, 2006, Stüben	ZFMK-DNA-JJ0137, ZFMK-TIS-cC0170ver	EU286324 EU286488
Onyxacalles verrucosus (Wollaston 1863)	Spain: Canary Islands, Tenerife, 6 km N La Laguna, Monte de las Mercedes; N28°31′50″ W16°17′09″, 950 m; laurisilva, 2003, Stüben	ZFMK-DNA-JJ0509, ZFMK-TIS-cE570ver	--------- GU987937
Onyxacalles verrucosus (Wollaston 1863)	Spain: Canary Islands, Tenerife, 4 km S Los Silos, Teno Mts., Monte del Aqua; N28°19′20″ W16°49′14″, 700 m; laurisilva, 2003, Stüben	ZFMK-DNA-JJ0510, ZFMK-TIS-cE571ver	GU988373 GU987938
Onyxacalles verrucosus (Wollaston 1863)	Spain: Canary Islands, Tenerife, NE La Laguna, Anaga Mts. near Moquinal; N28°31′55″ W16°17′24″, 840 m; laurisilva, 2008, Astrin and Stüben	ZFMK-DNA-JJ0604, ZFMK-TIS-cE677ver	GU988433 GU987995
Onyxacalles verrucosus (Wollaston 1863)	Spain: Canary Islands, Tenerife, NE La Laguna, Anaga Mts. near Chinobre; N28°33′21″ W16°10′46″, 808 m; Laurus, Ixanthus viscosus, 2008, Astrin and Stüben	ZFMK-DNA-JJ0605, ZFMK-TIS-cE678ver	GU988434 GU987996
Onyxacalles verrucosus (Wollaston 1863)	Spain: Canary Islands, Tenerife, SW Los Silos, Teno Mts., Monte del Agua, Chupadero; N28°19′23″ W16°49′12″, 940 m; Laurus novocanariensis, 2008, Astrin, Stüben, Behne and Floren	ZFMK-DNA-JJ0776, ZFMK-TIS-cE813ver	GU988524 GU988085
Onyxacalles vilae sp.n. (*O. luigionii*)	France: Isère, 2 km SE Lans en Vercors, Montagne de Lans; N45°06′45″ E05°36′21″, 1352 m; Abies, Fagus, Fraxinus, 2005, Stüben	ZFMK-DNA-JJ0025, ZFMK-TIS-cI0027lui	EU286286 EU286451
Onyxacalles vilae sp.n. (*O. luigionii*)	Croatia: Dalmatian, 8 km E Karlobag, Velebit Mts., Stupacinovo; N44°32′41″ E15°09′58″, 1049 m; Fagus, 2007, Stüben	ZFMK-DNA-JJ0195, ZFMK-TIS-cHR0339lui	EU286348 EU286512

gaps. All of these were included into phylogenetic analysis. We implemented the GTR+I+Γ [29] model of nucleotide substitution for both genes in Bayesian MCMC analyses, run in MrBayes ver. 3.1.2 [30]. Only COI was included for the new sequences. We ran two independent replicates for 10 million generations per analysis (each with 1 cold chain and 3 chains of different temperature). Every 1′000th tree was sampled (20′000 trees retained). Negative log-likelihood score stabilisation was determined in a separate visualisation (in MS Excel). Accordingly, we retained 19.800 trees (after discarding burn-in), of which a 50%-majority rule consensus tree was built, with posterior probabilities (Figure 1).

4. Results and Discussion

4.1. A Gestalt Switch—Changing the Way You See: Kyklioacalles pyrenaeus (Boheman, 1844). The species of *Onyxacalles* are characterised by a particularly long and slender rostrum which is at least 3-4 times as long as wide between the insertions of the antennae. A further conspicuous feature (not typical for western Palearctic Cryptorhynchinae—excepting the species of the Macaronesian Islands) is the unusually long and slender (arachnoid) legs. The name of this genus refers to the hook-shaped tip of the aedeagus. No further species of the former accumulative genus *Acalles* or of other western Palearctic genera of the Cryptorhynchinae exhibit such a characteristic hook-shaped tip of the aedeagus (*onyx*; greek: hook, hook-shaped tool). Complex-sclerotised structures of the internal sac are absent or reduced to simple line- or bar-like structures. These structures have been significant, for instance, for the partly phylogeny-based classification and determination of the genera and species of *Dichromacalles* and *Kyklioacalles*. The strongly sclerotised median lobe exhibits either only unclear structures or none at all (see [31]).

As early as the beginning of the last century, in their ground-breaking revision of the western Palearctic species of *Acalles* s.l., A. and F. Solari placed the species *A. pyrenaeus*

Boheman, 1844, *A. henoni* Bedel, 1888, *A. croaticus* Brisout, 1867, and *A. luigionii* Solari, 1907—although together with further species—into the same group (see IV. group; [32]). Together with initially 7 further species (among it the above species denominated by A. and F. Solari, as well as 3 further species from the Canary Islands), they were transferred into the new genus *Onyxacalles* [1, page 186], a genus that currently comprises 20 species.

However, *Onyxacalles pyrenaeus* is a polymorphic species, with regard to the outline of the aedeagus as well as the more or less ovally rounded elytra [1, page 188]. This is—among others—the reason why we could not ascertain definitively whether the subspecies *Acalles pyrenaeus germanicus* Letzner, 1882 (= *Onyxacalles boehmei* Košťál & Holecová, 2001 [33] syn.) is really a junior synonym or not (even if the first author considers it a synonym; cf. [7, page 123]).

In addition to *Onyxacalles portusveneris* (Mayet, 1903) (see Figure 4), *O. pyrenaeus* has an exceptionally large distribution area (cf. [31]). This could explain the high genetic distances of the mitochondrial COI and 16S gene (e.g., France: Lans en Vercors—Austria: Merkersdorf, p-distances: COI = 8,5%, 16S = 2,4%). This species can be found from the Pyrenees to the mountains of Western, Central, and Eastern Europe to the Carpathians and can be beaten from the branches of different conifers, especially larch, but can also be sifted under deciduous trees (e.g., *Fagus*).

This does not coincide with the other *Onyxacalles* species, which live on different deciduous trees and never prefer conifers. Apart from these ecological conditions, we can establish that the above-mentioned differences to the other genera of the western Palearctic Cryptorhynchinae point to a closer relationship to the species of *Onyxacalles*, a view with which most authors concur (e.g., [33]).

In any case, the taxonomists did not pay attention to the distinct ecology and—in comparison with the other species of *Onyxacalles*—the clearly narrower aedeagus (cf. Figure 6(k) versus Figures 9(a)–9(l)). Are these peripheral characteristics? And in which genus of Cryptorhynchinae is it possible to place this species (without the need for a monotypic genus)? The first author had never imagined assigning this species to the genus *Kyklioacalles* Stüben, 1999. There was no reason for such a review, not even an "initial suspicion" (see below).

However, this presumed "*Onyxacalles*" species appears deeply nested within the genus *Kyklioacalles* in the dendrogram [34, Figure 1]. This inclusion within *Kyklioacalles* is maximally supported. Furthermore, it is obvious that *Acalles pyrenaeus* forms a clade with *K. fausti* (Meyer, 1896), *K. reginae* Stüben, 2003, *K. saccoi* (Colonnelli, 1973) [35], and *K. reinosae* (H. Brisout de Barneville, 1867) [10, 34]. Two species, *K. reginae* and *K. reinosae*, are distinguished from the other species by the completely different habitus, but chiefly belong and were allocated early on to the *K. fausti* group "only" on the basis of the endophallus. This allocation has been shown to be justified by molecular data [34].

But what would have happened if *Kyklioacalles* had not been defined initially on the basis of the cyclical structure of the endophallus, as the name implies [36], and as an immediate consequence, *Acalles pyrenaeus* would not have been eliminated? Putting it the other way round: what if the similarity between this species *Kyklioacalles saccoi* and *Kyklioacalles fausti* had been considered in a habitus-to-habitus comparison? That is not only a simple, retrospective, and dispensable "what-if" question, because in this case one brings the fact to mind that *A. pyrenaeus* must now be coercively placed among the *Kyklioacalles* species, Figures 8(f)–8(h).

The definition especially of a higher taxon is an arbitrary supposition. As taxonomists we always operate with *constructs* and as morphologists we *find* characteristics that are prominent to our eyes (and sometimes "we like to see"). It must be admitted that extremely rarely do we look for homologies, which in theory constitute the best criterion (e.g., [37]), but are in practice often difficult to find when dealing with cryptic and similar-looking species.

This change of mind and perception is similar to a Gestalt switch [22], a figure spinning in two directions: the contour line of the species is the same, but the species is not (as in the Gestalt psychology, the vase-face and duck-rabbit illusions). We cannot explain this Gestalt switch based on morphological research alone, and we cannot build and establish it *within* this framework. But we know the cause for this inconspicuous paradigm change: only extrinsic evidence from DNA analysis has *opened* the morphologist's *eyes* in this case—and this in the true sense of the word.

4.2. Integrative Taxonomy: Changing the Way You Look for Species. Integrative taxonomy sounds like an accumulation of different disciplines: morphology, molecular biology, ecology, ethology, and biogeography deliver the ingredients. But this is not invariably the case, and not so simple. It is a more eventful, reciprocal exchange of *evidence*, which an initial suspicion either confirms or rejects (comparable to an unsolved criminal case).

An initial morphological suspicion was already available when we discovered the new species *Onyxacalles nuraghi* (*O. henoni* group) in the humid *Quercus/Acer* forest in the mountains of Marghine on Sardinia. The differences from the well-known species of this group are obvious and easy to assemble (see below differential diagnosis of *O. nuraghi*). In the case of *Onyxacalles vilae* sp. n. (*O. luigionii* group) from the Velebit Mountains (Croatia), it was morphologically more ambiguous, but in case of the third species *Onyxacalles torre* sp.n. (*O. henoni* group) from Corsica, the specimens of the current type series remained completely unnoticed in the collection for years.

But the molecular results in view of these three (resp. four) species are obvious (see also Figures 1–4). With maximal support, *O. vilae* is widely separated in the tree topology from *O. nuraghi* and *O. torre* (*O. henoni* group; Figure 1). Together with *Onyxacalles* sp. 1, *O. vilae* groups with *O. luigionii* (*Onyxacalles* sp. 1 as sister taxon of the latter), but all these species are separated by considerable p-distances (>9% COI and >4% 16S; Figure 3). Interestingly, the *O. vilae* specimen from Croatia shares the haplotype of the French specimen. *O. torre* and *O. nuraghi* are genetically closer to each other (1,8% 16S; Figure 2), but not sister species.

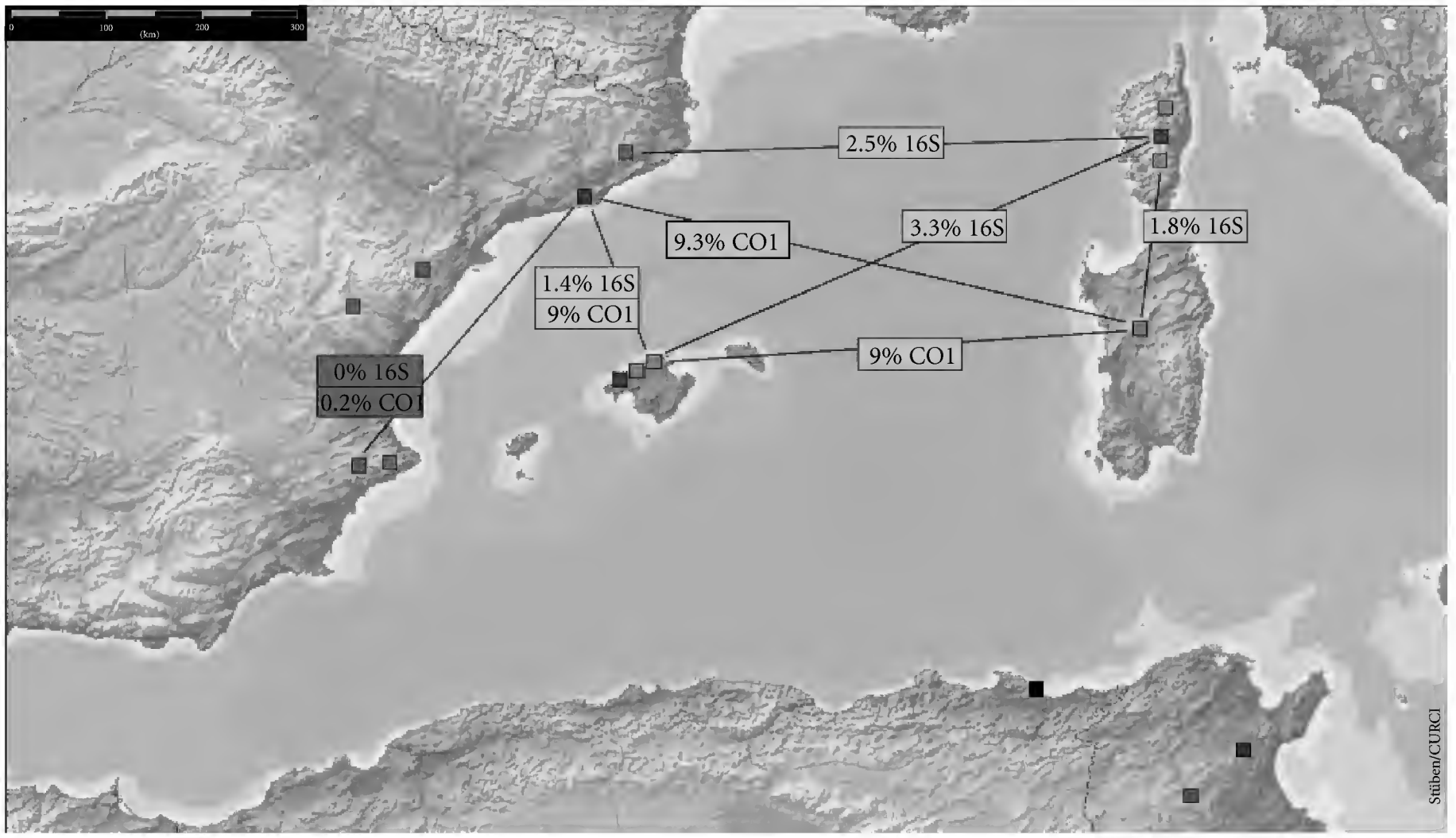

FIGURE 2: Distribution of related species of the West Mediterranean species of the ***O. henoni*** group (without *O. croaticus*, Eastern Europe) with values of COI and 16S p-distances.

The unambiguous molecular evidence (cf. Figures 2 and 3) prompts the morphological reinvestigation of the material and forces the morphologist to look for new characters (see below "taxonomy")—if they exist!

On the one hand—in the case of *Onyxacalles* species—we currently give more attention to the apex of the rostrum and its punctures, or of the more or less curved and hook-shaped apex of the aedeagus. Vice versa, we see in the conspicuous and serially placed elytral bristles and its erected tufts on the intervals "seductive anthropomorphisms" and analyses of yesteryears. The perception does not become more precise, and the tuning is not finer adjusted—rather the way we view characters has changed: the perspective regarding criterion and weighting has undergone a shift influenced by extrinsic data.

On the other hand, however, the few specimens in the immediate vicinity of the French village Sospel (Maritime Alps) attest that it does not succeed in all cases (see: Figure 1: *Onyxacalles* sp. 1). The high p-distances of the mitochondrial COI and 16S genes of these specimens compared to the Middle and South Italian populations of *O. luigionii* and to *O. vilae* from the Velebit Mountains and the French Isère indicate the plausibility of a new species (cf. Figure 3). However, morphological differences could not be diagnosed. More findings and larger series of comparison material should establish a clearer picture in future. Therefore, it is premature to postulate a cryptic species and contrast it in a differential diagnosis by nothing more than molecular characters at this stage. Furthermore, this action would have the direct disadvantage that all previous existing records from the French and Italian Maritime Alps could not be allocated to the above-mentioned species. Nevertheless, this could shift in the future, should in depth "DNA barcodes" become available for all applied entomological disciplines.

The first step in this development has already been taken: the **M**olecular **W**eevil **I**dentification-Project (ZFMK, CURCI), which is going to establish a molecular (DNA barcodes) and photographic database (stacked images) as well as the highly important associated reference collections for European Curculionoidae (ca. 6000 species). Only by meticulous cross-vouchering can misidentifications be corrected, and molecular results can be linked to the more than 250 years history of entomology. Integrative taxonomy is not just an accumulative or encyclopaedic "furthering of knowledge," but rather—as in this case—an interactive process in an interdisciplinary dialogue.

5. Taxonomy

Family: Curculionidae Latreille, 1802.

Subfamily: Cryptorhynchinae Schoenherr, 1825.

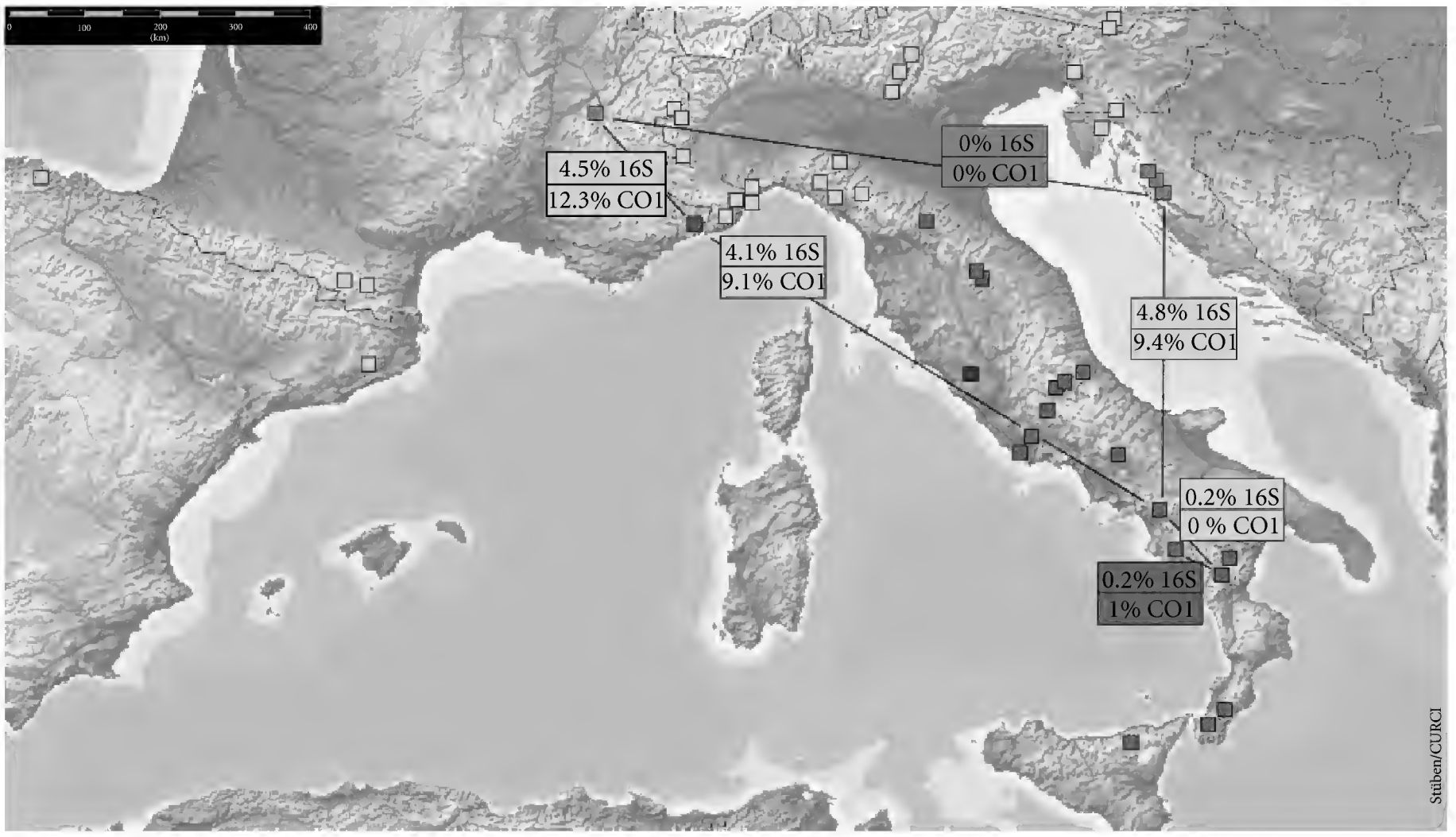

FIGURE 3: Distribution of related species of the ***O. luigionii*** group in the Mediterranean area with values of COI and 16S p-distances.

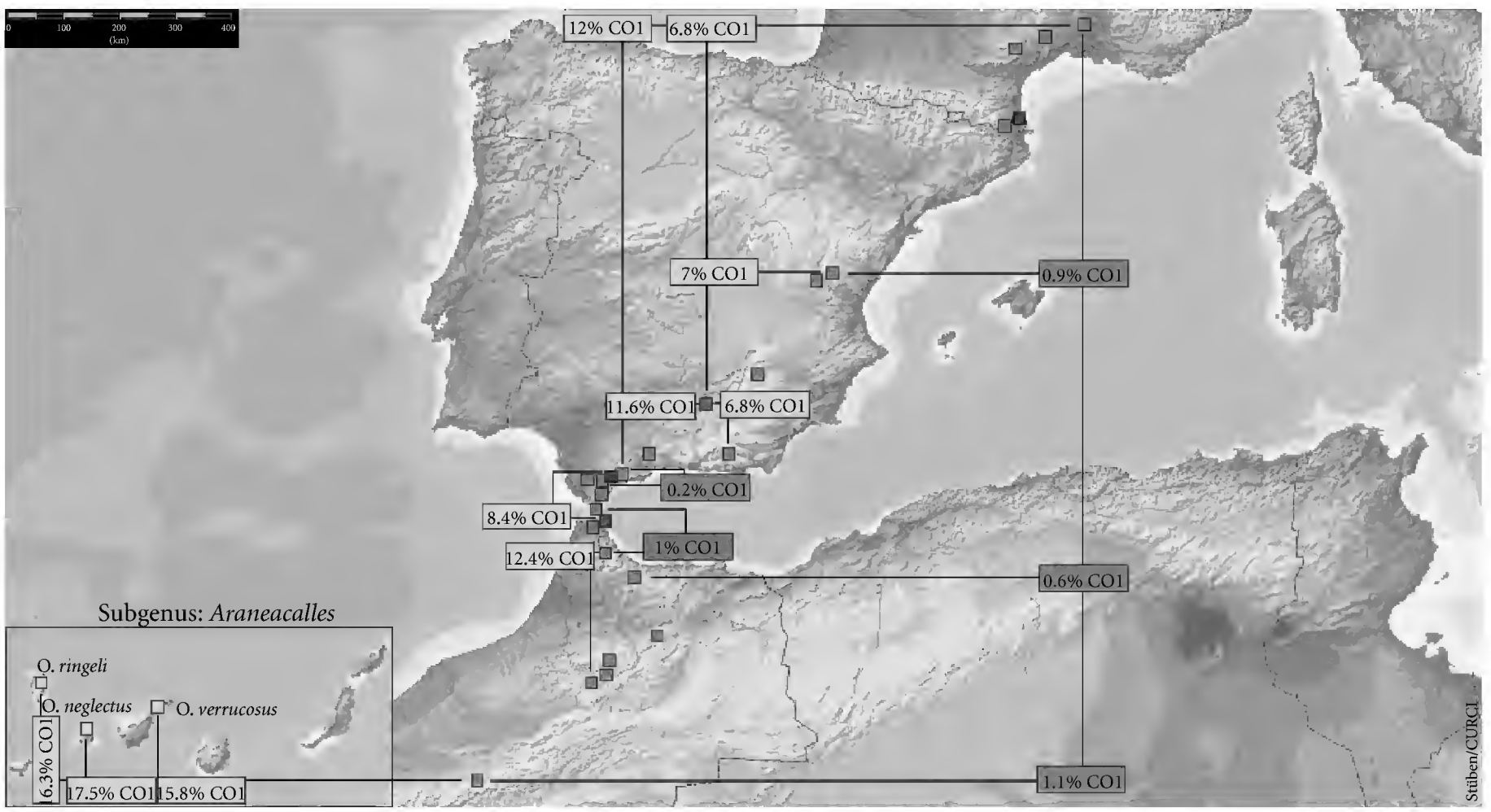

FIGURE 4: Distribution of related species of the ***O. portusveneris*** group in the Mediterranean area with values of COI p-distance (658 bp).

Genus: *Onyxacalles* Stüben, 1999, Type species: *Acalles luigionii* A. and F. Solari, 1907 (L.t.: Central Italy).

5.1. New Species of the Onyxacalles henoni Group

5.1.1. Onyxacalles nuraghi Stüben sp. n.

(Figures 5(a)–5(c), 5(i), 6(l)).

Type Material

Holotype (1♂). Italy: Sardinia, Macomer, Lei, N40°19′54″ E08°53′49″, 1020 m, *Quercus, Acer monspesulanus*, 4.10.2010, leg. Stüben-27-, coll. CURCULIO-Institut, D-Mönchengladbach.

Paratypes (1♂). Data as for holotype, coll. Stüben; **4♂**, **1♀**: Italy: Sardinia, Macomer, Lei, N40°19′17″ E08°53′52″, 586 m, *Quercus* ilex, 7.10.2010, leg. Stüben-33-, coll. Stüben, CURCULIO-Institut, D-Mönchengladbach (1♀), coll ZFMK, 1♂: ZFMK-DNA-JJ1097, ZFMK-TIS-cIT1097.

DNAtype (1♂). Data as for holotype, coll. ZFMK: ZFMK-DNA-JJ1090, ZFMK-TIS-cIT1090; GenBank Acc. no COI: JN121399, 16S: JN642097.

Differential Diagnosis. The new species from the south-facing slope of the Chain of Marghine (Italy: Sardinia) belongs—from a morphological and molecular perspective—to the *Onyxacalles henoni* group and should be compared with the most closely related species from Majorca (Spain): *Onyxacalles balearicus* Stüben, 2005.

Onyxacalles nuraghi

(1) Disc of pronotum with a channel from the base towards the flat sector in front of the fore-margin; with tufts of bristles on both sides of the channel (Figure 5(i)).

(2) Bristles on the elytral intervals at least 2x as long as wide; shaping tufts with big gaps; their distances range from 3x the length of bristles.

(3) Elytra of male with parallel sides in the middle sector (dorsal view); contour line of elytra forms almost a semicircle in lateral view (Figure 5(a)).

(4) Apex ("hook") of the aedeagus (in ventral view) smaller (Figure 5(c)).

Onyxacalles balearicus

(1*) Disc of prontoum without a channel and without tufts of bristles (Figure 5(h)).

(2*) The free-standing bristles on the intervals shorter, 1.3x as long as wide; placed in a single row, not forming tufts.

(3*) Elytra of male broader and stronger (short ovally) rounded (slightly "egg shaped"); contour line of elytra flatter or slightly rounded behind the base in lateral view (Figure 5(g)).

(4*) Apex (hook) of the aedeagus (in ventral view) broader (Figure 5(j)).

The new species from Sardinia is different from *Onyxacalles henoni* (Bedel, 1888) [38] (Algeria: Mt. Edough, *loc. typ.*), with which it has the tufts of bristles on the elytral intervals in common, by (1) darker elytral integument (Figure 5(a) versus 5(k)), (2) finer and longer white bristles on the femora, and (3) longer apex ("hook") of the aedeagus (lateral view, see Figure 5(c) versus 5(n)). It can be distinguished from *Onyxacalles valencianus* Germann, 2005 from the Spanish mainland (Barcelona: Villarana, *loc. typ.*) by (1) elytral tufts of bristles (versus single bristles), (2) deep channel of the pronotum (versus without channel), and (3) longer apex of aedeagus (lateral view, see Figure 5(c) versus 9(h)).

For a comparison with all other species see below the "Key to the species, of *Onyxacalles* Stüben, 1999".

Description

Length. 2.60–3.40 mm (without rostrum).

Head and Rostrum. Eyes large; rounded ovally towards front and acuminate towards underside of rostrum; frons between eyes more slender than the base of rostrum; rostrum reddish brown, closely covered with white scales at the base; rostrum of male reaching 3/4 length of pronotum and finely punctuated towards apex; rostrum of females reaching 4/5 length of pronotum, slender, shiny, and even more finely punctuated. The last three funicles of antennae short ovally rounded; the first two funicles elongated; the club clearly separated from funicles.

Pronotum. Widest at the end of the first third of the pronotum (holotype: 1.17x as wide as long); well rounded laterally towards the fore-margin and the base; with a deep depression at the sides directly behind the fore-margin; disk of pronotum strongly arced, with a channel in the middle from the base towards the flat sector in front of the fore-margin. The integument is rich in contrast consisting of round black scales on the disk and oval, white/brown scales on the flanks of the pronotum. Elongated and black bristles in an upright position in the middle of the disk on both sides of the channel; with a similar, but white tuft of bristles on each side of the pronotum; the deep punctures always covered with scales.

Elytra. Oblong (holotype: 1.29x as long as wide); widest in the middle and there with nearly parallel sides; only slightly rounded directly in front of the base; short ovally rounded towards the apex. Contour line of elytra strongly arced, almost forming a semi circle. The shiny and predominantly dark brown integument with a beige/white crescent-shaped fascia in front of the base and on the elytral slope. Bristles on the first and third interval (excluding the suture stripe)

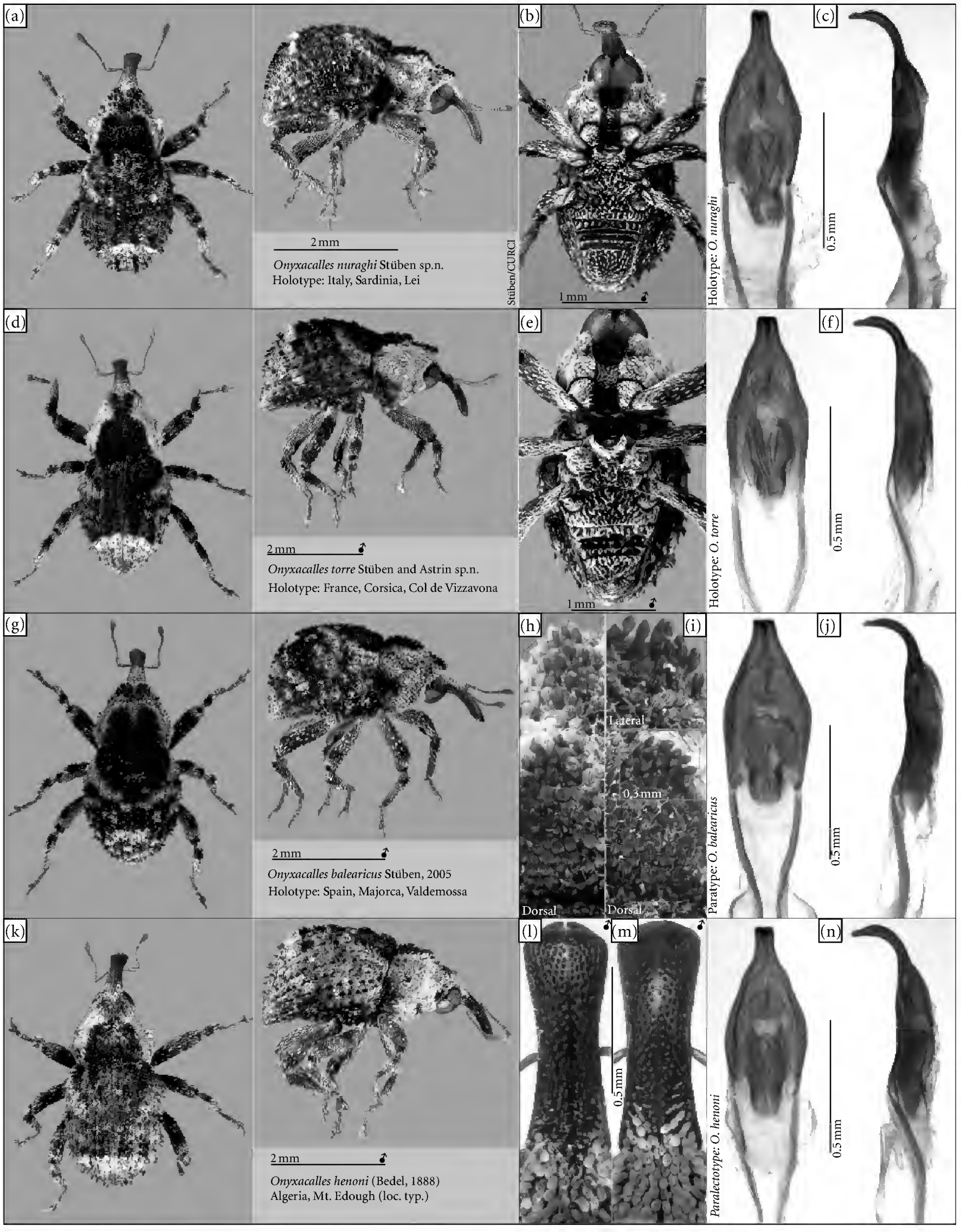

FIGURE 5: (a)–(c) *O. nuraghi* sp.n.—habitus (dor./lat./ven.), aedeagus (ven./lat.). (d–f) *O. torre* sp. n.—habitus (dor./lat./ven.), aedeagus (ven./lat.); (g, j) *O. balearicus*—habitus (dor./lat.), aedeagus (ven./lat.); (k, n) *O. henoni*—habitus (dor./lat.), aedeagus (ven./lat.). By comparison, bristle of pronotum—*O. balearicus* (h) versus *O. nuraghi* (i); rostrum—*O. henoni* (l) versus *O. torre* (m).

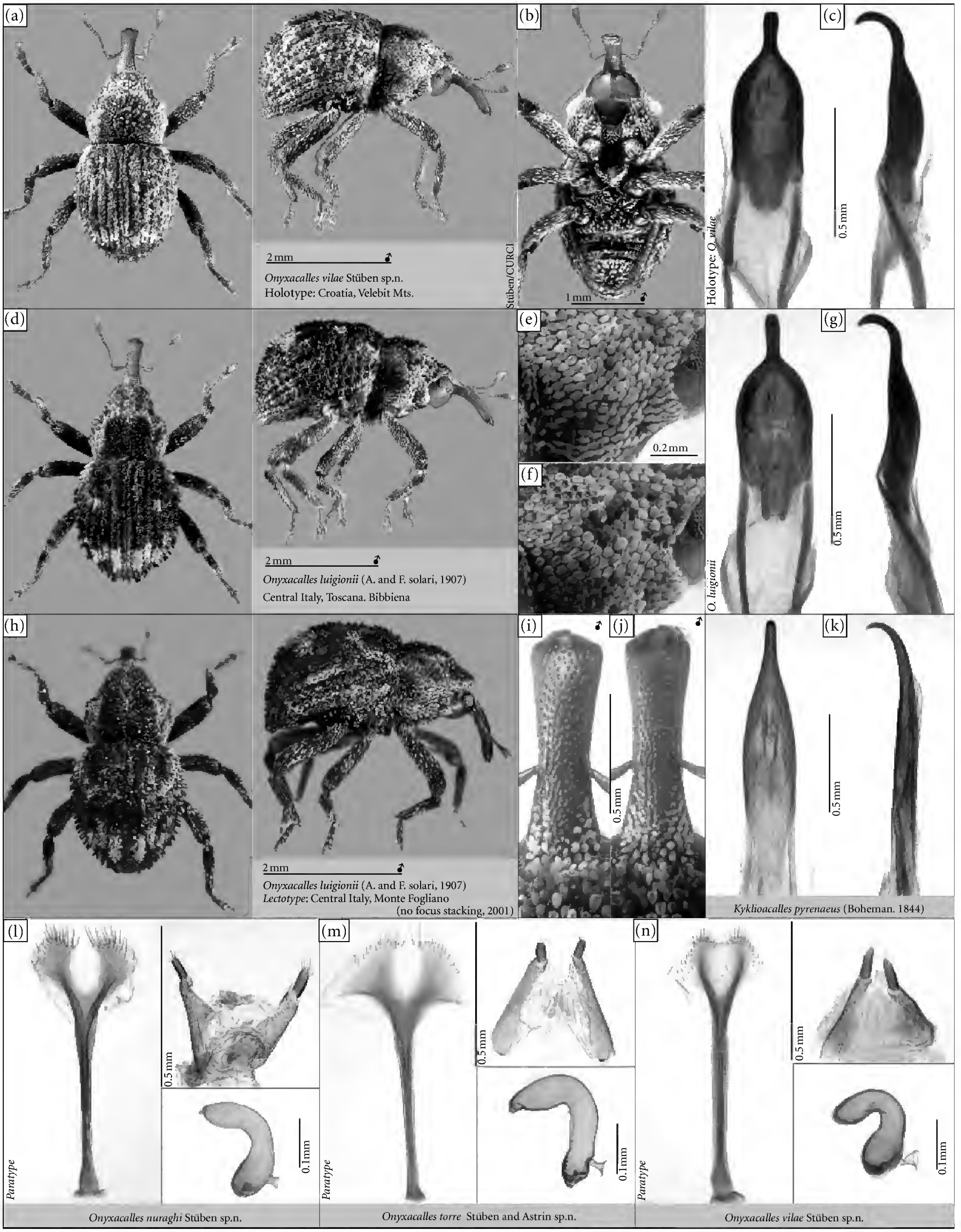

FIGURE 6: (a–c) *O. vilae* sp. n.—habitus (dor./lat./ven.), aedeagus (ven./lat.); (d, g) *O. luigionii*—habitus (dor./lat.), aedeagus (ven./lat.); (h) *O. luigionii* (lectotype)—habitus (dor./lat.); (k) *Kyklioacalles pyrenaeus*—aedeagus (ven./lat.); (l–n) female genital (spiculum ventrale, ovipositor, spermatheca) of *O. nuraghi* sp.n. (l), *O. torre* (m) and *O. vilae* (n). By comparison, bristle of pronotum (lat.)—*O. vilae* (e) versus *O. luigionii* (f); rostrum—*O. vilae* (j) versus *O. luigionii* (j).

at most 2x longer than wide, forming flattened tufts, which have big gaps between them (their distances range from three times the length of bristle); bristles sparse on the second and fourth intervals; striae on the disc and at the sides of elytra clearly narrower than intervals, punctures oblong.

Legs. Long; the marginal front femora reach the base of the rostrum; the hind femora reach the end of the elytral apex. They are covered with predominantly dark brown scales; tibia with long, white, and laterally protruding bristles.

Venter. The 2nd strip-type sternite only a little bit longer than the 3rd, but not longer than sternites 3 and 4 together. 1st sternite of male with a broad depression (Figure 5(b)).

Female Genital. See Figure 6(l).

Aedeagus. Apex ("hook") of the aedeagus (in ventral view) small, see Figure 5(c).

Etymology. The species name refers to the Nuragic civilization of Sardinia, lasting from the Bronze Age (18th century BC) to the 2nd century AD.

Ecology. Onyxacalles nuraghi was discovered by the first author on Sardinia near Lei (Macomer) in the mountains of Marghine in 2010. The seven specimens were shifted under *Quercus* and *Acer* between 500 and 1000 m above sea level.

Distribution. This species is so far only known from the Chain of Marghine, Figure 2.

5.1.2. Onyxacalles torre Stüben and Astrin sp. n.

(Figures 5(d)–5(f), 5(m), and 6(m)).

Type Material

Holotype (1♂). France, Corsica (Haute-Corse): Col de Vizzavona, 22 km S Corte, 1100 m, 8.10.2001, 42°06′45″N 09°06′49″E, *Fagus* (sift), leg. Stüben-4-, coll. CURCULIO-Institut, D-Mönchengladbach.

Paratypes (8♂, 9♀). Data as for holotype, coll. Stüben, CURCULIO-Institut, D-Mönchengladbach (1♀), Zoologisches Forschungsmuseum Alexander Koenig, D-Bonn (1♂, 1♀).

DNAtype (1♀). Data as for holotype, coll. ZFMK: ZFMK-DNA-JJ0882, ZFMK-TIS-cF479; GenBank Acc. no 16S: GU988592.

Further Material (1♂). France, Corsica (Haute-Corse): Caporalino 10 km N Corte, 350 m, 7.10.2001, 42°23′08″N 09°11′37″E, *Alnus, Fraxinus, Quercus* (sift), leg. Stüben-2-, coll. Stüben; **2♂**: France, Corsica (Haute-Corse): Tattone, 18 km S Corte, 750 m, 8.10.2001, 42°09′21″N 09°09′43″E, *Castanea* (sift), leg. Stüben-3-, coll. Stüben; **1♂**: France, Corsica (Corse-du-Sud): Radicale, 20 km E Ajaccio, 400 m, 9.10.2001, 41°55′31″N 08°58′10″E, 9.X.2001, *Quercus ilex* (sift), leg. Stüben-9-, coll. Stüben; **2♂**: France, Corsica (Corse-du-Sud): Cozzano 2 km NE Zicavo, 750 m, 10.10.2001, 41°55′31″N 09°08′31″E, *Castanea* (sift), leg. Stüben-12-, coll. Stüben; **4♂**, **4♀**: France, Corsica (Corse-du-Sud): Coll de La Vaccia N, 9,5 km S Zicavo, 1150 m, 10.10.2001, 41°49′19″N 09°05′04″E, *Fagus* (sift), leg. Stüben-14-, coll. Stüben.

Differential Diagnosis. The new species from Corsica (France) belongs—morphologically and molecularly—to the *Onyxacalles henoni* group and is distinguished from *Onyxacalles henoni* (Bedel, 1888) [38] from Algeria (Mt. Edough, *loc. typ.*) by the following characteristics.

Onyxacalles torre

(1) Rostrum finely punctuated towards apex (Figure 5(m)).

(2) Scales of the elytra predominantly dark brown or black (Figure 5(d)).

(3) Apex ("hook") of the aedeagus broader in ventral view and strongly curved (nearly rectangular) in lateral view (Figure 5(f)).

Onyxacalles henoni

(1*) Rostrum coarsely and densely punctuated towards apex (Figure 5(l)).

(2*) Scales of the elytra predominantly bright: white, beige, or brown (Figure 5(k)).

(3*) Apex ("hook") of the aedeagus smaller in ventral view and not so strongly curved in lateral view (Figure 5(n)).

The new species from Corsica can be distinguished from *Onyxacalles nuraghi* from Sardinia (see above) by (1) contour line of elytra behind the base flatter (in lateral view, Figure 5(d) versus 5(a)), (2) elytra more egg-shaped towards the apex (Figure 5(d) versus 5(a)), and (3) apex ("hook") of the aedeagus (in ventral view) shorter and wider (Figure 5(f) versus 5(c)). For a comparison with all other species, see the "Key to the species of *Onyxacalles* Stüben, 1999" below.

Description

Length. 3.00-4.00 mm (without rostrum).

Head and Rostrum. Eyes large; rounded towards front and acuminate towards underside of rostrum; frons between eyes as wide as the base of rostrum; rostrum reddish brown, closely covered with white scales at the base; rostrum of male reaching 2/3 length of pronotum and finely punctuated towards apex (Figure 5(m)); rostrum of females reaching 3/4

length of pronotum, slender, shiny, and even more finely punctuated. The last three funicles of the antennae short ovally rounded, the fourth 1.5x, the third 2x, the second 4.5x, and the first conical funicle 2x longer than wide; the elongated club clearly separated from funicles.

Pronotum. Widest at the end of the first third of the pronotum (holotype: 1.17x as wide as long); strongly rounded laterally towards the fore-margin and the base; with a depression at the sides directly behind the fore-margin; disk of pronotum arced, in the middle sometimes with a slight channel-like depression in front of the base. The integument is rich in contrast consisting of round black and dark-brown scales on the disk and in front of the fore-margin, and more or less oval, white scales on the flanks of the pronotum. In the middle of the disk on both sides of the flat depression with elongated, studded, and black bristles in an upright position; with similar placed, but shorter and white bristles on each side of the pronotum; the punctures always covered with scales.

Elytra. Oblong (holotype: 1.31x as long as wide); widest in front of the middle, here with parallel sides or slightly egg-shaped towards the apex; strongly curved in front of the base. Contour line of elytra flatter behind the base in lateral view, the contour line of the elytral slope forming an arc towards the apex. The shiny and predominantly dark brown or black integument with a beige/white crescent-shaped fascia in front of the base and on the elytral slope; sometimes the whole apex can be light brown. Bristles on first and third interval (excluding the suture stripe) 1.5x longer than wide, flattened, and shaping tufts; their distances range from the 2x length of bristles. Bristles sparse on the second and fourth intervals; striae on the disc and at the sides of elytra are small strips, clearly narrower than the intervals, punctures on the disc oblong, round at the sides.

Legs. Long; the marginal front femora reach the base of the rostrum, and the hind femora reach the end of the elytral apex. They are covered with predominantly dark brown and elongated scales; tibia with white/brown and laterally protruding bristles forming fasciae.

Venter. The 2nd strip-type sternite only a little bit longer than the 3rd, but not longer than sternites 3 and 4 together. 1st sternite of male with a broad depression (Figure 5(e)).

Female Genital. See Figure 6(m).

Aedeagus. Apex (hook) of the aedeagus broad in ventral view and strongly curved (nearly rectangular) in lateral view; see Figure 5(f).

Etymology. The species name refers to the Torrean civilization in Corsica during the second millenium BC. The characteristic building of this culture is the "Torre" (tower), the Corsican counterpart of the Sardinian "Nuraghe."

Ecology. *Onyxacalles torre* was sifted by the first author in the mountains of Corsica and is a nocturnal inhabitant of the dark and shady forests like all other *Onyxacalles*.

Distribution. This species is so far only known from Corsica (France); Figure 2.

5.2. New Species of the *Onyxacalles luigionii* Group

5.2.1. *Onyxacalles vilae* Stüben sp. n.

(Figures 6(a)–6(c), 6(e), 6(i), and 6(n)).

Type Material

Holotype (1♂). Croatia: 20 km S Krasno Polje, Northern Velebit Mts., N44°38′14″ E15°05′13″, 1185 m, limestone: *Fagus*, 26.7.2004, leg. Stüben-10-, coll. CURCULIO-Institut, D-Mönchengladbach.

Paratypes (1♂, 2♀). Data as for holotype, coll. Stüben, CURCULIO-Institut, D-Mönchengladbach (1♀), Zoologisches Forschungsmuseum Alexander Koenig, D-Bonn (1♀); **1♀**: Croatia: Krasno Polje, Northern Velebit Mts., N44°49′40″ E15°01′49″, 838 m, limestone: *Fagus, Quercus*, 25.7.2004 leg. Stüben-7-, coll. Stüben; **1♂**: Croatia: 5 km W Krasno Polje, Northern Nord-Velebit Mts., N44°48′49″ E14°58′36″, 1534 m, limestone: *Fagus*, 26.7.2004, leg. Stüben-8-, coll. Stüben; **1♀**: Croatia: 6 km W Krasno Polje, Northern Velebit Mts., N44°48′56″ E14°58′08″, 1494 m, limestone: *Fagus*, 26.7.2004, leg. Stüben-9-, coll. Stüben; **1♂**: Croatia: 12 km S Krasno Polje; Northern Velebit Mts., N44°43′00″ E14°59′42″, 1414 m, limestone: *Fagus*, 27.7.2004, leg. Stüben-14-, coll. Stüben.

DNAtype (1♂). Croatia: 8 km E Karlobag, Velebit Mts., Stupacinovo, N44°32′41″ E15°09′58″, 1049 m, limestone: *Fagus*, 14.07.2007, leg. Stüben-27-, coll. ZFMK: ZFMK-DNA-JJ0195, ZFMK-TIS-cHR339; GenBank Acc. no COI: EU286512, 16S: EU286348.

Further Material (9♂, 7♀). France, Isère, 2 km SE Lans en Vercors, Montagne de Lans, 45°06′40″N 05°36′25″E, 1391 m, 23.7.2011, Kalk: *Fagus* (beaten), leg. Stüben-2-, coll. Stüben; **3♂, 3♀**: France, Isère, NW Lans en Vercors: near Autrars, Parc Regional du Vercors, 45°14′12″N 05°34′58″E, 1370 m, 23.7.2011, Kalk: *Fagus* (beaten), leg. Stüben-5-, coll. Stüben.

Differential Diagnosis. The new species from Croatia (Velebit Mts., *loc. typ.*) belongs—morphologically and molecularly—to the *Onyxacalles luigionii* group and is distinguished from *Onyxacalles luigionii* (A. & F. Solari, 1907) [32] from Central Italy (Monte Fogliano, *loc. typ.*) by the following characteristics.

Onyxacalles vilae

(1) Rostrum of the male broader, 2.8x as long as wide (as measured by apex); punctures not so densely packed

in front of the apex (separated by flat intervals) Figure 6(i).

(2) Bristles of the low-contrast elytra on first and third intervals (excluding the sutural stripe) longer and more slender; their distance is larger (Figure 6(a)).

(3) Scales of the white fascia at the sides of the pronotum (behind the base) predominantly oblong (Figure 6(e)).

(4) Median lobe of aedeagus smaller, 1.64x as long as wide; apex ("hook") a little bit shorter (in ventral view), flatter, and not so strongly curved in lateral view (Figure 6(c)).

Onyxacalles luigionii

(1*) Rostrum of the male more slender, 3.1x as long as wide; punctures mainly dense towards the apex (only separated by small ridges) Figure 6(j).

(2*) Bristles of high-contrast elytra on the on first and third intervals shorter, broader (towards the apex of bristle), and more dense, clearly visible on the white fascia of the elytral slope (Figures 6(d) and 6(h)).

(3*) Scales of the white fascia at the sides of the pronotum (behind the base) predominantly round (Figure 6(f)).

(4*) Median lobe of aedeagus broader, 1.93x as long as wide; apex ("hook") of the aedeagus longer (in ventral view), strongly (nearly rectangular) curved in lateral view. Internal structure (endophallus) of the sac different (Figure 6(g)).

For a comparison with all other species see below the "Key to the species of *Onyxacalles* Stüben, 1999."

Description

Length. 2.40–3.20 mm (without rostrum).

Head and Rostrum. Eyes large; rounded ovally towards front and acuminate towards underside of rostrum; frons between eyes as wide as the base of rostrum; rostrum reddish brown, closely covered with white and oval scales at the base; rostrum of male 2.8x as long as wide (as measured by apex) and finely punctuated towards apex, here separated by flat intervals (Figure 6(i)); rostrum of female clearly longer, slender, shiny, and even more finely punctuated (without punctures in front of the apex). The last funicles of antennae nearly trapezoidal, the funicles 4–6 short oval, the third funicle 1.3x, the second 3x, and the first conical funicle 1.5x longer than wide; the elongated club not clearly separated from the 7th trapezoid funicle.

Pronotum. Widest at the end of the first third of the pronotum (holotype: 1.12x as wide as long); well rounded laterally towards the fore-margin and the base; with a slight depression at the sides directly behind the fore-margin; disk of pronotum arced, without a channel or a flat depression in the middle. The integument not so rich in contrast, consisting of round, brown scales on the disk and off-white, predominantly oblong scales at the sides of the pronotum (behind the base, Figure 6(e)). In the middle of the disk with elongated brown bristles in an upright position; with a similar, but white tuft of bristles on each side of the pronotum; the deep and dense punctures covered with scales.

Elytra. Short oval (holotype: 1.19x as long as wide); widest at the end of the first fourth in front of the elytral base; here laterally strongly rounded directly in front of the base; ovally rounded towards the apex. Contour-line of elytra flatter behind the base in lateral view, the contour line of the elytral slope forming a circular arc towards the apex. Bristles of the low-contrast elytra on first and third intervals (excluding the sutural stripe) slender, 2x-3x as long as wide, their distance reaching the double length of bristle, and forming tufts only in front of the base and on the elytral slope; the uprightly protruding bristles on the second and fourth intervals have wider gaps between them (their distances range from three- to fourfold length of bristle); the scales on the intervals do not cover the underground completely; striae on the disc broad, but not broader than the intervals, reaching the width of the intervals at the sides of elytra (but often covered by scales); punctures deep and rounded.

Legs. Long; the marginal front femora reach the base of the rostrum, and the hind femora reach the end of the elytral apex. They are covered with predominantly brown scales; tibia with short, white, and laterally protruding bristles.

Venter. The 2nd strip-type sternite only a little bit longer than the 3rd, but not longer than sternites 3 and 4 together. 1st sternite of male with a broad depression (Figure 6(b)).

Female Genital. See Figure 6(n).

Aedeagus. Median lobe of aedeagus small, 1.64x as long as wide; see Figure 6(c).

Etymology. The species name refers to a "Vila" (*fairy*) in the Velebit Mts. This massif has a similar relevance for Croatians to Olympus for Greeks or the Fuijiyama for the Japanese. In Croatia, the mystical Velebit Mts. range is famous for its fairies, the most celebrated called "Vila Velebita" (*The Fairy of Velebit*).

Ecology. Onyxacalles vilae was sifted by the first author in the Velebit mountains of Croatia under *Fagus* and *Quercus* between 800 and 1600 m above sea level.

Distribution. A complete distribution map will be given in a separate faunistic study in the future, but this species was also sifted by the author in the Montagne de Lans near Lans en Vercors (France: Isère)—on limestone and under *Fagus*, too (N45°06′45″ E05°36′21″, 1352 m). Working hypothesis: It could be possible that all specimens of the "Alpine Arc"—between Grenoble and the Velebit mountains—belong to this

new species and can be separated from the Central Italian populations of *Onyxacalles luigionii* (cf. Figure 3).

6. Key to the Species of *Onyxacalles* Stüben, 1999

(1) Smaller species with an ovally rounded habitus, legs shorter, femora reach the base of the rostrum; rostrum shorter and broader; if tufts of bristles exist on the elytral slope, these are only densely placed (not tapered). Distribution: continent of Western Palaearctic.

Subgenus: *Onyxacalles* s. str **2**

(1*) Larger species with a more "elliptical" habitus and with long legs, femora reach the insertions of the antennae; rostrum very long and slender; the tapered tufts of bristles on the elytral slope strongly protruding (Figures 8(b)–8(d)). Distribution: Western Canary Islands.

Subgenus: *Araneacalles* Stüben and Astrin, 2010 ... **16**

(2) Apex of the aedeagus regularly rounded in lateral view: Figures 6(c) and 6(g) (both species without distinctive tufts of bristles on the uneven elytral intervals, only with densely placed bristles in one or two rows).

***Luigionii* group** **2**

(2*) Apex of the aedeagus with a second, separated peak in lateral view (cf. Figures 9(a)–9(l)) (most species with more or less characteristic tufts of bristles on the elytra).

..... **4**

— *Luigionii* group —

(3) Rostrum of the male more slender, 3.1x as long as wide; punctures mainly dense towards the apex (only separated by small ridges) (Figure 6(j)). Bristles of high-contrast elytra on the 1st and 3rd intervals shorter, broader (towards the apex of bristle), and more dense (Figures 6(d) and 6(h)). Median lobus of aedeagus broader, 1.93x as long as wide; apex ("hook") of the aedeagus longer (in ventral view), strongly (nearly rectangular) curved in lateral view. Internal structure (endophallus) of the sac different (Figure 6(g)). Distribution: Central and Southern Italy (Figure 3).

***Onyxacalles luigionii* (A. & F. Solari, 1907) [32]**
= *? Onyxacalles porcheti* [39] (Figure 8(e), Pyrenees)

(3*) Rostrum of the male broader, 2.8x as long as wide (as measured by apex); punctures not so densely packed in front of the apex (separated by flat intervals) (Figure 6(i)). Bristles of the low-contrast elytra on the 1st and 3rd intervals (excluding the sutural stripe) longer and more slender; their distance larger (Figure 6(a)). Median lobus of aedeagus smaller, 1.64x as long as wide; apex ("hook") a little bit shorter (in ventral view), flatter, not so strongly curved in lateral view (Figure 6(c)). Distribution: Croatia (l.t.), France: Isère (Figure 3).

***Onyxacalles vilae* Stüben sp. n.**

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(4) Pronotum levelled, "triangular" and stubby, strongly broadened just behind the base; the base 2x longer than the fore-margin (this characteristic is not so pronounced in *O. gibraltarensis*; however, this species can be clearly separated from all other *Onyxacalles* s. str. by the completely rounded sides of the aedeagus (Figure 9(a)), see digit 5). Distribution: Southern France, Iberian Peninsula, Morocco.

***Portusveneris* group** **5**

(4*) Pronotum more arched and marginally broader than long, widest at the end of the first third; the base at most 1.5x longer than the fore-margin. Distribution: Algeria, Tunisia, Western Spain, West Mediterranean Islands, Southeastern Europe, Turkey.

..... **9**

— *Portusveneris* group —

(5) Pronotum more slender, clearly separated from elytra; body outline broadly similar to the species of the *henoni* group (see digit 4*), but easy to distinguish from these species by the completely rounded sides of the aedeagus. Habitus (Figure 7(a)). Aedeagus (Figure 9(a)). Distribution: Southern Spain, Northern Morocco (Figure 4).

***Onyxacalles gibraltarensis* Stüben, 2002**

(5*) Pronotum widest directly behind the base and elytra widest directly in front of the base; therefore, pronotum and elytra do not seem separated (Figures 7(b)–7(e)).

..... **6**

(6) Elytra egg-shaped towards the apex (Figure 7(b)); Aedeagus (Figure 9(b)). Distribution: Southern France, Iberian Peninsula, Morocco (Figure 4).

***Onyxacalles portusveneris* (Mayet, 1903) [27]**

(6*) Elytra oval or with more or less parallel sides (Figures 7(c)–7(e)).

..... **7**

(7) Male with a split midtibia spine at the apex; uneven elytral intervals without tufts of bristles. Habitus (Figure 7(c)). Aedeagus (Figure 9(c)). Distribution: Southern Spain (Figure 4).

***Onyxacalles seguraensis* Stüben, 2003**

(7*) Male without a split midtibia spine at the apex; uneven elytral intervals with tufts of bristles.

..... **8**

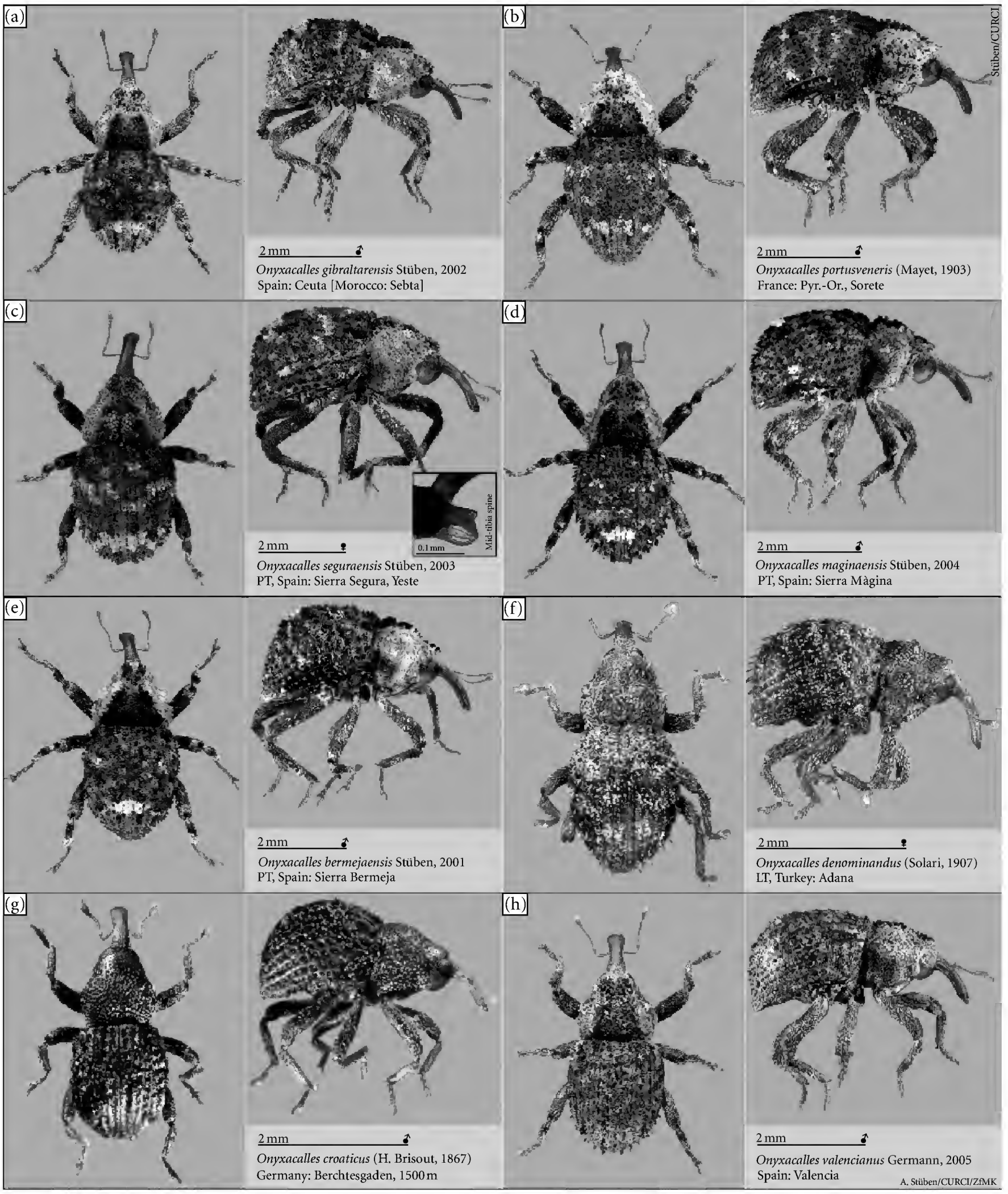

FIGURE 7: (a–e): ***Portusveneris* group**—*O. gibraltarensis* (a), *O. portusveneris* (b), *O. seguraensis* (c), *O. maginaensis* (d), and *O. bermejaensis* (e); (f): **Incertae sedis**—*O. denominandus*; (g-h): ***Henoni* group** (see also next figures)—*O. croaticus* (g) and *O. valencianus* (h); all habitus (dor./lat.).

(8) Punctures at the sides of elytra fine and slender; bristles on the first four intervals of the elytral slope in single row. Habitus (Figure 7(d)). Aedeagus (Figure 6(d)). Distribution: Southern Spain (Figure 4).

***Onyxacalles maginaensis* Stüben, 2004**

(8*) Punctures at the sides of elytra broader and deeper; bristles on the third interval of the elytral slope densely placed, forming a pronounced tuft at the level of the white fascia. Habitus (Figure 7(e)). Aedeagus (Figure 9(e)). Distribution: Southern Spain (Figure 4).

***Onyxacalles bermejaensis* Stüben, 2001**

—

(9) Pronotum with a deep midgroove and with strong concavities on each side; a species from Turkey.

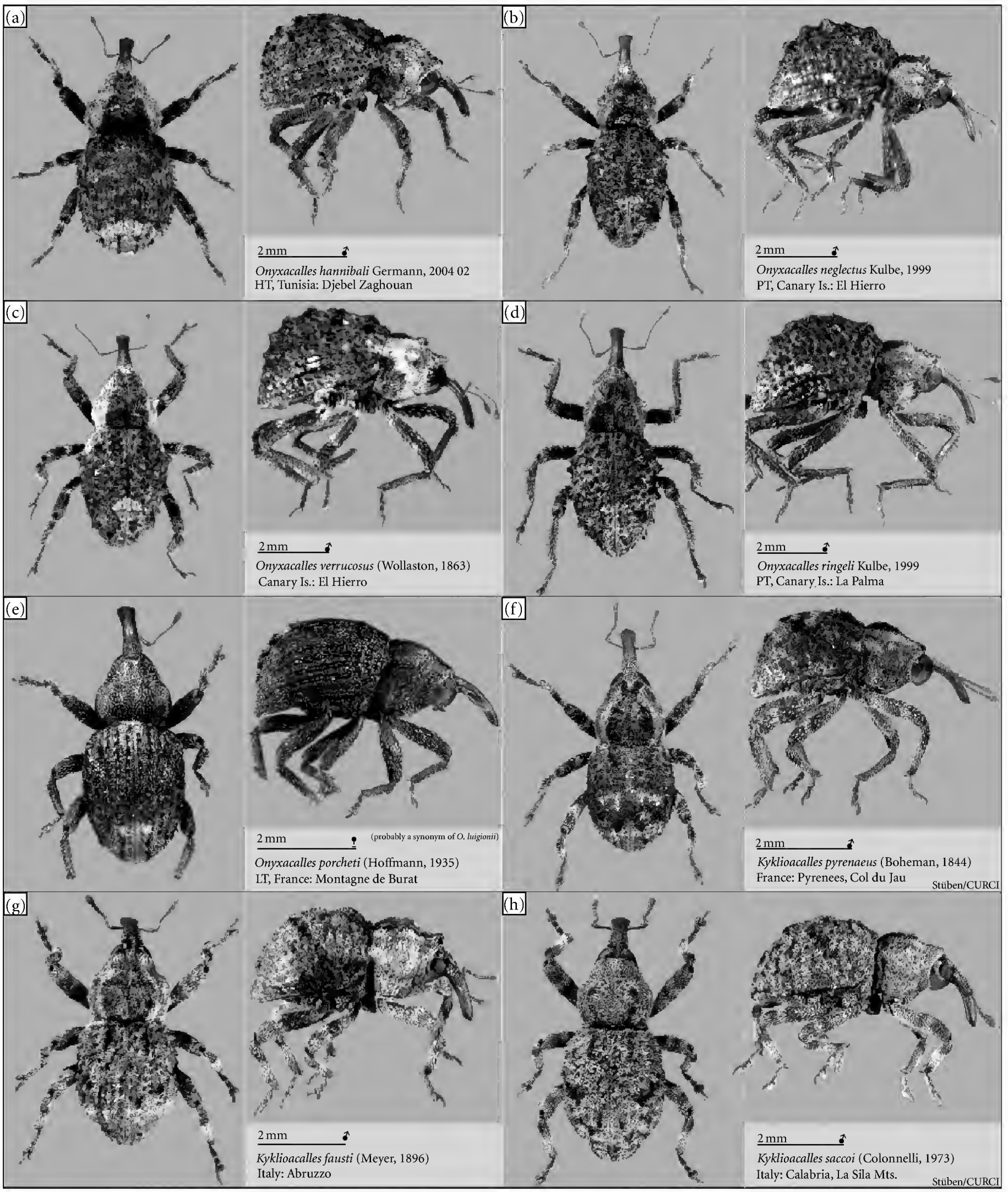

FIGURE 8: (a): ***Henoni* group**—*O. hannibali* (see also the other species of this group: Figures 5(a), 5(d), 5(g), 5(k)); (b–d): subgenus: ***Araneacalles***—*O. neglectus* (b), *O. verrucosus* (c), and *O. ringeli* (d); (e): *O. porcheti* (perhaps *O. luigionii*); (f): *Kyklioacalles pyrenaeus*; (g) *K. fausti*; (h) *K. saccoi*; all habitus (dor./lat.).

Habitus (Figure 7(f)). Aedeagus (Figure 9(f)). Distribution: Turkey.

***Onyxacalles denominandus* (A. & F. Solari, 1907) [32]**
= *? Onyxacalles amasyaensis* Wolf, 2001

(9) Pronotum behind the base at most with a flat depression or a hinted channel; mainly West Mediterranean species, only one species from southeastern Europe.

***Henoni* group** **10**

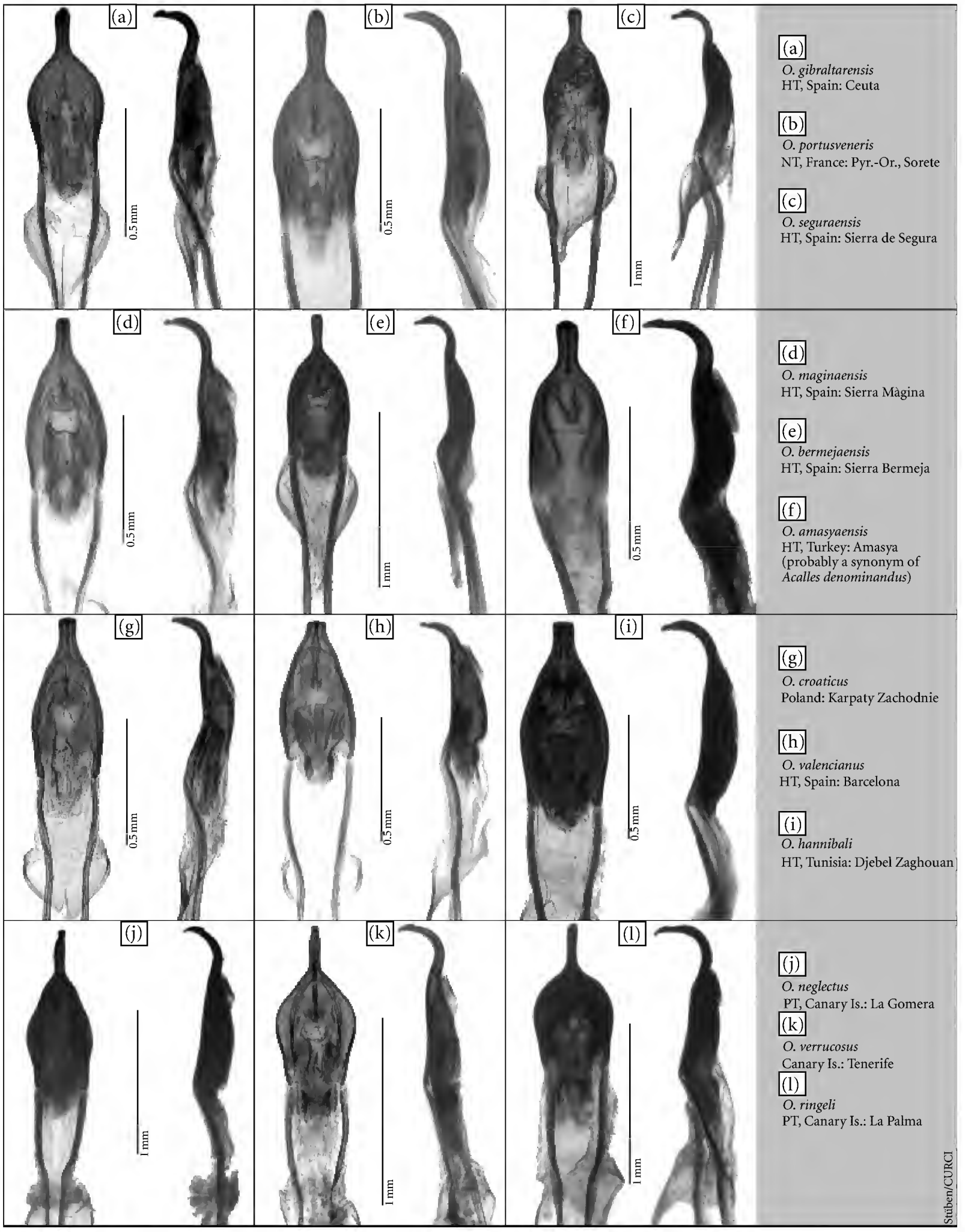

FIGURE 9: (a–d): ***Portusveneris* group**—*O. gibraltarensis* (a), *O. portusveneris* (b), *O. seguraensis* (c), *O. maginaensis* (d), and *O. bermejaensis* (e); (f): **Incertae sedis**—*O. amasyaensis*; (g–i): ***Henoni* group**—*O. croaticus* (g), *O. valencianus* (h), *O. hannibali* (i) (see also the other species of this group: Figures 5(c), 5(f), 5(j), 5(n)); (j–l): subgenus: ***Araneacalles***—*O. neglectus* (j), *O. verrucosus* (k), and *O. ringeli* (l).

— *Henoni* group —

(10) Elytra with superelevated and in tubercles dissected intervals; a species from southeastern Europe, which is added to the *henoni* group preliminary on the basis of a similar form of the aedeagus. Habitus (Figure 7(g)). Aedeagus (Figure 9(g)). Distribution: East and Southeast Europe [31].

***Onyxacalles croaticus* (H. Brisout de Barneville, 1867) [10]**

(10) Elytra flat, without tubercles.

..... **11**

(11) Elytra and pronotum (almost) without tufts of bristles; these single, beaded bristles placed in a row.

..... **12**

(11*) Elytra on the intervals 1 and 3 and pronotum at the sides with tufts of bristles; these bristles densely placed in 2-3 rows, forming tufts at regular intervals.

..... **13**

(12) Elytral bristles short, shovel-shaped and densely placed (at most 1.3x as long as wide); elytra rich in contrast (colored); apex of aedeagus strongly curved in lateral view. Habitus (Figure 5(g)). Aedeagus (Figure 5(j)). Distribution: Spain, Majorca (Figure 2).

***Onyxacalles balearicus* Stüben, 2005**

(12*) Elytral bristles more slender, at least 2x as long as wide and their distance large; elytra poor in contrast; apex of aedeagus flatter curved in lateral view. Habitus (Figure 6(n)). Aedeagus (Figure 9(h)). Distribution: Eastern Spain (Figure 2).

***Onyxacalles valencianus* Germann, 2005**

(13) Disc of pronotum with a channel from the base towards the flat sector in front of the fore-margin; elytra of male with parallel sides in the middle sector (dorsal view). Habitus (Figure 5(a)). Aedeagus (Figure 5(c)). Distribution: Italy, Sardinia (Figure 2).

***Onyxacalles nuraghi* Stüben sp. n.**

(13) Disc of pronotum at most with a flat hallow behind the base; elytra of male broader and stronger (short ovally) rounded (slightly "egg-shaped").

..... **14**

(14) Elytral intervals only with a few bristles and small tufts; elytra poor in contrast. Habitus (Figure 8(a)). Aedeagus (Figure 9(i)). Distribution: Tunisia (Figure 2).

***Onyxacalles hannibali* Germann, 2004**

(14*) Elytral intervals studded with bristles and with numerous distinctive tufts; elytra rich in contrast.

..... **15**

(15) Rostrum coarsely and densely punctuated towards apex (Figure 5(l)); scales of the elytra predominantly bright: white, beige, or brown (Figure 5(k)); Apex ("hook") of the aedeagus smaller in ventral view and not so strongly curved in lateral view (Figure 5(n)). Distribution: Algeria, Mt. Edough (Figure 2).

***Onyxacalles henoni* [38]**

(15*) Rostrum finely punctuated towards apex (Figure 5(m)); scales of the elytra predominantly dark brown or black (Figure 5(d)); apex ("hook") of the aedeagus broader in ventral view and strongly curved (nearly rectangular) in lateral view (Figure 5(f)). Distribution: France, Corsica (Figure 2).

***Onyxacalles torre* Stüben and Astrin sp. n.**

— Subgenus: *Araneacalles* —

(16) Punctures of the 1st and 2nd elytral striae rounded, pothole-like, and as wide as the intervals; the underground of elytra in front of the middle on the 8th and 9th intervals with scales, not shiny; fore-margin of pronotum with a curved up collar. Habitus (Figure 8(b)). Aedeagus (Figure 9(j)). Distribution: Canary Is., La Gomera (l.t.), El Hierro (Figure 4).

***Onyxacalles neglectus* Kulbe, 1999**

(16*) Punctures of the 1st and 2nd elytral striae elongated, clearly smaller than intervals; the underground of elytra in front of the middle on the 8th and 9th intervals without scales, shiny; fore-margin of pronotum without a curved up collar.

..... **17**

(17) Punctures at the extreme striae slender, the intervals broader; elytra widest in front of the middle, egg-shaped. Habitus (Figure 8(c)). Aedeagus (Figure 9(k)). Distribution: Canary Is., Tenerife (l.t.), El Hierro (Figure 4).

***Onyxacalles verrucosus* (Wollaston, 1863) [40]**

(17*) Punctures at the extreme striae larger and rounded, not broader than intervals; elytra oval, widest in the middle. Habitus (Figure 8(d)). Aedeagus (60). Distribution: Canary Is., La Palma (Figure 4).

***Onyxacalles ringeli* Kulbe, 1999**

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Research Article

Attractant Pheromone of the Neotropical Species *Neomegalotomus parvus* (Westwood) (Heteroptera: Alydidae)

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The Neotropical broad-headed bug, *Neomegalotomus parvus* (Westwood), is adapted to various leguminous crops and is considered a pest in common bean and soybean. The chemical communication of this species was studied in order to identify an attractant pheromone. Males and females of *N. parvus* produce several short-chain esters and acids, and their antennae showed electrophysiological responses to five of these compounds, three common to both sexes (hexyl butanoate, 4-methylhexyl butanoate, and hexyl hexanoate), and two female-specific compounds (4-methylhexyl pentanoate and hexyl pentanoate). Both aeration extracts of females and a solution containing five synthetic compounds mimicking the natural blend were attractive to males and females *N. parvus* in a laboratory bioassay. Aspects of the chemical ecology of the broad-headed bugs and the possibility to use pheromone-baited traps in the field for monitoring are discussed.

1. Introduction

Neomegalotomus parvus, or broad-headed bug (Heteroptera: Alydidae), subfamily Alydinae, is native to South America. As other alydines, *N. parvus* is an oligophagous bug that feeds on immature seeds of legumes [1, 2]. The taxonomic status of Neotropical alydine bugs was reviewed by Schaefer and Panizzi [3], Schaffner and Schaefer [4], Schaefer [5], and Schaefer and Ahmad [6]. From these works, Neotropical species formerly classified in the genus *Megalotomus* are now grouped in the genus *Neomegalotomus*, and South American species of *Neomegalotomus* were synonymized as *N. parvus*.

N. parvus has been adapted to various leguminous crops such as lablab beans, *Dolichus lablab* L. [7], pigeon pea, *Cajanus cajan* (L.) Mill., pig bean, *Canavalia ensiformis* (L.) DC., and indigo, *Sesamum indicum* L. [8, 9]. However, it is the common bean, *Phaseolus vulgaris* L. [10, 11] and soybean, *Glycine max* (L.) Merrill [12], that this bug is an economically important pest.

Insect feeding causes direct damage to crops and, in beans, is responsible for reduction of seed mass and high seedling mortality [9, 11]. Santos and Panizzi [12] showed that artificial infestation of soybean plants during the pod-filing stage causes a reduction in seed vigor and viability and has a negative effect on seed quality when infestation is in advanced stage. However, asynchrony between vulnerable stages of soybean seed development and *N. parvus* populations allows soybean crops to usually escape severe injury from this insect in the field [9].

Currently, control of *N. parvus* is exclusively insecticidal, and application timing is not based on the accurate population monitoring. These insects are easily disturbed and highly mobile, so the sampling cloth technique normally used to survey heteropteran populations in the field [13] is not a reliable. In Brazil, sweep-netting is the recommended monitoring method but it is laborious and time consuming; therefore, most growers prefer to use calendar-based application of insecticides. Pheromone-baited traps would be

an alternative, more precise sampling technique to help minimize pesticide applications and increase efficacy.

Semiochemicals of Alydidae have been described for *Alydus* [14, 15], *Megalotomus* [14], *Riptortus* [16–18], and *Leptocorisa* [19] species, and for some of these bugs both adults and nymphs are reportedly attracted. For example, field experiments showed that *Riptortus clavatus* (Thunberg) could be efficiently captured in traps baited with their aggregation pheromone [20–22].

For *N. parvus*, traps baited with live males captured significantly more males that unbaited traps [23]. In addition, it is known from field observations that some bugs are attracted to cow urine [24], and traps baited with cow urine or NH_4OH solutions captured *N. parvus* in the field [25]. Thus, traps are potentially useful for population monitoring of *N. parvus*, but the efficiency of trap-based monitoring could be greatly improved if more specific and powerful attractants were available. The objective of this work was to determine if *N. parvus* males and/or females produce specific compounds that could be used as pheromone.

2. Materials and Methods

2.1. Insects Rearing. A laboratory colony of *N. parvus* was established from adults and nymphs field-collected from 2009 to 2011 from beans fields near Embrapa Genetic Resources and Biotechnology, Brasília, DF, Brazil (15° 47′ S and 47° 55′ W). Bugs were reared in 8 L plastic containers, on a diet of green beans pods (*Phaseolus vulgaris* (L.)), branches with flowers, and pods of pigeon pea (*Cajanus cajan* (L.)) Millsp, dried pigeon pea seed, and water at 26° ± 1°C and 65% r.h. a 14 light: 10 dark photoperiod (light 06:00–20:00 h). The food supply was renewed twice a week. Males and females were grouped for mating, with pieces of cotton placed in containers for oviposition. Eggs were conditioned in plastic containers and, after emergence, nymphs were maintained similarly to adults. Males and females used in the experiments were separated after the imaginal molt and cuticular hardening to prevent mating. Sexually mature 8–15-day-old adults were used for all experimental bioassays and volatile collections since at this age insects started to mate.

2.2. Collection of Volatiles. Volatiles were collected ($N = 6$ extracts) from groups of 20–30 males or females *N. parvus*. To minimize emission of defensive compounds [26] the insects were carefully introduced into 1 liter glass containers shortly after the end of scotophase when they were quiescent. Air was drawn into the container through a bed of 4–12 mesh activated charcoal (Fisher Scientific, Pittsburgh, PA, USA), and out of the container through two traps (15 cm × 1.5 cm OD) containing Super Q (100 mg each; Alltech Associates, Inc., Deerfield, IL, USA) by a suction pump (~1 L/min). Insects were fed fresh green beans daily, and aerated continuously for 7–10 d, and a sample taken every 24 h. The Adsorbent traps were eluted with 0.5 mL hexane, and the eluates were stored at −20°C until needed for chemical analysis or behavioral bioassays. Extracts were concentrated under a gentle stream of N_2 to yield a solution of approximately 0.1 bug-equivalent/μL/24 hours of solution (~500 μL) to be tested.

2.3. Analysis and Derivatization of Extracts. For quantitative analysis, 1 μL crude extracts and fractions thereof were analyzed by gas chromatography flame ionization detector ((GC-FID), Shimadzu 17A GC) (Kyoto-Japan) equipped with DB5 column (30 m × 0.25 mm ID, 0.25 μm film; J&W Scientific, Folsom, CA) on a temperature ramp of 50°C/2 min, then 8°C/min to 250°C/10 min. Injections were made in splitless mode. To quantify the pheromone released per insect, 5 aeration samples of females were selected, and 1 μL of isobutyl acetate (1 mg/mL hexane solution) was added as internal standard (IS) at a final concentration of 0.02 μg/mL. One microliter of each sample was injected into the GC in the splitless mode, with helium as carrier gas. Amounts of volatiles released by the insects per day were calculated in relation to the area of the internal standard. Data were collected with Class GC software (Class CG-10 Version 2.01, Shimadzu (Kyoto, Japan)) and were handled using Excel (Microsoft Corporation 2003).

For qualitative analysis, selected extracts were analyzed using an Agilent MSD 5975 instrument equipped with a quadrupole analyzer coupled to a GC 7890, a splitless injector, and helium as the carrier gas. Ionization was by electron impact (70-eV, source temperature 200°C) using the same column and conditions described above for GC-FID analysis. Chemical ionization (CI) MS spectra were obtained using the same GC-MS equipment using methane (CH_4) as the reagent gas with the same column and conditions described above for GC-FID analysis.

Compounds were identified comparing their mass spectra with database spectra (NIST 2008 library), retention indices, and coinjection with authentic standards.

Five aeration samples of females were combined, and concentrated to dryness under gentle N_2 flow, and submitted to alkaline hydrolysis by adding 50 μL of methanol and 50 μL of 2 M NaOH in a 1.5 mL glass conical vial. The sample was kept at room temperature for 2 hours. After, water (100 μL) was added and organic phase was extract with hexane (200 μL tree times). The combined organic phases were concentrated under nitrogen flow to ~50 μL, and the extract was analyzed by GC-MS by electron impact as described above.

2.4. Coupled Gas Chromatography-Electrophysiology. GC-electroantennography (EAG) was used to pinpoint compounds within mixtures that were detected by the antennae of males and females.

A GC Perkin Elmer Autosystem XL (NY, USA) was coupled to an EAG detector (Syntech, Inc., Hilversum, The Netherlands). The GC was equipped with a nonpolar DB-5 column (30 m × 0.25 mm ID, 0.25 μm film, J&W Scientific, Folsom, CA, USA), and a splitless injector with helium as the carrier gas (1 mL/min). The column temperature was programmed to 80°C (2 min), then heated to 250°C at 8°C/min, and held for 10 min. The effluent temperature to EAG system was kept at 195°C. The antenna of one male or one female were cut and immediately placed in stainless steel

electrodes, the base of the antenna was placed in the reference electrode and the distal ends of the antenna was placed in the recording electrode, the electric connection was achieved using conductive gel. The electrodes were connected to an Autospike interface box and an AC/DC amplifier IDAC-2 (Syntech, Inc.). Preparations were held in a continuous humidified air flow (1 L/min) with a Stimulus Controller CS-55 (Syntech, Inc.). The females and males antennae of *N. parvus* were tested using a female extracts of *N. parvus* ($N = 5$) containing all compounds identified in the volatiles collections and for a solution containing the synthetic compounds, that showed electrophysiology response from the antenna in crude extract, hexyl butanoate (0.05 mg/mL), (*S*)-4-methylhexyl butanoate (0.005 mg/mL), hexyl pentanoate (0.0075 mg/mL), (*S*)-4-methylhexyl pentanoate (0.002 mg/mL), and hexyl hexanoate (0.0075 mg/mL) ($N = 3$). Only peaks that showed the polarization and depolarization of the antenna were considered as EAG responses, and only compounds that elicited response in all antennas tested ($N = 5$) were considered electrophysiologically active.

2.5. Synthesis of 4-(S)-Methylhexyl Pentanoate and 4-(S)-Methylhexyl Butanoate. (*S*)-4-methyl-1-hexanol (TCI America, Boston, MA, USA) 11.6 mg (0.1 mmol) was treated with butyryl chloride (10.4 μL, 0.1 mmol) in the presence of pyridine (8 μL,/500 μL methylene chloride). The mixture was poured into ice-water, extracted with methylene chloride ($3 \times 100\,\mu$L), the organic extract was washed with 0.1 M HCl, water, and dried with Na_2SO_4. A similar procedure was conducted using valeryl chloride to prepare 4-(*S*)-methylhexyl pentanoate. The structures of synthesized compounds were confirmed by mass spectrometry analysis.

4-(S)-Methylhexyl Butanoate MS. *m/z* = 129 (12), 115(4), 98 (28), 89 (82), 83(8), 71 (82), 70 (100), 69(49), 57 (58), 56 (36), 55(31), 43(55), 42(15), 41(49).

4-(S)-Methylhexyl Pentanoate MS. *m/z* = 143(10), 115(5), 103(79), 98(35), 97(6), 85(58), 83(9), 70(100), 69(44), 57(95), 56(34), 55(31), 43(16), 42(13), 41(43).

Source of Compounds. Super *Q* (80/100 mesh) was purchased from Alltech (PA, USA). The sources of chemical as follows: camphene, 6-methyl-5-hepten-2-one, hexanoic acid limonene, undecane, nonanal, dodecane, decanal, tridecane (Sigma Aldrich, Steinheim, Germany), hexyl acetate (TCI-America, portland, USA). Butyl butanoate, pentyl butanoate, hexyl butanoate, hexyl pentanoate, and hexyl hexanoate were provided by Jeffrey Aldrich (USDA-ARS, Invasive Insect and Behavior Laboratory, MD, USA).

2.6. Olfactometer Bioassays. A two-choice olfactometer modified from Borges and Aldrich ("W-olfactometer"; [27]) was used to test the biological activity of *N. parvus* aeration extracts and synthetic compounds. The olfactometer release chamber was a 500-mL three-neck, round-bottom flask (all 24/40 joints, Kontes, Vineland, New Jersey). Two 250 mL rotary evaporator trap adapters (24/40 joints) were attached to each side arm of the release flask (the treatment and control arms). A charcoal (20/40 mesh) filter column (130 mm × 10 mm ID) was attached to the side arms using two 40 cm long pieces of a silicone tubing (3/16 I.D. × 5/15 E.D. VWR Scientific Corporation, Darmstadt, Germany), inserted in a "Y" connector of the same diameter and connected to adapters (24/40 joint) on each side arm of the olfactometer. The air was humidified by passage through a container of distilled water between the charcoal filter and the arms of the olfactometer. The middle neck of the flask was connected to the vacuum pump with an adapter, and the air flow was adjusted with a "Clear Flow Rotameter" (Accura Flow Products, Warminster, Pennsylvania 18974-0100) to a flow of 0.8 L/min. The apparatus was positioned horizontally on a countertop in a room with bright fluorescent lights (2 × 36 W, daylight (6500 K) lamps Sylvania Activa 172, Sylvania, Danvers, MA, USA) during photophase. The temperature in the bioassay room was maintained at 26.0 ± 1.0°C. The positions of the olfactometer arms were inverted between control and treatments after each three repetitions to avoid any positional bias. The apparatus was cleaned with fragrance-free liquid soap, rinsed thoroughly with water, and dried at 120°C after every five replicates. The insects were placed in the round-bottom flask (release chamber), and the treatments were placed at the end of the reducing adapter chamber (treatment arms).

A single *N. parvus* adult (male or female) was gently introduced into the release chamber of the Y-tube olfactometer with the aid of an artist's paint brush (Camel Hair, number 1), and its pattern of behavior (response) was recorded for 10 min/replicate. The duration of each bioassay replicate was monitored using a stopwatch. Prior to testing, the insects were allowed to acclimate for a short period (ca. 3 min) in the release chamber while assembling the treatment chambers. The first choice of the insect was recorded, that is, the first arm of olfactometer that the insect chose, entered and remained in for at least 100 sec. The test insects were used only once during the bioassays.

The bioassay procedures described above were used to compare the biological activity of aeration extracts of females, and synthetic standards prepared in proportions matching that produced by *N. parvus* females. For aeration extracts, the solution of test stimulus was 1 individual equivalent/24 hours (IE) spotted on a strip of filter paper (1.5 cm long and 0.5 cm wide); controls consisted of filter papers treated with hexane. Forty-five bioassays were performed for each sex (males and females). Bioassays were conducted using a 5 μL of a synthetic solution containing the five synthetic compounds that showed EAG responses ($N = 35$ for females and $N = 40$ form males) (hexyl butanoate (0.005 mg/mL), (*S*)-4-methylhexyl butanoate (0.0005 mg/mL), hexyl pentanoate (0.00075 mg/mL), (*S*)-4-methylhexyl pentanoate (0.0002 mg/mL), and hexyl hexanoate (0.00075 mg/mL)).

2.7. Statistical Analysis. Choices made by the insects in the bioassays were analysed using logistic regression. The fitted model contained a factor for the side (left or right) on which the stimuli were presented to control for this variability. We tested the hypothesis of no preference (50% first choice to

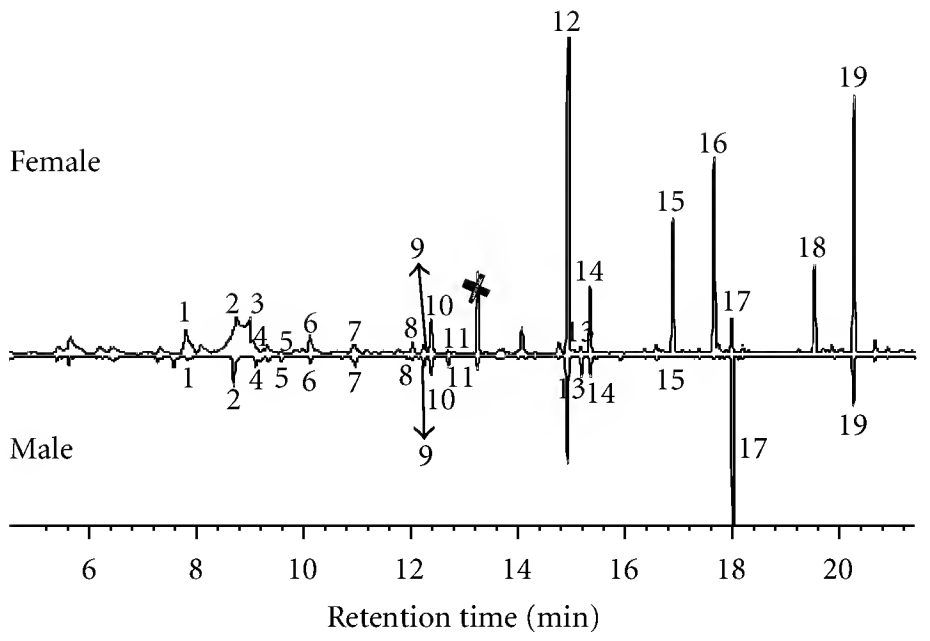

Figure 1: Gas chromatogram profile of an air-entrainment extract of males and females of *Neomegalotomus parvus*. (1) Camphene, (2) 6-methy-5-hepten-2-one, (3) hexanoic acid, (4) butyl butanoate, (5) hexyl acetate, (6) limonene, (7) (*E*)-2-octen-1-ol, (8) pentyl butanoate, (9) undecane, (10) nonanal, (11) unknown compound, (12) hexyl butanoate, (13) dodecane, (14) decanal, (15) 4-methylhexyl-butanoate, (16) hexyl pentanoate, (17) tridecane, (18) 4-methylhexyl-pentanoate, and (19) hexyl hexanoate.

each vibratory signal) using a chi-square Wald test. All tests were conducted using the *R* programming language [28].

3. Results

3.1. Chemical Analysis. The chemical analysis of males and females of *N. parvus* extracts obtained from volatile collection showed quantitative and qualitative differences between the extracts. Quantification of extracts showed that females produce higher amounts of several compounds (Table 1, Figure 1) compared to males and females also release some specific compounds that were not found in the extracts of males, such as hexyl pentanoate and 4-methylhexyl pentanoate.

The mass spectra and the retention times of compounds **15** and **18** (Figure 1) did not match those of any compound from the database and/or the literature. The mass spectra of **15** (*m/z*, relative abundance): 129(11), 115(4), 98(28), 89(78), 83(9), 71(76), 70(100), 69(47), 57(53), 56(34), 43(49), 41(42), and compound **18**: 143(9), 115(5), 103 (76), 98(35), 97(6), 85(57), 83(7), 70(100), 69(30), 57(89), 56(32), 55(28), 43(15), 42(12), 41(40) suggested ester homologues. CI-MS analysis of the female crude extract showed that compounds **15** and **18** had a molecular adduct ions ($[M+H]^+$) at 187 and 201, thus providing additional evidence that these two compounds could have a similar chemical structure differing by one methyl group. In order to obtain more information about the chemical structure of these two esters, a pooled female extract was submitted to alkaline hydrolysis. The GC-MS analysis of the hydrolyzed crude extract showed that the peaks corresponding to the esters disappeared and new peaks were generated, one of which matched the synthetic standard of 4-methyl-1-hexanol. These results, combined with retention index data (Table 1), suggested that compounds 15 and 18 could be 4-methylhexyl butanoate and 4-methylhexyl pentanoate, respectively. Indeed, the mass spectra and retention indices of synthetic (*S*)-4-methylhexyl butanoate and (*S*)-4-methylhexyl pentanoate matched those of esters found in female crude extract.

The other volatile compounds identified from both males and females are common to Alydidae; mainly short-chain esters and acids, and one alcohol [(*E*)-2-octen-1-ol] that is a common defensive compound in several Pentatomidae (Table 1).

3.2. Coupled Gas Chromatography-Electrophysiology. In GC-EAG experiments the antennae of *N. parvus* males and females responded to only five components present in the extract of females (Figure 2) that were identified as hexyl butanoate, 4-methylhexyl butanoate, hexyl pentanoate, 4-methylhexyl pentanoate, and hexyl hexanoate. When a blend containing these five synthetic compounds (hexyl butanoate, (*S*)-4-methylhexyl butanoate, hexyl pentanoate, (*S*)-4-methylhexyl pentanoate and hexyl hexanoate) was tested, the antennae of males and females responded in a similar way as to aeration extracts of females.

3.3. Bioassays. In W-olfactometer bioassays with aeration extract of females, both *N. parvus* males and females were significantly attracted to the extract treatment arm of the olfactometer (Figure 3). Similarly, males and females were significantly attracted to the treatment arm containing the five-component synthetic blend active in EAG experiments (Figure 4).

4. Discussion

Males and females of *N. parvus* produce several short chain esters and acids, most of which were previously reported for others species of Alydidae from the metathoracic scent glands [14, 15, 17, 18, 22] (Table 2); however, this is the first report of pentanoates from Alydidae. The antennae of *N. parvus* showed electrophysiological responses to five of these esters, three common to both adult sexes (hexyl butanoate, 4-methylhexyl butanoate and hexyl hexanoate), and two female-specific compounds (4-methylhexyl pentanoate and hexyl pentanoate). Both males and females were attracted to aeration extracts of females, and to the synthetic blend of the five EAD-active compounds in proportions mimicking those of the compounds produced by females.

Interestingly, in the Alydidae either the male [17, 18] or female [15–19] can emit the attractant pheromone, depending on the genus. In the rice alydid bug, *Leptocorisa chinensis* (Dallas), although there were no detectable qualitative differences in aeration extracts of males versus females, only males were attracted to a 5 : 1 blend of *(E)*-2-octenyl acetate and octanol [19]. In the alydid *R. clavatus*, the attractant pheromone is produced by males and attracts females, males, and nymphs, plus an egg parasitoid [17]. Furthermore, it has been established for *R. clavatus* that two of the three essential pheromone components ((*E*)-2-hexenyl (*E*)-2-hexenoate and (*E*)-2-hexenyl (*Z*)-3-hexenoate) are produced in the enlarged lateral accessory glands of males that are attached to the metathoracic scent gland reservoir. However, the third

Table 1: Amounts of the compounds identified in extracts obtained from air-entrainment of males and females of *N. parvus* ($N = 5$).

Compounds	Retention index (DB-5)*	(ng/24 hours/insect)	
		Males	Females
(1) Camphene	954	0.02 ± 0.01	1.7 ± 0.02
(2) 6-Methyl-5-hepten-2-one	980	1.12 ± 0.85	1.05 ± 0.78
(3) Hexanoic acid	981	0.59 ± 0.37	1.19 ± 0.74
(4) Butyl butanoate	995	1.11 ± 0.73	0.01 ± 0.01
(5) Hexyl acetate	1008	1.26 ± 0.91	1.37 ± 0.79
(6) Limonene	1035	0.06 ± 0.01	1.2 ± 0.52
(7) (*E*)-2-Octen-1-ol	1059	0.06 ± 0.02	0.01 ± 0.01
(8) Pentyl butanoate	1093	1.23 ± 1.17	0.66 ± 0.16
(9) Undecane	1100	2.14 ± 0.98	1.67 ± 0.92
(10) Nonanal	1104	1.28 ± 0.51	1.48 ± 0.45
(11) Unknown compound	1165	0.14 ± 0.13	0.33 ± 0.12
(12) Hexyl butanoate	1193	2.15 ± 1.92	43.58 ± 10.12
(13) Dodecane	1200	2.21 ± 1.49	2.89 ± 1.48
(14) Decanal	1207	1.68 ± 0.49	2.08 ± 0.64
(15) 4-methyl hexyl-butanoate	1262	0.11 ± 0.07	4.49 ± 1.64
(16) Hexyl pentanoate	1288	—	6.84 ± 2.11
(17) Tridecane	1300	15.06 ± 14.05	5.11 ± 2.21
(18) 4-methylhexyl-pentanoate	1364	—	0.49 ± 0.15
(19) Hexyl hexanoate	1386	1.20 ± 0.84	1.50 ± 0.52

* Retention index was calculated using the retention time obtained in GC-FID analysis using a DB-5 column with a temperature program of 50°C/2 min, then 8°C/min to 250°C/10 min.

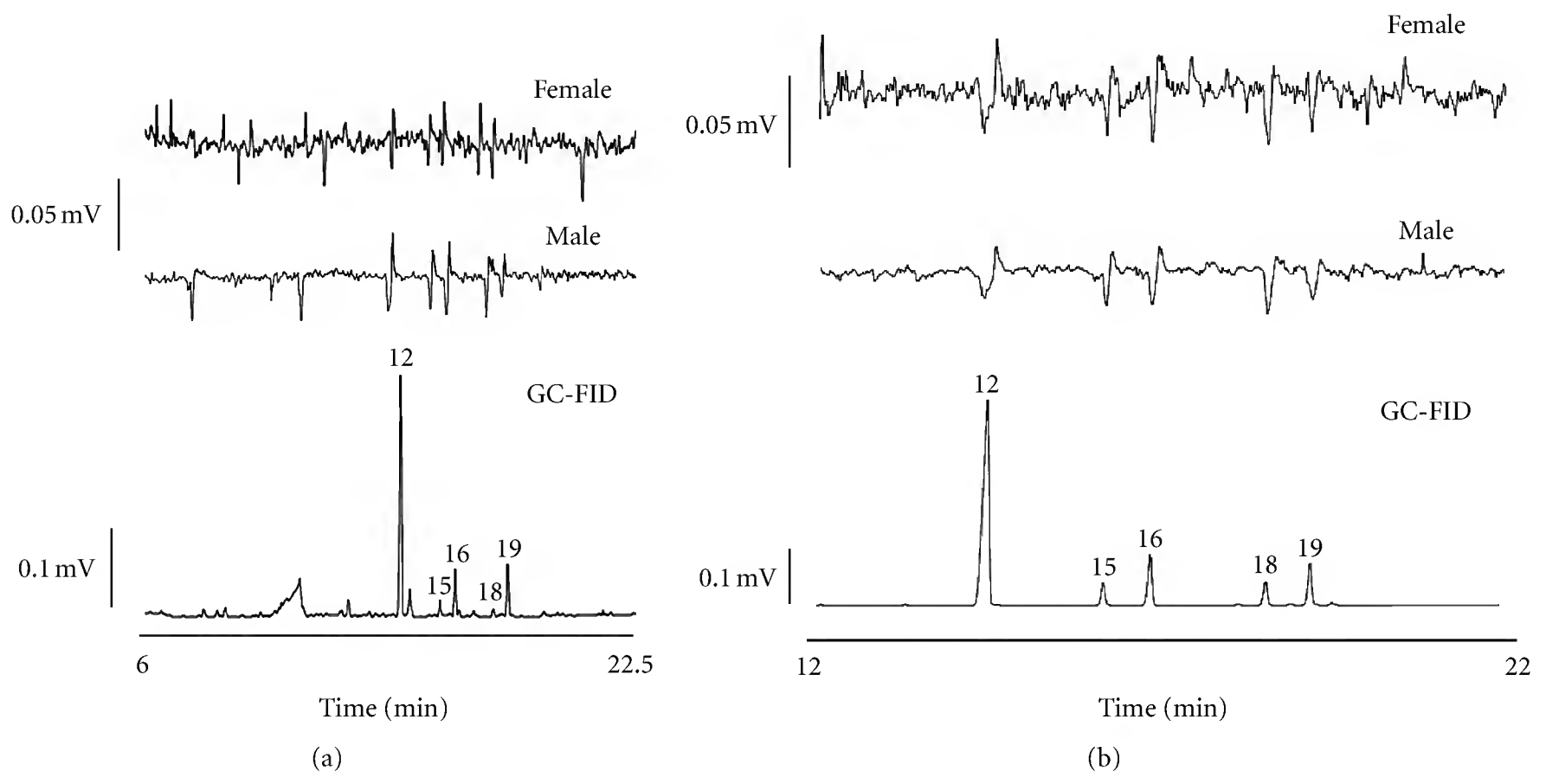

Figure 2: (a) A typical response to crude extracts of female aerations of antenna of *Neomegalotomus parvus* female and male in GC-EAG analyses. (b) A typical response to a synthetic mixture of compounds found in *N. parvus* aerations and scent glands of antenna of *N. parvus* female and male in GC-EAG analyses. (12) hexyl butanoate, (15) 4-methylhexyl-butanoate, (16) hexyl pentanoate, (18) 4-methylhexyl-pentanoate, and (19) hexyl hexanoate. In (b) the synthetic mixture tested was (12) hexyl butanoate (0.05 mg/mL), (15) 4-*S*-methylhexyl-butanoate (0.005 mg/mL), (16) hexyl pentanoate (0.0075 mg/mL), (18) 4-*S*-methylhexyl-pentanoate (0.002 mg/mL), and (19) hexyl hexanoate (0.0075 mg/mL).

Table 2: Principal compounds found in metathoracic scent gland and volatile collection of Alydidae species.

Compounds	*M. scutellaris* [18]	*R. serripes* [17]	*R. clavatus* [21, 22]	*M. quinque-spinosus* [14]	*A. eurinus* [14, 15]	*A. pilosulus* [14]	*L. chinensis* [19]	*N. parvus* (this work)
Butanal					MF	MF		
Nonanal							MF	
2-Methyl-butanal				MF				
Methyl propanal				MF				
(*E*)-2-Hexenal	MF	MF		MF	MF	MF		
(*E*)-2-Octenal	F						MF	MF
Octanol							MF*	
(*E*)-2-Hexen-1-ol	MF				MF	MF		
(*E*)-2-Octen-1-ol	MF						MF	MF
2-Methyl-butanoic acid				MF				
Butanoic acid	M			MF				
Hexanoic acid	M	M			MF	MF		MF
(*E*)-2-Hexanoic acid		M						
(*Z*)-3-Hexanoic acid		M						
(*E*)-2-Octanoic acid	M							
Hexyl-acetate								MF
Octyl acetate							MF	
(*E*)-2-Octenyl acetate							MF*	
(*Z*)-3-Octenyl acetate							MF	
Isobutyl 2-methyl-propanoate				MF				
2-Methyl-butyl 2-methyl-propanoate				MF				
2-Methyl-butyl butanoate				MF	MF			
(*E*)-2-hexenyl butanoate	M	M						
Butyl butanoate				MF	MF	MF		
(*E*)-2-Methyl-butenyl-butanoate					F*			
2-Methyl-butyl-butanoate					F*			
Butyl-butanoate								MF
Pentyl-butanoate								MF
Hexyl-butanoate					MF	MF		MF*
4-Methyl-hexyl-butanoate								MF*
(*E*)-2-Hexenyl-butanoate	F	F						
Hexyl-pentanoate								F*
4-Methyl-hexyl-pentanoate								F*
Butyl-hexanoate				MF				
2-Methyl-butyl-hexanoate				MF				
(*E*)-2-Hexenyl-(*Z*)-3-hexenoate	M	M	M*					
(*E*)-2-Hexenyl-(*E*)-2-octenoate		M						
(*E*)-2-Hexenyl-(*E*)-2 hexenoate		M	M*			MF		
(*E*)-2-Hexenyl-(*Z*)-2-hexenoate		M	M*					
Hexyl hexanoate								MF*
Tetradecyl-2-methyl-propanoate			M*					
Octadecyl-2-methyl-propanoate			MF					

Note: compounds labeled with F are female specific, labeled with M are male specific, and labeled with MF were identified in both gender. Compounds labeled with asterisk are those for which attraction function were confirmed in laboratory or field assays. Aldrich et al. [18], Leal et al. [17], Endo et al. [21], Yasuda et al. [22], Aldrich and Yonke [14], Aldrich et al. [15].

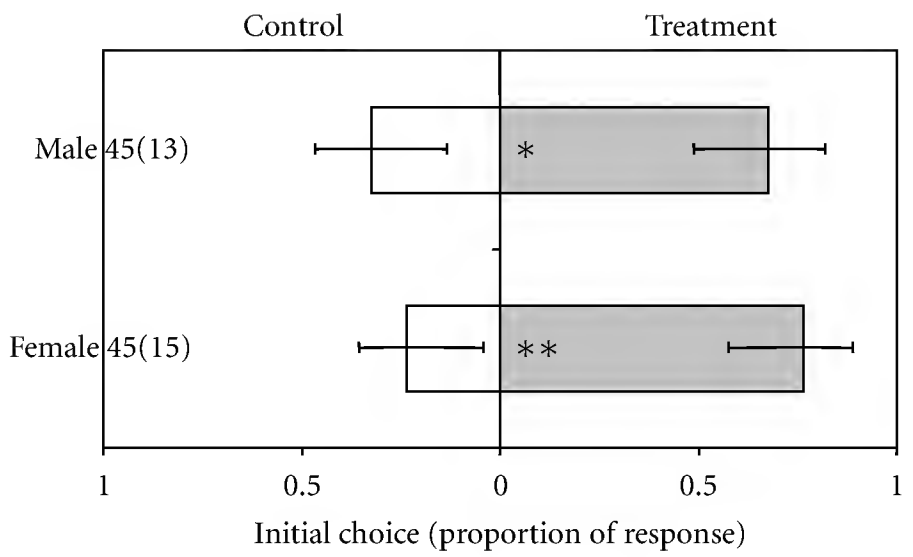

FIGURE 3: Initial choice (mean ± confidence interval (CI) 95%) of *Neomegalotomus parvus* males and females to crude extract of female aeration. Analyses of initial choices were carried out by logistic regression and χ^2 Wald test. *indicates $P < 0.05$ and **indicates $P < 0.01$. Numbers at left indicate total number of insects tested and numbers in brackets indicate the number of insects that did not make a choice after 5 minutes.

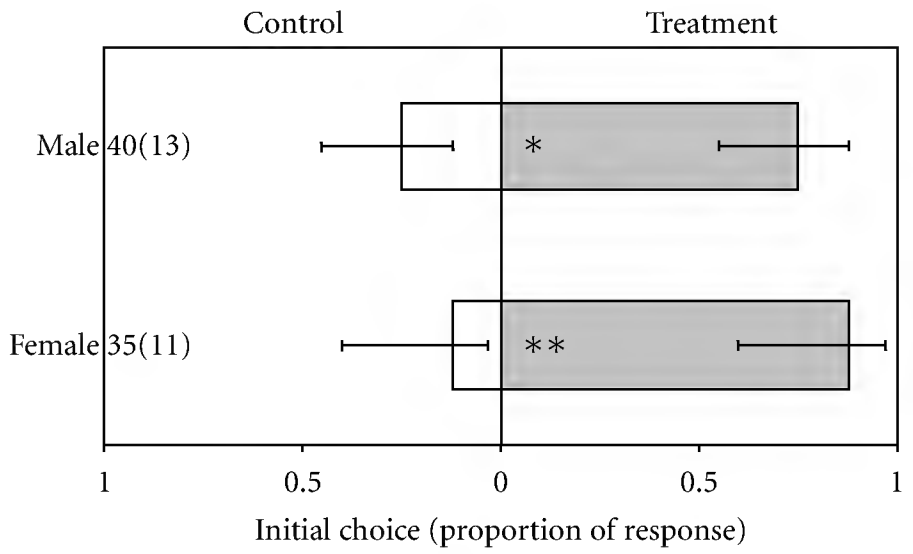

FIGURE 4: Initial choice (mean ± confidence interval (CI) 95%) of *Neomegalotomus parvus* males and females to 5 uL of a solution containing the synthetic authentic standards found in females extracts (hexyl butanoate (0.005 mg/mL), 4-(*S*)-methylhexyl-butanoate (0.0005 mg/mL), hexyl pentanoate (0.00075 mg/mL), 4-(*S*)-methylhexyl-pentanoate (0.0002 mg/mL), hexyl hexanoate (0.00075 mg/mL). Analyses of initial choices were carried out by logistic regression and χ^2 Wald test. * indicates $P < 0.05$ and ** indicates $P < 0.01$. Numbers at left indicate total number of insects tested and numbers in brackets indicate the number of insects that did not make a choice after 5 minutes.

essential component (myristyl isobutyrate) is produced from cells in the abdominal sternum of males [15].

Of the known alydid pheromone systems, *N. parvus* attractant pheromone appears most similar to that of *Alydus eurinus* (Say), where females produce a pheromone from the nonsexually dimorphic lateral accessory glands of the metathoracic scent gland complex with essential components being (*S*)-(-)-2-methylbutyl butanoate and *(E)*-2-methyl-2-butenyl butanoate. Although the chirality of 4-methylhexyl hexanoate and 4-methylhexyl pentanoate in *N. parvus* has not been unequivocally established ((*R*)-4-methyl-1-hexanol was not available), the biological activity of a blend containing synthetic esters with *S* absolute configuration combined with the fact that only (*S*)-(-)-2-methylbutyl butanoate was active for *A. eurinus* is suggestive that the 4-methylhexyl esters in *N. parvus* may also have *S* configuration. Dissections of adult *N. parvus* by one of us (J. Aldrich) indicated that the lateral accessory glands are enlarged in females and contain the key pheromone components (unpublished data). In *A. eurinus*, the female-produced pheromone attracts males, and to lesser extents females and nymphs; further tests are needed to establish whether the *N. parvus* pheromone will exhibit a similar pattern in the field.

Interestingly, a communication system similar to that of alydids occurs in plant bugs (Miridae) where sex pheromones consisting of aliphatic esters are produced in metathoracic scent glands of females [29–31], and in certain seed bugs (Lygaeidae) where males produce chemically similar aggregation pheromones from sexually dimorphic metathoracic scent glands [32].

In summary, *N. parvus* females produce two specific compounds that were not identified in male extracts and these compounds are pentanoates, the first time observed in Alydidae species, the other esters identified, hexanoates, and butanoates, were previously reported for others species of Alydidae. Five of these esters, hexyl butanoate, (*S*)-4-methylhexyl butanoate, hexyl pentanoate, (*S*)-4-methylhexyl pentanoate, and hexyl hexanoate stimulate an electrophysiological and behavioral response from males and females, indicating a possible function as aggregation pheromone. The identification of an attractant pheromone for *N. parvus* may lead to a more effective monitoring system for this pest, to more accurately guide insecticidal programs against this pest. Further field testing is necessary to make this potential application a reality.

Acknowledgments

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Review Article

The Role of Silk in the Behaviour and Sociality of Spiders

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This article describes the links between the production of silk by spiders and their behaviour. Silk allows the spider to change its physical environment, which in turn leads to behavioural changes and impacts in the new environment. The feedback between silk and the animal producer can explain the architecture of spider webs and their adaptation to the environment, by referring only to stereotypic stimulus-response reactions without necessarily resorting to a "representation" by the animal of the structure it builds. Silk can act as a means of protection against environmental stress, a snare for prey, a means of locomotion, and also as support for chemical signals or to act as a vector of vibratory signals. These last two functions have undoubtedly played a key role in spider socialization and explains the phenomena of group cohesion, collective decision making, and the coordination of activities, without resorting to mental "representations" for the overall situation. The bulk of this review describes silk as the chief agent directing the construction of traps, communication, social cohesion, and cooperation amongst its producers.

1. The Production of Silk: A Capacity Widespread amongst Arthropods

Only arthropods produce silk, and insects of almost every order secrete silk-like proteins. For example, Dictyopteran mantids enclose their eggs in a capsule that has a chemical composition similar to silk. However, only a few groups have developed an advanced behaviour for silk weaving (Hymenoptera, Lepidoptera, Embioptera, Thysanoptera, Trichoptera, and some larvae of Diptera) [1]. This ability is widespread among arachnids, particularly spiders, including some fossil species [2, 3], and may have evolved from an onychophoran-like ancestor [1, 4].

The only animal silk used commercially to date is from a moth caterpillar, the silkworm *Bombyx mori*. But many other Lepidopteran larvae produce a silk cocoon into which they withdraw during metamorphosis. Some social larvae construct silken shelters to house the colony [5, 6]. Silk production during pupation is also characteristic of the larvae of Hymenoptera, while some adults are also capable of producing silk, such as the Sphecidae [7]. This larval capacity also occurs in the workers of certain ant species. The best known are *Oecophylla* which use their larvae to "sew" leaves together, thereby forming a compact nest for the colony. Some other ant species also build silken nests [8–12]. *Camponotus senex* is a South American tree ant that has behaviour similar to *Oecophylla* [13]. However, for the ant *Melissotarsus emeryi* of South Africa, it is the adults that directly produce silk from glands located in the oral cavity [14].

Many other more cryptic arthropods produce silk. The aquatic larvae of Trichoptera (caddisflies) build bags assembled from sand grains or plant fragments using silk. Some of them (*Hydropsyche*) even develop net-like traps to capture small prey driven towards them by the current [15, 16]. The social Embioptera use silk secreted from metatarsal glands on their front pair of legs to produce silk-lined shelters [17–20]. The social Thysanoptera sew leaves together to form a common shelter [21]. Social psocids (*Archipsocus*) weave silken roofs over their feeding area [22].

Among the arachnids, spiders are the most famous producers of silk. But some mites also spin webs as a nest to house the group [23–26]. Male pseudoscorpions guide females to their spermatophore by weaving a net, as a kind of corridor narrowing towards the tip and composed of silken threads [27, 28]. They also build nest protections using silk glands at the end of their chelicerae [29].

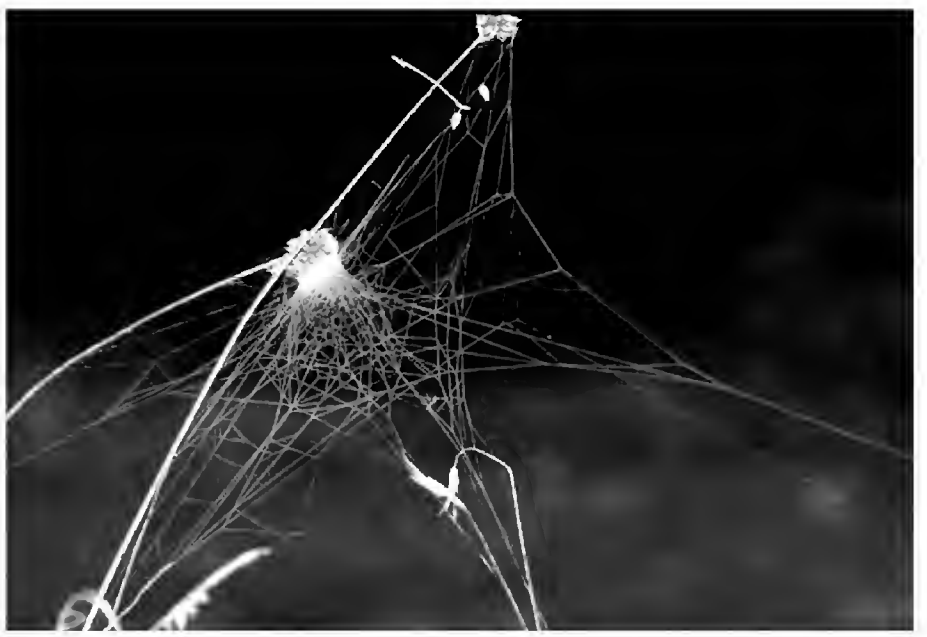

Figure 1: The retreat of *Araneus quadratus*. The threads of the web are revealed by highlighting them with colour (Photo Krafft).

The variety of species that produce silk in such a variety of unrelated groups demonstrates the adaptive significance of this material in the prevailing environment. A variety of structures are produced, ranging from simple protective packaging (cocoons) to the geometric orbs of certain spiders, through to web sheets and three-dimensional structures where several aspects are curiously reminiscent of the architecture of man-made structures such as suspension bridges [16].

Spiders produce different types of silk, such as a non-sticky dry thread that tracks the spiders progress (any spider in the Araneomorphae that moves leaves this security thread behind it), strong threads for the framework and rays of geometric webs, sticky spiralling threads for capturing prey, cribellum silk whose structure is particularly complex and which adheres to the prey like "velcro," parchment silk, and silk used in the manufacture of cottony egg sacs. A spider can produce up to 8 different kinds of silk. Their functions are as varied as the structures produced [30]. Furthermore, the spider can attach a thread to any substrate or existing thread with ease. This diversity and flexibility of use justifies the extensive research conducted on the fine structure of silk [16, 31–36], and its physicochemical properties [37–46].

2. Silk as a Protective Material

The original function of the silk secreted by spiders was probably to make cocoons to protect eggs and build individual shelters. Many solitary spiders build silken retreats that are bell-shaped tubes within vegetation or under stones [30] (Figure 1). The walls of these structures provide effective protection against wind and rain. Silk linings can also resist water, allowing some spiders to live in areas subjected to flooding by forming an air bubble in which to live until the water recedes. *Argyrodes* even lives permanently under water within a silky diving bell. This spider traps a bubble of air, and gas exchange between the air and water occurs through the wall of silk, which allows sufficient oxygenation and carbon dioxide removal.

Silk is also a material that can modify certain elements of the environment. Many spiders live within the cubicles made from leaves bonded with silk. This is particularly the case for *Larinioides cornutus*, which frequently lives amongst reeds lining ponds. The reed's leaves are bent by the spider using a succession of silken threads which, by narrowing, increase the curvature so that the distal part of the leaf becomes located near the proximal end. Caterpillars use the same technique.

All spiders enclose their eggs within a silken egg sac of varying complexity. In temperate regions, the young of most species hatch in autumn (fall) but remain in hibernation within the egg sac until they emerge in spring. They spread out a few days later after a short gregarious period. These egg sacs are often composed of several types of silk, as is the case for *Argiope bruennichi*. The outer part of the egg sac is a tough leathery shell, followed by a red silken wad (red-brown floss-silk), and finally an envelope of white silk containing the eggs. *Agelena labyrinthica* manufactures a large cell in which the female stands until her death in autumn and includes a second chamber of the same shape that contains a basket of eggs.

Silk is used to protect the colonies of social spiders [47–49]. *Anelosimus eximius* is a social spider from Guyana that constructs large complex webs with volumes ranging from 0.001 to 1,000 m^3 [50] (Figure 2). Colonies reaching 100 m^3 are common. These structures function primarily to capture prey. If the web is disturbed by a predator such as ants, predatory wasps, and other species of spiders, their vibrations are readily transmitted through the silky network allowing *A. eximius* to mount a coordinated defence. Small emigrant populations (20 to 50 individuals) with incomplete webs can be quickly decimated by Ponerine ants, while these attacks are useless when the web becomes functional. The arrival of a predatory wasp often attracts a large number of spiders, which makes it dangerous for the wasp to continue its attack. To capture a spider, the predator must first isolate an individual. On the other hand, the senior author has observed in Gabon the total destruction of a colony of over 20 m^3 of another species of social spider, *Cyrtophora*, in the space of one night by raiding Magnan ants. The protection of social spiders within these large webs can also be ineffective against some vertebrates, especially birds that remove silk from the colonies for their own nests or catch spiders for food.

Nests of social spiders of the genus *Stegodyphus* are more compact. The spiders live within a dense mass of silk whose protective effect against vertebrate predators improves when the nest is larger [51, 52]. Contrary to popular belief, these nests do not protect against changes in temperature and humidity. At most, they offer some protection against the wind and rain [53].

3. Silk Used to Build a Snare and to Exploit the Environment

Many species of spiders are wanderers that pursue their prey using visual information or the vibrations produced by prey as they move through the air or upon soil [54]. They often

FIGURE 2: Colony of *Anelosimus eximius* from Guyana (5 m long, 3 m high; Photo A. Bernard).

FIGURE 3: The orb-web of *Araneus quadratus* (Photo Krafft).

build silken retreats in vegetation, or under bark or stones, but may move frequently.

It is generally accepted that the web-spinning species evolved from an extension of these benefits in the use of silk. In developing a trap, the spider changes its environment in order to exploit certain food resources. These traps are static nets that can only catch mobile prey, thereby requiring the spider to select a site that is sufficiently rich in resources for web construction. Despite the fact that spiders can change sites if necessary, the construction of a web is an important investment by sedentary web-spinning species. The architecture of these traps is varied [16, 55, 56].

The function of some webs is simply to provide a warning. The thread, or a sheet of silk attached to a retirement, transmits the vibrations produced by prey to the spider. The trap allows the prey to stay on its course and does not restrain it. The most notable examples are spiders from the genera *Segestria* and *Uroctea*. Several threads radiating from their retirement run one or two millimetres above the substrate, supported by small columns of silk. These posts tend to alternate their attachment to the thread left and right as is sometimes the case for the overhead lines of electric trains. The threads are not sticky but made of dry silk, yet perform their warning function perfectly because they lack any contact with the substrate.

Other traps have the function of both warning and interception. Many traps consist of a three-dimensional network of dry threads above a horizontal web sheet. This is the case for the Agelenidae and Linyphiidae. In some cases, the sheet may be a radiating geometric structure as in *Cyrtophora*. The prey is temporarily halted by the tent-like network of threads and may eventually fall onto the web sheet. Although the threads are not sticky, they adhere to the prey (especially if it is hairy or adorned with spines) like cotton wool [57–59]. But the retention time of the trap is short so the prey can escape if the spider is slow or absent. These webs are designed as permanent traps. The spider simply repairs them every day. However, it sometimes abandons the web to find a new location and construct a new trap [60, 61]. Such reconstructions are expensive and can require 19 times more energy than is expended by simply maintaining an existing web [62].

Finally, some webs have the function of warning, interception and retention. These include the classical two-dimensional webs of the orb spiders (Figure 3), such as *Araneus diadematus* and *Argiope bruennichi*. The orbs are constructed from a set of spokes that support a sticky spiralling silk thread covered with droplets of glue. The complex geometry of these structures has captured the imagination and allows the spider to weave large webs with the minimum of silk. The capture area is huge compared to the amount of material used. However, this trap is fragile and deteriorates quickly. Any prey that is caught leaves a hole in the web. It is, therefore, rebuilt regularly, often daily. But as these spiders ingest the old (nutritious) web before building a new one, the energy expended is no greater than if the web was simply maintained [63, 64], which also allows them to then change sites easily if there is a reduction in the availability of prey [65]. Some cribellate species (e.g., *Uloborus*) develop similar traps except that the sticky spiral is replaced with a cribellum thread (hackled band), which relies on fine fibres on the threads to entangle rather than adhere the prey [66]. As well as orbs, many permanent three-dimensional traps have similar functions. Some sections of the network of threads can be sticky (Theridionidae) or composed of cribellum silk (Dictynidae, Amaurobiidae). This allows smaller prey to be captured permanently, even if the spider is not immediately involved. While there are differences in the capacity of different adhesive silks, these are largely offset by the response of the spider, usually rapid in traps with short retention times and slower for others [59].

All of these web-designs in silk have a significant impact on the behaviour of spiders. Not only do webs allow the capture of prey, but they give the spider an expanded perceptual field. The spider not only discerns the objects it touches or that pass nearby, but also anything that touches the web. Rain, wind, a leaf falling on the web, or large prey that passes through the web can all be detected. Webs are a true extension of the sensory organs of the spider, thus giving it improved control over its environment [67, 68].

In web-spinning species, the spider is notified of the arrival of prey by the broadcast of vibrations through the

silky network upon prey impact and its attempts at escape [67, 69]. These vibrations are complex phenomena. An impact produces a strong transient signal, beating wings produce sinusoidal vibrations, and web rupture from struggling prey causes sudden variations and sporadic signals. The spider can probably identify the type of prey on the basis of these signals, as shown experimentally by reproducing the same response at given frequencies [70–74]. In geometric webs, these vibrations converge on the hub [75] being borne mainly by the rays [67]. Spiders are well endowed with mechanoreceptors such as cleft bodies and lyriform organs that are sensitive to vibrations [76–79] and allow the spider to locate its prey. The distribution of vibration receptors on eight legs facilitates orientation as each appendage will measure slightly different distances and signal intensities according to the exact positioning of the prey [80]. In orb-weaving spiders, this orientation is facilitated even further through the rays [77, 81, 82]. At its hub, the spider can easily detect the area of web with prey. In contrast for species exploiting a web sheet, locating prey may require two to four shifts in direction by the spider before it can find its victim [83]. These reassessments are made during short stops. An immobile prey cannot be located, unless the spider sends vibrations through the web by pulling its threads. This process, also used by orb-weaving spiders, is akin to echolocation used by bats and not only locates the object on the web but also helps to evaluate its mass [77].

When the spider comes into contact with its prey, it paralyzes the prey by injecting venom through the chelicerae. Further, some species such as *Araneus*, and more dramatically *Argiope*, wrap the prey in broad ribbons of silk. The wrapping may occur before or after the bite. Faced with prey that have their own defensive systems (e.g., Orthoptera equipped with spiny legs, or stinging Hymenoptera), *Argiope* immobilizes its prey using a silky "straitjacket" before moving closer to bite.

In some cases, as with *Argiope*, the web is decorated with wide bands of white silk in the shape of a zigzag or disk (the stabilimentum) on or near the hub [30]. This intriguing structure has been likened to a system that might protect against predators [84, 85]. It can make the spider appear larger or provide camouflage to hide the exact location of the spider [86]. The stabilimentum is frequent and large in cobwebs where spiders are well fed while the reverse is true for poorly fed spiders. Its presence can reduce the catch rate of webs by 30%. In contrast, 70% of the webs lacking a stabilimentum can be destroyed by the passage of birds compared to 30% when a stabilementum is present [87, 88]. Other results, partly based on laboratory experiments, suggest that the stabilimentum acts as a system that attracts prey because it reflects ultraviolet light [89–92]. However, some prey are able to avoid webs based on visual information [93, 94], and the function of the stabilmentum is still controversial [86].

The production of a fixed web that can only capture mobile prey has an important effect on the behaviour of spiders. Made sedentary by the web, it is imperative that the spider selects sites rich in prey. A few changes in sites, about once a month for *Agelenopsis aperta* (web sheet builder), is especially costly since it involves the complete reconstruction of the web and retirement. However, it allows the spider to adapt to possible variations in ecological conditions and food requirements. The selection of sites based on physical characteristics of the environment and its fauna has been demonstrated in *Agelenopsis aperta* [60] and some orb-weaving species [95], as the sites selected by the spider were different from random sites. The sites differ especially by their richness in prey, the presence of bushes, land depressions, and attractive elements for prey such as flowering plants or organic waste. Moreover, during its development, the spider seems to become increasingly demanding in its selection. But all of the information that spiders take into account are poorly known.

Prey caught by a web is lost to its neighbour. There is, therefore, a potential risk of competition that has been verified. Under certain conditions of population density, spiders do indeed show an even spatial distribution [60, 96–98]. In *Agelenopsis aperta*, these "territories," which can reach several square metres, have a surface size inversely proportional to the amount of prey available and if by chance two spiders exploit an area equivalent to one "territory," this can result in a 40% reduction in the catch rate for each individual. One of these individuals will then leave the location earlier than might be expected under normal circumstances [60, 99]. Because of these movements within a population, there are relatively frequent meetings between spiders, and one may attempt to steal the web of the same species. The protagonists can use vibratory signals as threats during agonistic interactions. If size differences are not too great, this dialogue of vibration escalates until one gives way [61].

By its presence in the environment, the web reveals the site chosen by the spider based on ecological characteristics. It also tells us about the perception that the spider has of its environment. In fact, spiders are able to change the size of the web as a function of prey availability [100]. In prey-rich environments, the webs are smaller than in poorer environments. This adaptability in web size suggests that the spider can make an assessment of prey availability, leading to "decisions" on whether to build a large or small web. This adaptation can be explained by a simple mechanism, not involving complex cognitive processes, at least in the spiders that build sheet webs (see below: construction of the web).

Spiders are not the only arthropods that can weave silk snares. Some aquatic larvae of caddisflies construct traps between stones and capture prey driven by the current [15, 16]. There is also the rather exceptional case of a predatory larva of Diptera in Australia *Arachnocampa luminosa* [1, 101], which weaves a trap made of sticky threads hanging from a horizontal silk line.

4. Silk as a Means of Communication

Like many animal species, spiders communicate via chemical signals or pheromones [102]. Those with well-developed vision (Salticidae and Lycosidae) also display visual signals such as by waving their legs in specific choreography [103–105]. Tactile stimuli between spiders are often mentioned incidentally in the literature but have not been studied

objectively. At first glance, these modes of communication do not differ from those of most other animal species.

The unique properties of spiders arise primarily from the exploitation of their remarkable sensitivity to vibrations [77]. Indeed, this sensitivity to vibration is used to detect prey and also serves in social and sexual communication. Many species communicate while wandering, reconciling sexual and agonistic encounters, through the vibratory signals produced by the movement of their legs or abdomen and as transmitted by the substrate [106–115]. Web-spinning species have taken advantage of the capabilities of the silken thread to transmit all kinds of vibrations. Males coming into contact with a female's web produce specific vibrational signals, by pulling the threads or by vibrating their abdomens, that is to say, by using the same type of behaviour as wandering spiders [116–124]. These signals are specific and may lead to acceptance by the female if she is receptive. Vibratory communication at distance allows the male to decide whether to approach or avoid the female. Often a dialogue is established between partners, strengthening the system of partner identification within a species and improving its effectiveness as a species barrier.

Silk also has an influence on chemical communication in spiders. Females are capable of depositing a chemical trace on the ground or substrate, which initiates courtship behaviour in male *Lycosides*, without allowing their orientation toward the female [125]. Their originality lies in the fact that females of almost all species can combine their sex pheromones with their travelling thread and the threads of their web. A male is thus able to identify a thread of silk produced by a female adult, sometimes even a female subadult, and to monitor them using both chemical and mechanical information. This method is used by both wandering and web-spinning species [118–120, 122, 124, 126–142].

While this mechanism seems well suited to wandering spiders, it begs the question of effectiveness in sedentary web-spinning spiders. However, observations in the laboratory on *Tegenaria* showed that while remaining faithful to their web home females regularly leave it and move about their immediate environment for short periods. During these wanderings females leave a trail of threads behind, which converge towards the web allowing the orientation of males [143]. In orb-weaving spiders, many radiant threads have no direct connection with the web but lead directly to their retirement [98].

It is plausible that the threads also influence the territorial distribution of spiders. It has indeed been demonstrated in some Salticidae spinners, that females are able to distinguish between their own threads and those of a foreign female of the same species [144, 145]. In a choice situation, they spend more time on their own silk. But they also spend more time on the silk of an unknown female than of a known congener. Orb-weaving spiders could also use the threads to detect the arrival of an intruder.

Sexual communication in spiders is even more complex because it often combines chemical, visual, and vibratory communication. Due to the dispersion of individuals in the environment (except in social species) and their reduced mobility compared with flying arthropods, encounters between the sexes cannot be left to chance. It is usually the male that travels in search of the female, which is easily explained in web-spinning species because, after mating, the female needs to remain with its web and feed for egg maturation and the construction of the egg sac. Selecting a new location would be expensive. Although not researched in the literature, it is apparent that the males of wandering spiders also spend more time searching for females, even though the females are less bound to a specific site. In all cases studied so far, the coming together of the sexes is based upon the use of chemical signals, volatile pheromones, and/or an association with silk. In the latter case, the male also uses mechanical information in silk. As the partners converge, they can also utilise vibrational and visual communication. Visual communication or vibration through the substrate is utilised by Lycosidae, visual communication is used by the wandering Salticidae, and vibratory communication through silken threads is used by the web-spinning species. It is therefore, an addition of signals using different vectors, which refines the identification of partners and avoids disruption from the vagaries of the environment. In *Tegenaria* species (spinning species), a combination of chemical signals and vibrations enhances the species barrier, and the absence of one type of signal can be partially compensated for by the presence of another [116].

Social life has apparently changed this system of communication among nonterritorial species. By living in a "telephone network," it is easy to see that the males of social, territorial, or nonterritorial species use vibrational signals to woo females, as is the case for the solitary web-spinning species [146–148]. However, it appears that chemical signals associated with female silk have no effect in *Mallos gregalis* (social nonterritorial), which seems quite logical given that the males live permanently within the webs woven by females [149].

5. Silk as an Organiser of the Behaviour Needed to Build a Trap

A web's geometry provides a record of the behaviour of the spider during its manufacture. The complexity of the architecture and the consistency of its production have captured the imagination of biologists, who suggested in the early 1900s that the spider must have a strict plan or instinctive map for construction in its "head." It is true that construction takes place through a succession of specific acts [150–152].

Before building its web, the spider explores the media available. It then weaves the primary rays and first threads of the framework, leading to a draft hub that marks the convergence of the primary rays. It then constructs the secondary rays to complete the frame and finally it lays the tertiary rays. Then, beginning at the hub, it begins constructing the provisional spiral thread, which increases in pitch from the hub to the periphery and is made of dry (non sticky) threads (Figure 4). Finally, returning from the periphery to the hub, the spider weaves a sticky spiral thread which is neither regular nor tight. These steps lead the spider

FIGURE 4: Web showing the primary spiral (Photo Krafft).

to produce a structure of complex architecture that has been extensively described in the literature [153–157].

Nevertheless, as it was difficult to accept that a "representation" of this complex architecture and its implementation could be contained within the head of a spider, we tried to explain this construction through a succession of strictly stereotyped and sequential acts. We attempted to describe the spider as an example of automatic genetic programming where each stage of construction was well planned. But this rigid concept of their behaviour had to be abandoned in light of the experiments that were conducted.

Despite its exploratory behaviour before the construction of a web, the spider does not seem to develop a "representation" of the available media. Indeed, the first web built in a new environment is smaller and more uneven than in the finished product (the peripheral attachment points for the rays are not in the same plane). Only gradually does the spider adjust the attachment points of the main threads to produce a larger and flatter web. The spider seems to require a succession of tests before reaching its perfect construction [158, 159]. We can at least deduce that the spider does not "anticipate" the final design of its web from the beginning. During successive reconstructions, it modifies some threads in the frame while maintaining others as aids for travel. As the spider always leaves a thread behind it, one can imagine that flatness improves spontaneously when it takes shortcuts between points previously separated by a third thread that lies outside the plane. This is only a hypothesis, but one that deserves testing, especially in light of what we know about the construction of web sheets (see below).

Independence between the successive steps involved in web construction was highlighted long ago. However, the importance of this phenomenon was not always understood due to the lack of appropriate concepts. The classic experiment was to cut rays during the construction phase. If the spider was following stereotypical acts according to a strict sequence, the spider that had placed the expected number of rays should continue its work according to a genetic program and, therefore, produce an incomplete web containing the damage. However, the spider detects the missing rays and replaces them. Conversely, if we add artificial rays the spider builds fewer of its own. In addition, the spider is able to revert to earlier constructional behaviours as required. If we destroy rays while it is engaged in the latter steps of constructing the temporary spiral, it replaces the missing rays [160, 161]. This suggests that it is the work already performed that controls manufacturing behaviour. The behaviour of the spider is guided by the silky structures already present. König, therefore, proposed that all the elements needed to explain web construction were processes of stigmergy. This idea was supported at the time by Szlep [162] who demonstrated the absence of a strict sequential organization in web manufacturing behaviour, that is, there was independence of action between each step. The concept of stigmergy was still to be established [163].

Another important observation has not had the impact that it deserves. A spider placed on a partially developed web prepared by a congener continues to work upon the web by immediately adopting the appropriate behaviour needed for its completion [162]. This confirms the independence of the acts (the spider notes its environment before engaging in the steps needed for web construction) and the fact that they are controlled by the existing web structure. Further, it shows that the behaviour of an individual can be driven by the silky structures built by another individual, which is the very principle of social stigmergy. These results have been confirmed by showing that a small spider placed on the partial construction of a large web will also finish the design larger than it normally would [164]. Nobody at the time realized that these results also demonstrate that solitary spiders could potentially cooperate in web building, as even though the individuals do not work simultaneously, the sum of their efforts is a completed web. One might ask why they do not cooperate spontaneously in nature. A possible explanation is territorialism, which is partly based on genetic determination [165–167] and was probably heavily selected during the evolution of some spiders. But the geometry of the web is another possible explanation. The two spirals are each composed of a single thread that can only be installed by one individual at a time.

Also, the hub is a strategic point that can be occupied by only one spider. Intrigued by the scarcity of social species, biologists have wondered about the origin of sociality, but with little or no reference to the mechanisms responsible for their individualism.

The work conducted 60 years ago on the construction of webs was repeated 20 years later, and confirmed that the spider gains information from the silken structures already in place. For example, it takes into account the angle between adjacent rays, between rays and the provisional spiral, and the distances between the rays and successive spiral turns [168]. They also confirmed the flexibility of the manufacturing behaviour, however, stereotypical the acts [169]. This flexibility is due to the independence of action between

different acts, due to the fact that the spider primarily uses information from local existing structures to "decide" its correct behaviour [170]. The winding direction of the provisional spiral, for example, influences the placement of the sticky spiral [171]. These processes allow, among other things, the spider's web to adapt to environmental conditions [172].

Factors other than the existing silken structure also have an influence on web building behaviour. Gravity is responsible for centring the hub in the upper part of the web [66, 173]. The surface area of the web above the hub is always less than the surface area below. Spiders with weights glued to their backs increase this asymmetry [174]. The structure of the environment, such as the materials available, also influences the construction of the web [172].

However, it is impossible to reduce web manufacturing to a simple automatic response to external factors. The web of each species has a specific architecture and the webs of similar species have more in common than those of species from different genera [175]. Some characteristics of the webs are independent of age and size of the spider [176]. Moreover, the construction of a web by a young spider requires maturation of the behavioural mechanisms [177, 178]. Thus, we cannot exclude an internal programming that at least prevents the spider from reacting in a random manner to stimuli. Even in the context of stimulus/response reactions, the information collected is processed and will help generate a response. Information processing can be altered by drugs that cause specific alterations to the architecture of the web, a change in the positioning of the hub or the appearance of irregularities [179–181]. It may also depend on the situation. Gravity has an influence on the laying of the provisional spiral, but not the sticky spiral, probably because the spider bases its weaving of the sticky spiral on the presence of the provisional spiral [171]. Moreover, the provisional spiral maintains constant angles with the rays and does change pitch, while the sticky spiral maintains a constant spacing between successive turns which do not change pitch [182]. These phenomena again demonstrate the role of existing structures in determining subsequent behaviour. The spider may also partially compensate for physical disabilities. After a leg is lost, it regenerates at the next moult but with reduced size. This reduction in the length of one or more legs affects the spacing between the turns of the sticky spiral but has no effect on the installation of the provisional spiral. These results demonstrate that the behavioural rules governing the installation of the two spirals are different [182]. For the installation of the provisional spiral, the spider takes account of the angle with the rays, while for the sticky spiral, it weaves according to the distance that separates it from the previous spiral. Finally, the amount of silk available in the silk glands is another factor that determines the size of the web [183].

With improved modelling programs, it may be possible to explain these variations by simple adjustments to the various quantitative or qualitative feedback stimulus/response reactions involved.

The web is the geometric reflection of complex animal behaviour, and thus the material of choice for studying this behaviour. It is not surprising that several researchers have attempted to model the web building behaviour of spiders. In 1965, Witt stressed the importance of this biological model while adding, however, that while it was easy in principle to model the manufacturing behaviour, that is to say, to develop a model mimicking the construction of a web, it would also be difficult to validate the different stages of programming [176]. The first test model assumed that the spider took into account the angles between the spiral, rays, and the previous lap of the spiral [184]. The model weaved the author's virtual web and it resembled natural webs with particular "Greek" architectures (when the spider comes too close to the frame in the upper part of the web, it turns around; it, therefore, produces more spiral thread in the lower than upper part of the web). The author stresses that this model does not prove that the rules introduced in the model are identical to the behavioural rules of spiders. But this result successfully demonstrates that it was possible to obtain a complex structure on the basis of simple principles.

It was only recently that attempts at modelling have grown. Based on previous results, Vollrath developed a model of virtual spider manufacturing that takes into account the angles between the threads and radii, distances between successive turns of the spiral, leg length, and other parameters of varying complexity gleaned from the architecture of the web [185, 186]. Nevertheless, all the rules introduced into the model are simple rules based on the responses of the virtual spider to local information, apart from the memory of the last segment of the thread's run [187]. The model produces very similar formations to natural webs, even though it contains no rules on the overall architecture of the web. Moreover, the artificial webs obtained are suitable for the space available, so, therefore, accommodate local landscape features [188]. The architecture of the web is an emergent property of the system [187]. Finally, the virtual spider which has one leg "regenerated" constructs a web in accordance with those woven by living spiders that have undergone the same treatment [189]. A set of simple and stereotyped responses to local information makes it possible to achieve an overall plastic behaviour, demonstrating that rigid acts in isolation do not prevent flexibility and confers robustness to the system.

This model successfully demonstrates that it is possible to obtain a complex geometric structure from simple behaviours, without having to explicitly specify the overall final architecture of the web, and that the spider's response to local information is sufficient to adapt the web to environmental conditions. However, doubt remains as to the validity of the stimulus/response reactions in the model. The behavioural rules used in the model were derived mainly from the geometry of the web and observations of spider behaviour, and are not, or only slightly, based on stimulus/response interactions verified experimentally. But we must recognize that the web geometry is complex and it is technically difficult to identify all the elementary interactions governing their construction.

The geometric orb web is traditionally opposed to those webs labelled "irregular." These irregular webs, however, also obey architectural rules as it is possible to distinguish the webs of the different species. But these rules would seem to

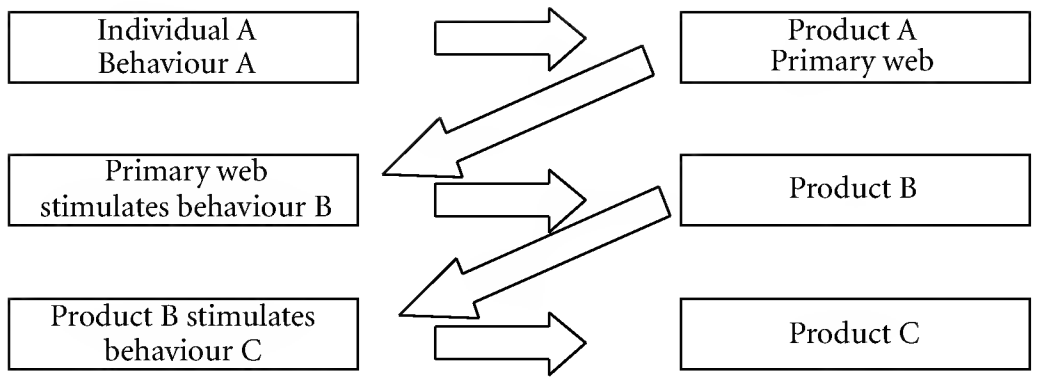

FIGURE 5: Processes in self-stigmergy. The behaviour of an individual is driven by the product of its previous actions (based on Grasse [163]).

be simpler, which makes them more suitable for modelling [190]. Unfortunately, few researchers have been interested in their construction [191].

The web of *Agelena labyrinthica* consists of a horizontal sheet topped with a three-dimensional network. A retreat tube open at both ends leads onto the horizontal sheet. Every thread of this trap is made of dry silk. The network intercepts airborne prey that fall rapidly onto the sheet where they are captured by the spider.

This web is constructed in several stages. In the laboratory, in a horizontal square glass box empty of any support structures, the spider firstly weaves an outline of its retirement amongst a tangle of threads in a corner, and an outline of a sheet in the form of a framework of threads that are more or less horizontal. Construction continues through the completion of the retirement and the final sheet layer. The sequential order of these structures is variable, which reveals a certain independence of action.

As in the case of the construction of geometric orb webs, structures built beforehand steer the subsequent behaviour of the spider. Because of the shape of the box, the spider builds its retirement in one of the 4 corners of the box. If we allow a spider to build an outline of its retreat in one corner, then remove the spider and turn the box 180° in a horizontal plane to eliminate any possible action from external factors, and replace the spider which in the meantime has built a complete web in another box, it uses the existing draft outline to develop its final retreat.

The same approach during the construction of the framework for the horizontal sheet shows that the height at which the spider weaves the first draft of its web determines the subsequent position of the final web.

Independence between the various steps necessary for construction is also demonstrated. If the web is partially destroyed, the spider will rebuild the missing part, web, or retreat, without changing those parts that were intact.

These results suggest the involvement of a process similar to the stigmergy described by Grasse [163] for explaining the construction of nests and mounds by termites, except that in spiders it uses interactions between itself and the structures it has already built rather than between these structures and congeners (Figure 5). It is, therefore, a "self-stigmergy" and not a real social stigmergy, as is also the case in birds [192]. This distinction may seem subtle, but it makes sense when one examines the origin of cooperative processes in the emergence of sociality (see below).

These simple mechanisms do not explain all of the architecture of the web, such as the appearance of the sheet, network and retreat. Surveys conducted in nature show a relationship between the provision of materials and these three elements. An analysis of the vertical plane of a web reveals a heterogeneous distribution of vegetation, the latter being less numerous above than below the web.

The sheet is built on the border of a medium density of vegetation below, and a more open environment above. In addition, the height of the three-dimensional web is correlated with the height of overhanging vegetation. The webs may, therefore, take different forms depending on the configuration of the environment.

Any spider that moves leaves a thread along its path. During these trips, it often fixes this thread to a support. On a flat surface, the spider has a constant probability of fixing this thread at a constant distance, as is the case in the social spider *Anelosimus eximius* [193]. But the presence of bumps on the surface increases the frequency of attachment, as shown by measuring the lengths between each attachment point. Roughness, therefore, encourages a moving spider to fix its thread to supports.

It is easy to demonstrate the influence of these environmental factors on web building by placing spiders in an artificial environment composed of a high density of small supports and a low density of large supports. In such environments, the spider systematically builds its web at the border between the dense environment represented by small supports and the open environment represented by the large supports. A change in the height of the small supports causes a change in the height of the web sheet, while the height of the network is determined by the height difference between small and large supports. In addition, a cardboard shelter mimicking a rolled leaf systematically determines the position of the retreat. This shelter can also have a slight influence on the position of the sheet. If positioned below the boundary between dense and open environment, the spider will lower the level of the sheet.

We can, therefore, formulate the following hypothesis. Moving randomly in the environment, the spider becomes trapped by a particular density of vegetation due to the frequency by which it fixes its thread to supports, leading to the construction of a draft retreat. Radiating from this site, it would reach the tops of the supporting plants and its thread would join them together. The most numerous threads appear at the border between the medium density

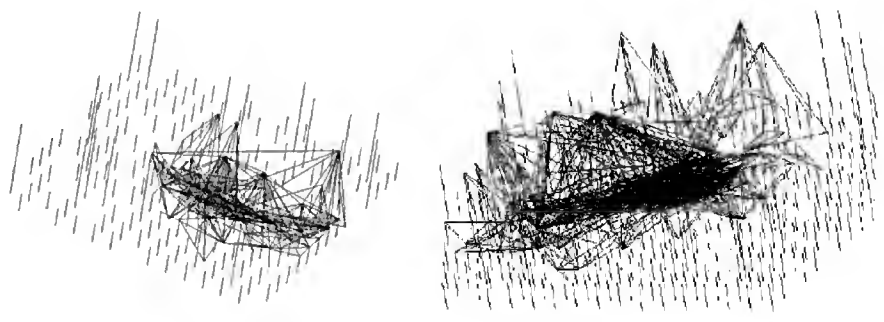

FIGURE 6: Silken structures produced by the model, where vertical lines represent vegetation with differing heights. Showing a horizontal density (sheet), topped by a network [190].

of plant cover and the open. This appears a draft sheet. The location of the fewer overhead supports in the more open area is responsible for the appearance of the irregular network which tops the sheet. While it is easy to develop such a hypothesis, which implies that the spider has no knowledge of the architecture of the structure it will build, it is more difficult to verify experimentally. In this situation, modelling can be of great assistance.

The proposed model consists of a virtual environment with three types of supports, supports with zero height corresponding to flat ground, small supports that occur in high frequency, and fewer large supports, thus mimicking natural conditions [190, 194]. The rules imposed on the virtual spider are very simple. It may move at random, fixing the thread that traces its journey over the tops of the supports, in agreement with previous results (high probability of fixing to the tops of supports and low probability of fixing close to the flat ground). Unlike attempts to model the construction of geometric webs, these rules do not imply any assessment of the angles between the threads but assume it is the environment that determines the architecture of the web. In addition, these steps all correspond to the stimulus/response reactions that have been demonstrated in spiders. Despite their simplicity, these rules are sufficient to produce a structure that has some similarities with the "irregular" webs observed in nature. It is sufficient to produce a draft sheet and network.

The result nevertheless is far from perfect as the virtual structure occupies all of the space available when in nature webs have a finite size within infinite space, and while the network appears accurate, the virtual sheet remains a loose dishevelled framework. This suggests that there must be a mechanism that also limits the movement of spiders. One can certainly imagine various rules such as "return to shelter regularly." But knowing the role of silk in spiders, we can also assume that they are trapped by their own silken structures because of a possible attraction to silk.

By introducing only one additional rule in the model, a factor for the attraction of silk, we can vary the size of the web and obtain a virtual web that is limited in size and where the sheet has strong demarcation (Figure 6) [190]. This attraction leads to a certain probability that the spider will choose to follow an existing thread. Placed on a base already connected by a thread to one of eight adjacent supports, the spider is more likely to approach that support over the remaining seven. This rule "traps" the spider in such a way that it remains within existing silken structures. The strength of this attraction modulates the size of the web. Moreover, for a given number of excursions, it determines the density of the sheet.

The attraction for silk has been demonstrated in *Agelena labyrinthica*, validating the model. In a binary choice situation (T-maze with the two arms providing the test options), the spider spends more time on a substrate covered with its own silk than on a blank substrate. This attraction for silk is further regulated by the state of satiation of the spider. A satiated spider spends more time on a silken substrate than a hungry individual, while the frequency of travel within the experimental device does not change (the spider is not simply resting on the silk). As the silk used is produced by spiders reared under standard conditions, one can deduce that it is the responsiveness of individuals to the silk that has changed, rather than there being any differences in the silk "bait."

We know that spiders are able to integrate a variety of information to select their construction site [60, 61, 156, 195, 196] and to modulate the structure of their web based on various environmental factors [197–200] such as prey availability [201–203] and frequency of damage from large nonprey animals [65]. Finally, in orb-weaving spiders, the presence of prey induces the spider to anticipate the time when it should build its web [204]. These studies concern spiders that rebuild their geometric webs daily and can therefore easily adapt this behaviour to changing environmental conditions.

These skills in adaptability have been little studied in species that produce irregular webs that are built to last. Nevertheless, several environmental factors (rain, wind, prey) constantly destroy or damage these webs and can reduce their volume by 7 to 11% each day. A vacant web can be completely destroyed in 15 days. This damage forces the spider to weave every day to maintain its trap, producing 5 to 15% of the silk originally invested in its total construction [62].

However, it has been shown for a species of Eresidae from Namibia, that while its web is regularly destroyed by wind and sand it also adjusts the size of the trap according to the quantity of prey available, without changing the size of its retreat [205]. Riechert noted that *Agelenopsis aperta* webs tend to be larger in areas with limited prey, but there are no quantitative results to confirm this impression [206].

These results suggest an "evaluation" of prey availability and a comparison with information on the amount of prey captured previously, according to memory. Without excluding this possibility out of hand, the regulation of the attraction for silk by the state of satiety could reveal a much simpler mechanism than one involving highly complex cognitive processes. To compensate for daily reductions in the surface and volume of its web, or to expand its web, the spider must temporarily leave the web to reach neighbouring supports. But the attraction for silk determines the probability of the spider leaving its web, and therefore the rate of daily expansion. If this probability is high (due to a reduced attraction to silk), the extensions to the surface area and volume of the web may exceed daily destruction and lead to a net increase in the size of the trap. A low probability (in the case of well fed spiders, more attracted by silk), however,

causes a reduction in the size of the web. The spider might as well settle for a state of balance between adjusting the size of the web with the availability of prey.

It has been shown in the field [190] that by providing extra food to some spiders and not others (the latter being limited to naturally captured prey) for four consecutive days, the web surface area of individuals fed artificially decreased slightly while those in the control group increased significantly. A similar result is found if volume rather than surface area is measured.

These results support the hypothesis of an adaptation where the size of webs is modified according to prey availability or satiety. When the amount of prey caught is enough to satiate the spider, it becomes more responsive to the attraction of silk. In a way, it becomes trapped by its own silken structures, which limits the ability of the spider to extend its web. A hungry spider lacking in prey, however, will be less responsive to its attraction for silk, and will have a greater likelihood of leaving the confines of its structure and further expanding the size of its web. The same mechanism, not involving complex cognitive processes, could explain why spiders abandon webs, as they need to search for a new construction site.

This simple model is certainly not perfect. It relies on quantitative stigmergy, but during construction there are also qualitative changes in behaviour that are not being taken into account. When the basic frame for the web is completed, the spider changes its behaviour by producing bundles of silk rather than mere threads. The draft shelter becomes replaced by a tubular retreat that is open at both ends. These initial structures generated on the basis of a quantitative stigmergy acquire, from a certain state of completion, further stimulating properties resulting in a new type of behaviour. But this model has successfully demonstrated that it is possible to obtain a coherent silken structure tailored to the individual and obeying certain architectural rules without the spider needing to have a "representation" of the overall structure. The spider's response to local information is sufficient.

6. Silk as an Agent of Social Cohesion

Of the approximately 40,000 species of spiders currently known, only about fifty are more or less social species [29, 47–49, 166, 207–216], yet biologists have focused on the causes for the emergence of sociality rather than the reasons for the individualistic nature of most species. The term social spider should be qualified. It consists in fact of those species that show parental behaviour, where the juveniles live for a limited time in the web developed by the parent and then form associations of individual webs (social but territorial), and species that collectively operate a common web. The latter group includes what is usually called the nonterritorial social spiders. They cooperate in the construction of the web, prey capture, the care of young, and colony defence.

Although web-spinning spiders show pronounced individualistic behaviour, coupled with an intolerance leading to cannibalism, there are occasions when they form associations with their individual webs, sheets, or networks. These aggregations may depend on the structure of the environment when it offers a limited number of materials for attaching webs. The materials play the role of attractor. They also depend on the quantity of available prey [202]. *Nephila* can form aggregations in environments rich in prey, where the webs are attached to each other. However, when prey becomes scarce, agonistic interactions increase and lead to the dispersal of individuals [217]. Some species of the genus *Metepeira* are gregarious or solitary according to the availability of prey [196].

One may wonder about the origin of these associations. Attracting spiders to areas rich in prey appears unlikely because this would suggest that they can remotely sense that an area will be more abundant in prey. It is more likely that spiders select suitable sites during their travels, and effectively become trapped by their rich environments, where they can remain grouped because of a temporary reduction of their intolerance. Tolerance between conspecifics can indeed be experimentally altered by manipulating diet [218–220]. But these groups can also be explained by an attraction to the webs of congeners or even of different species [221].

Some species show varying levels of maternal behaviour. Sometimes, the juveniles live for a period of time in the web of the mother, without gaining much care from her, but which nevertheless leads to energy savings in terms of silk production [222]. More often, the mother feeds her young with trophic eggs, by regurgitation or by abandoning her prey [219, 223–227]. The mother can even offer its own body as a food source to the young [228–230]. Sometimes, juveniles will remain grouped into the subadult stage.

Communal structures begin when the juveniles construct their individual webs near each other and the mother. This is probably the origin of spider sociality in social territorial species, and while Uetz [166, 196] suggests that spiders of the genus *Metepeira* could come together as adults, this has not been proven. The communal web, therefore, consists of a large number of individual webs that are attached to each other. If the colony survives long enough, there may be an overlap of several generations [207, 231, 232]. But within these colonies, there remains a regular distribution of webs, modulated by genetic and environmental factors. The distances between individuals vary according to population size and prey availability. Juveniles from egg sacs collected in poor environments and bred in the laboratory under standard conditions show interindividual distances greater than those from egg sacs collected in rich environments. But even within a population, these distances vary with fluctuations in prey [166, 167, 233]. This regular distribution of webs is the result of agonistic interactions expressed mainly by an exchange of vibratory signals [232, 234, 235].

Individuals of *Parawixia bistriata* (a social territorial species) spend the day in a compact silky nest but at night move to their individual geometric webs which all hang from a vast network of common retaining threads. The construction of these webs is strictly individual, suggesting that the geometry of the web is an obstacle to cooperation. The capture of small prey is also individual, but occasionally large insects may attract many spiders onto the same web. They then cooperate to capture the prey [236, 237].

These various social organizations offer both advantages and disadvantages. Merely staying temporarily in the irregular web built by the mother allows *Holocnemus pluchei* juveniles to save energy by reducing their need to produce silk [222]. The exploitation of existing webs promotes the survival of young [238]. Maternal behaviour also has a beneficial effect on juvenile development, particularly from the time the mother feeds her young [225, 227–230, 238].

The advantage of gregariousness among territorial social spiders seems less obvious since the capture of prey is essentially performed individually. However, in *Metabus gravidus*, building a web within a common network takes less time than if the spider was isolated [48, 231]. The colonial structures also increase the catch rate per individual for *Cyrtophora citricola* and *Metepeira spinipes* [166, 196, 239–241]. The combination of individual webs can create a ricochet effect, where a prey that pierces one web because of its kinetic energy is likely to be stopped by a nearby web [242]. A ricochet effect has also been suggested in large communal colonies of *Anelosimus eximius* where "bystanders," spiders that surround a prey being subdued by "catchers," become catchers themselves if the prey temporarily breaks free and moves nearer to them [243].

One of the main benefits of nonterritorial social organization seems to be the ability to capture large prey, which would, otherwise, be uncontrollable for solitary spiders. Nentwig [244] has demonstrated that social spiders, however small, capture prey much larger than solitary species in tropical environments, increasing their range of available prey. In absolute terms, *Anelosimus eximius* captures more small prey (70%) than large prey (30%), but large prey provides 80% of the energy for the colony. Furthermore, the catch rate is 50% in small colonies compared to 76% in large colonies. These spiders are capable of capturing prey 700 times heavier than themselves [245]. The greater abundance of large prey in lowland tropical rainforest rather than at higher elevations helps to explain why *Anelosimus eximius* favours the lowlands [246]. The prey is also controlled more rapidly in *Stegodyphus mimosarum* if several individuals are involved in its capture [247]. If the colony becomes too large prey capture per capita falls due to the reduction of colony surface area compared to volume, triggering dispersal [248].

The society also provides an economy of silk. Frequent storms in the tropics regularly destroy webs. Reconstruction is less frequent among isolated than grouped individuals of *Agelena consociata* because of the relatively higher cost per individual in silk production and reduced social stimulation. Moreover, the expenditure of silk per spider decreases in large colonies, so that survival in small colonies is less than in large colonies [249, 250]. This economy is also seen in the silk of *Mallos gregalis* [251]. The size of the web grows less rapidly than population size, which is offset by an increased efficiency in predation.

The larger web structures also provide some protective effects against weather and predators [51–53]. In *Stegodyphus dumicola,* social groups are less often attacked by ants, birds, and other species of spiders than isolated individuals [252]. The juveniles of *Anelosimus eximius* have a better chance of survival in large than small colonies [253].

Sociality also has its drawbacks. The chances of female *Anelosimus eximius* being able to reproduce decreases with increasing colony size [253]. There can also be competition between individuals, where some have better access to prey and reproduction [51, 254, 255]. These constraints in social life are also demonstrated by the fact that isolated individuals produce more eggs than individuals within a society [256–258]. However, for *Stegodyphus dumicola*, the survival of the offspring that are produced increases [258], due in part to shared parenting [259].

All these phenomena are the consequence of group living, which itself is explained by the existence of a mechanism for group cohesion that reduces the possibility of dispersal by individuals. Unfortunately, researchers are more concerned about the possible external causes and consequences of these associations than in the mechanisms involved at the individual level. Group cohesion seems relatively loose amongst territorial social spiders, which allows some plasticity in the distribution of individuals, so that one sometimes finds spiders that have become isolated. The nonterritorial social spiders show more pronounced group cohesion, which has been stressed by many authors but rarely studied. The term interattraction as defined by Grasse [260, 261], of attraction between individuals of the same species and sex, can be used as one of the criteria for defining nonterritorial social spiders [262]. Sociality in these spiders seems to be the general rule and the few isolated individuals sometimes found near web settlements are probably migrants seeking to establish a new colony. When a loose group of individuals of *Agelena consociata* or *Anelosimus eximius* are placed on a bush or in an empty chamber, they combine to form a new colony [50, 263].

Although there is an attraction produced by air-borne pheromones in *Anelosimus eximius* [190], silk, whose function is essential in bringing the sexes together also appears to be the main factor behind group cohesion. Those solitary spiders, whose webs sometimes show a distribution approaching the gregarious type, seem attracted to the silky structures of their congeners and even those of different species [221]. The aggregated geometric webs of *Zygiella x-notata* can be explained by an attraction for silk [264, 265]. This attraction may even occur between social and solitary species in the genus *Stegodyphus* [208, 209]. Different results have been obtained in *Dictynides*, a genus that also contains solitary and social species. Silk is attractive to social species, but not the solitary species [266]. For these species, individuals do not seem to distinguish between their own silk and that of congeners. There are exceptions as *Portia labiata* (Salticidae solitary species) discriminates between its silk and its congeners and even between the silk of known and unknown congeners [144, 145].

Regarding nonterritorial social species, laboratory experiments have shown that a silken substrate is attractive to *Agelena consociata* and leads to clustering by individuals [263]. This attraction depends on a pheromone associated with the silk in *Diaea socialis*, but individual responses to this stimulus varies depending on their physiological state. Adult females that are ready to lay eggs are repelled by the silk of conspecifics, which explains their emigration and the founding of new colonies [267]. An attraction for silk also

overcomes repulsion between nestmates and nonnestmates in *Anelosimus eximius*, although colonies with both are less stable and less successful [268]. It seems that the attraction for silk is also modulated by the state of satiety in *Agelena consociata* and *Stegodyphus sarasinorum* as removing food will cause the spiders to leave their nest [269, 270]. In this case, individual emigrants sometimes travel in groups, as is also the case with *Anelosimus eximius* [271].

The cohesion of the travelling group seems to be due to the threads of silk deposited by member spiders. In *Anelosimus studiosus* individuals tend to follow the threads of silk produced by congeners [210]. When spiders (*Aebutina binotata*) are about to leave their colony, they unleash threads that cling to the surrounding vegetation. An individual who progresses along one of these threads without being followed by its congeners returns to the colony, so that eventually only one of these potential routes is followed by the group [272].

The ability of social species to follow the trail of congeners of the same sex was based mainly upon field observations, without being proven experimentally. Early demonstrations were based upon T-maze experiments offering binary choice. Female *Stegodyphus sarasinorum* follows the tracks left by a congener of the same sex. This guidance requires the presence of a silken thread [119, 132, 136]. A similar technique has shown that in *Anelosimus eximius* individuals spend more time on a silky substrate woven by a congener than on a blank substrate. Also, spiders do not distinguish between their own silk and that of conspecifics, even if they were from different colonies [190, 273].

This attraction is governed by two factors, the density of the silk and the satiety of the individuals tested. In T-maze choice experiments, spiders spend more time on a sustrate covered with dense silk than on one covered with a thin layer of silk, a mechanism that could focus the movement of individuals towards the densest regions of the nest under natural conditions and restrict their travel to the periphery. As for the solitary spider *Agelena labyrinthica*, the attraction for silk is modulated by satiety. Starving spiders spend less time on a silky substrate than satiated individuals. Presumably, this variation in attraction allows adjustment of the size of the trap as in solitary species, and in social species the emigration of individuals or groups [190, 269, 271–273].

The role of silken threads in the cohesion of moving groups has been demonstrated experimentally and modelled [193, 274, 275]. A binary choice experiment in the field showed that the probability of a spider following a pre-existing thread is higher than for any other path. When released from a container connected to two equivalent branches of a bush by yarn in the shape of a Y, all spiders grouped upon one of the two branches. The theoretical modelshows that there is an optimal probability to follow a pre-existing thread to maintain good cohesion. The attraction for silk therefore allows collective decision making (Figure 7) as is also the case in social caterpillars. A recent trace of silk within a choice of pathways becomes the route of priority [5, 6], which can be compared with the selection for chemical trails by ants [276]. Fernandez et al. [277] have modelled social spider attraction to silk in a sigmoidal way, where the

Figure 7: Given the choice between two branches of the shrub, spiders are attracted by threads to one of the branches (*Anelosimus eximius*; Photo Krafft).

number of founders in a colony has to exceed a critical value if settlement is to be successful and prevent dispersal.

The role of silk in social cohesion among spiders is further underlined by the fact that there are no social species without webs. Some authors have stressed the exceptions of *Diaea socialis* (Thomise) and *Delena cancerides* (Sparasside) where individuals live in a silky nest but outside are solitary hunters without webs [211, 212, 267]. In fact, these two examples only reinforce the idea that silk is essential for the social life of these spiders as sociality only occurs in the common nest.

7. Silk as an Agent for Coordination and Cooperation in Social Construction

By the 1940s, struck by the architecture of structures built by social insects and the many examples of cooperation during food harvesting, researchers saw these social insects as being fundamentally different to solitary insects. "The individual who belongs to a society differs in many respects to the solitary" [278]. The difference seemed so great that one wondered whether these social arthropods did not have a "global representation" of the structures they were to build or the task they were to realize (cooperative harvesting). This hypothesis was nevertheless difficult to accept because of the complexity and sometimes impressive size of the structures or complexities of actions compared to the apparently reduced cognitive abilities of the individuals involved. The theory of stigmergy developed by Grasse for termites [163, 279] lifted the veil on this mystery. Stigmergy is a mechanism where the structure created by an individual acquires stimulating properties that can control the behaviour of a congener by reducing its degrees of freedom. Although each step in the sequence of interactions is based on rigid stimulus/response reactions to local information only, this system enables a broader plasticity and the emergence of the phenomenon of self-organization, to produce complex and consistent structures, provided that the various acts are not organized into a hierarchical scheme of reactions. There

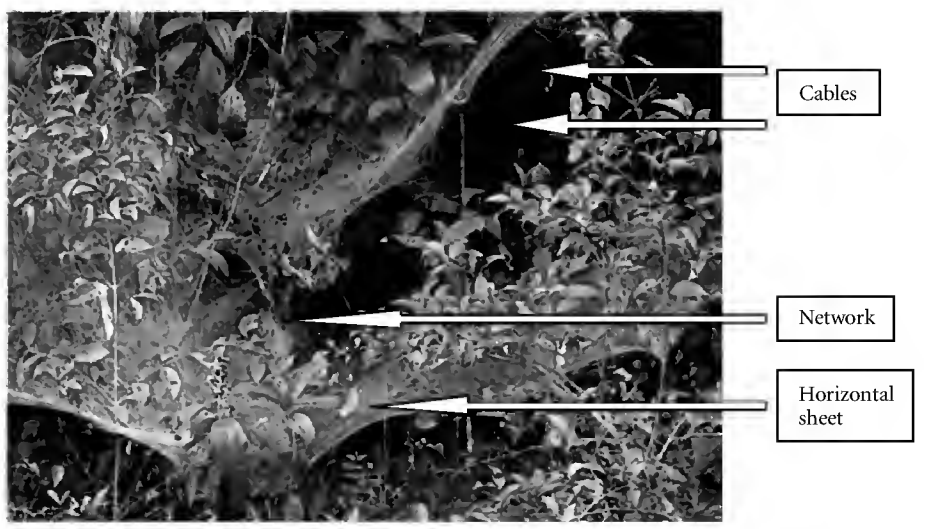

FIGURE 8: Portions of a web colony by *Anelosimus eximius* (Photo Krafft).

must be independence between each action. Widely adopted in recent years this theory has allowed us to understand the mechanisms of cooperation among ants, bees, wasps, and even some vertebrates [194, 276, 280–282].

All nonterritorial social spiders cooperate in building their irregular web trap, while the construction of geometric webs remains an individual activity amongst territorial social species. The architecture of the web can be a hindrance to cooperation and socialization, or it can promote these features [47, 55, 68, 119, 232, 235, 283–286]. As far as is known, there is only one nonterritorial social species from the orb-weaving spiders (a species of *Philoponella* in the Uloboridae) that cooperates in the construction of webs and prey capture. But the geometry of their webs has suffered. Their traps look more like the irregular structures of social species allowing nonterritorial and cooperative behaviour [287].

The traps constructed by *Anelosimus eximius* are impressive. They often reach a volume of 100 m^3. Although described as irregular, these traps obey certain architectural rules. The colony consists of one or several closely interconnected components each comprising a horizontal sheet, or hammock, above which is a silky three-dimensional network (Figure 8). Spiders are divided into groups under leaves embedded in the network [50, 271]. This structure is quite similar to that woven by *Agelena consociata* [49] and *Agelena labyrinthica* [190], with the difference being that the horizontal sheet does not rest upon a dense layer of vegetation but hangs below the plant material. It is built on the border between a dense medium above, and an open area underneath, which does not fundamentally change the problem relative to *A. labyrinthica*. But despite these rules, the overall shape of the structure varies considerably from one colony to another depending on the plant material utilised.

It is impossible to conceive that a 6 mm long spider has a representation of a three-dimensional structure tens of m^3 in size, along with the distribution of fixing points upon a range of substrates. Yet few studies address this aspect of social behaviour. Tietjen is probably the first to have considered a process of self-organization for the construction of the web by *Mallos gregalis* [288]. He noted that if we let spiders loose in an enclosure, the activity of individuals seems chaotic until the appearance of the first draft of a structure. The spiders then focus their constructive activity, which enables the rapid development of a communal nest. Aviles also noted the synchronization of construction activities by *Tapinillus* sp. [289]. It seems that during an initial period of inactivity, the mobilization of one individual can lead to the mobilization of others.

Yet the difference between solitary and social spiders seems so great that one is inclined to devise specific mechanisms of cooperation for the latter. However, the change from a solitary to social status has been made at least thirteen times by independent lineages [47, 290]. Despite the scarcity of social species, this transition has been frequent. This raises the problem of the ethological changes that would be needed for the emergence of cooperation during construction. Either these changes are important and numerous, which seems at odds with the frequency of the change amongst the lineages, or these changes are minimal, which seems difficult to accept because of the dramatic differences between the architecture of solitary and social structures.

However, the mechanisms governing the construction of the web does not seem fundamentally different between solitary and social species. If we drop *Anelosimus eximius* onto a small bush and leave the spider time to build the first draft of its web before removing it from the area, a second individual will expand and complete the construction rather than start anew. The first initial web built by an individual is able to control the behaviour of a fellow builder in accordance with the principles stigmergy (Figure 9). Furthermore, spiders are able to repair a hole in the hammock without changing its architecture, which shows independence between the various acts relating to construction, the essential principle of stigmergy [190].

This raises the question of whether social spiders simply would not use the mechanisms highlighted in solitary spiders (see web building by *Agelena labyrinthica*). However, in *Anelosimus eximius*, silk is attractive to congeners, which appears to be a characteristic of social species. But is it really a social characteristic? *Agelena labyrinthica* is attracted to its own silk as much as the silk of its congeners [190].

This is also the case for *Steatoda triangulosa* (Theridiidae) and *Tegenaria domestica* (Agelenidae), two solitary spiders that belong to families that also contain social species. It may therefore be hypothesized that solitary spiders have all the ethological mechanisms necessary for cooperation except the mutual tolerance that would allow them to work together on a common web.

This hypothesis is confirmed by the fact that it is possible to obtain a complete and fully functional web from *Agelena labyrinthica* when a succession of three individuals is placed separately on site. Each new spider continues their part of the work according to the structures already in place. The self-stigmergy demonstrated in this spider thus gives way to social stigmergy. This suggests that spiders can be unaware yet perfectly able to cooperate in the construction of a web.

Since this assumption is impossible to verify on biological material, it is necessary to use modelling to determine if it is at least sufficient to explain cooperation [190, 291, 292]. If, on the basis of behavioural rules identified in *Agelena labyrinthica*, we drop multiple virtual spiders in the virtual

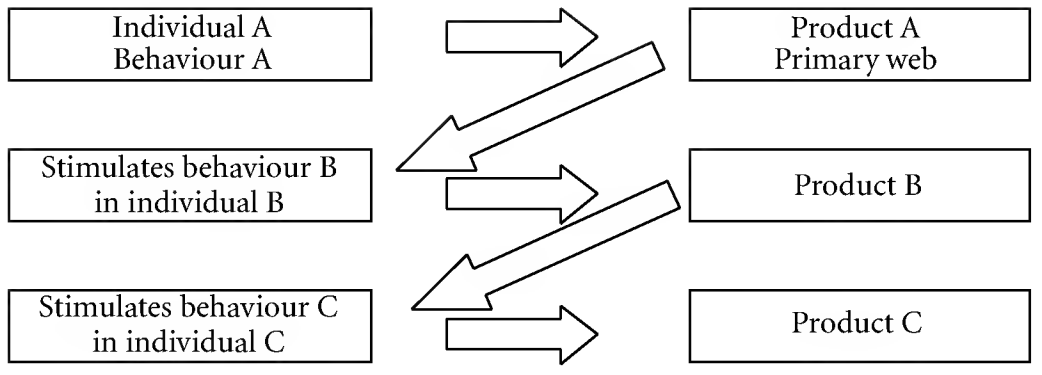

Figure 9: The stigmergy process. The behaviour of an individual is driven by the product of the behaviours of its congeners. Notice the difference with Figure 5.

environment, they build a common web provided that the probability of attraction for silk is adequate. If no attraction, the structure produced fills the entire space. With an average attraction, spiders gather gradually, grouped within a common structure. If another rule were added to the model that limits the life of a thread, the result would probably be even clearer because it would erase the first threads woven before the individuals began grouping. However, individual webs result if the attraction for silk is too strong, with each spider remaining trapped on its own structure.

As for the *Agelena labyrinthica* model, the size of the common web produced is inversely proportional to the attraction of silk. The validity of this model is further strengthened by the fact that, as with social spiders, the size of the structure produced grows less rapidly than population size. Both effects emerge as nonprogrammed features in the model.

None of the rules of this model involve direct interaction between individuals. These virtual spiders are unaware of any plan and ignore each other (i.e., no attraction, no aggression, and no signals between individuals), but simply take account of local information related to the presence of silk, whatever its origin. This suggests that cooperation during construction in social spiders is related to stigmergy based on ethological programs already present in solitary species, where the emergence of communal webs arises from a process of self-organization where individuals tolerate or can more simply ignore each other.

8. Silk as an Agent for Coordination and Cooperation during Prey Capture

Cooperation during prey capture is a feature of social spiders, at least in regard to nonterritorial social species [49, 247, 255, 272, 289, 293, 294]. Indeed, territorial social species that build sets of individual geometric webs do not cooperate in the control of prey [232, 234, 235], even though indirectly, these associations increase the catch rate per individual [241, 242]. An exception is that when individuals of *Parawixia bistriata* can access a web nearby, they will move in and help when the prey is large [236, 237]. The geometry of the web can be a hindrance to cooperation [47, 232, 235, 284], which is confirmed by the fact that the only species in the orb-weaving group that communally captures prey, *Philoponella* sp., became a nonterritorial social species that constructs irregular webs similar to that of *Anelosimus eximius* [287], while *Philoponella republicana*, which uses geometric webs, is a social territorial species.

The idea that silk is an agent of cooperation during predation is also reinforced by the example of certain social or subsocial species that do not spin webs. The Thomisid spider *Diaea socialis* builds a nest formed by assembling leaves together into a silken package. The young remain in this structure until adulthood, but adult gravid females leave [267]. To catch prey individuals leave the nest and hunt independently without a trap but share the prey brought back to the colony [211]. The sparassid spider *Delena cancerides* makes the same kind of structure, except that the nest may contain several females [212]. These authors mention that under artificial laboratory conditions, several individuals placed in a common enclosure can combine their efforts to capture prey. But there are no observations in nature to confirm this act, which should involve a coordinated movement of individuals through specific signals in a process similar to that of ants.

These findings suggest that the lack of a common trap prevents any form of cooperation during prey capture, or more precisely that it is the presence of a common trap that enables cooperation. What could be the mechanism? The descriptions of cooperative behaviour during predation among nonterritorial social spiders are relatively rare and scarce in detail. Bradoo claimed to have observed in *Stegodyphus sarasinorum* that the first individuals in contact with prey vibrated threads to emit recruitment signals [269]. But it is also likely that behaviours manifested by any spider facing prey only adds to the vibrations produced by the victim, which in turn facilitates its location by congeners. The latter seems more likely, as the original observations have not been confirmed [247]. While it is not certain that specific recruitment signals occur, simple cooperation also allows the efficient capture of large prey [244, 245].

There is, however, a curious behaviour in *Anelosimus eximius* which could lead to the recruitment of individuals towards prey. The movements of spiders towards prey are interspersed with short stops, which is probably to aid location and reorientation by individuals towards the prey as is the case with solitary species of *Agelenides*. A synchronization of these stops produces characteristic vibrational phenomena that can lead other individuals to the catch. During the

stationary phase, it is sufficient that when one of the participants moves that the others will follow, and these synchronized movements persist for several seconds after the disappearance of the prey [295]. But the role of these phenomena as a vibratory signal for recruitment is not demonstrated.

Prey vibrations were found to play a major role in the recruitment of spiders, where buzzing prey attracted more spiders than nonbuzzing prey [243, 296]. Prey length rather than prey mass also increased the number of spiders involved in prey transportation, as longer prey simply provided more sites upon which spiders can grip or pull [296]. Response to these simple cues precludes the need for the specific recruitment signals found in some social insects such as ants. Spiders provide an example of self-organising processes in group predation, where the movement of one spider engaged in group transport is likely to modify the stimuli perceived by the other group members (such as vibration produced, or indirectly, available sites on the prey), possibly producing in turn recruitment or departure of individuals [296].

Without *a priori* excluding the intervention of recruitment signals, it is possible to explain cooperation in social spiders during predation by referring to simple mechanisms based on stigmergy processes that favour silk. In *Anelosimus eximius*, spiders exhibit several types of behaviour when capturing prey. They throw sticky silk onto the victim to hinder it, followed by projections of dry silk to complete the immobilisation. Their bites paralyse the victim. For transportation towards their shelter, spiders fix threads to hoist the prey while cutting the threads that retard its progression [297]. By cutting these sequences of predation into four phases of equal duration, one finds that there is a sequential organization to the manifestation of these acts [298].

In phase 1, spiders project mainly sticky silk. Phase 2 is marked by projections of dry silk and biting. Bites become more important during phase 3 and transportation appears in phase 4 [298]. The partial recovery of these actions over time can be explained by the simultaneous intervention of several individuals. Despite appearances, there is not a rigid sequential organization of these various acts, nor the successive intervention of specialized individuals. Each spider is indeed able to demonstrate each of these acts if alone with the prey. However, within a group, the spider adjusts its behaviour to the situation encountered in terms of local information relevant to the state of the prey.

This independence of action, essential to the manifestation of stigmergy, allows the group to adjust its behaviour to the state of the prey. If we offer a killed prey to spiders and attract them using a set of short vibrations, they immediately manifest into the act of transportation. When applying a second set of vibrations (V2) spiders will project sticky silk and bite as in early capture. After stopping the vibrations spiders again engage in transportation.

The results of a theoretical model [298, 299] in which each spider would act independently of others and simply adjust its behaviour to the state of the prey resulting from previous actions, show a similar sequential organization of the different behaviours. As we know in solitary web-spinning spiders, predatory behaviour is mainly driven by the vibrations produced by prey. This adjustment could be a variation of probabilities that manifest a particular act in function according to the force with which the prey struggles. Indeed, during capture, the prey is exhausted and gradually moves less and less.

9. Conclusion: Silk Architecture and Its Factor in Socialization

The webs of spiders have caught the imagination. Interest at first centres upon the geometric webs, but then we also find that "irregular" webs obey specific architectural rules. It was then a simple step to imagine that the spider had a plan or instinct of how to build its web in its "head." The results mentioned above can, however, reject this hypothesis which is hardly compatible with the cognitive abilities of spiders. Processes of autostigmergy help explain the architecture of webs using simple stimulus/response reactions to local information without necessarily involving reference to a representation of the overall situation. The spider, guided by the configuration of the environment, sets up the first silky structure and thereafter takes its guidance for the construction process according to stigmergy. It is likely that the same is true for geometric webs, although responses to various stimuli are not all identified. These responses, certainly different from one species to another, could explain the differences in architecture. For example, slit sensilla on the legs of spiders are like biological strain gauges [300], and their architecture may suggest an optimal level of tension in the webs of spiders, which in turn would suggest to the spider when to fix or join its web. It is not the spider that "decided" to build a web that would obey certain architectural features, but the silky structure itself that controls the behaviour of the manufacturer according to rules governing its stimulus/response reactions. The silk itself is the architect in the construction of the web.

The first authors who studied nonterritorial social spiders stressed the three behavioural characteristics that distinguish them from solitary species, mutual tolerance, interattraction, and cooperation [49, 257, 262, 263]. This concept of sociality in spiders has been widely adopted since [47, 48, 213, 214]. Based on the observation of differences between the most exemplary solitary and social species, without taking into account the ethological mechanisms behind these features, a disadvantage was the apparent widening of the gap between these two modes of life, and suggestions that the transition from solitary to social required the development of several behavioural innovations. However, despite the scarcity of social species, this transition has occurred in independent lineages at least thirteen times [47, 290], making this hypothesis implausible.

The existence of several species showing maternal behaviour more or less developed in families that also contain social species, such as Erésides [52, 209, 301, 302], Théridionides [223, 262], the Dictynides [213, 272], and Agélénides [227, 303], suggests a gradual evolution of sociality from the family group [215, 284, 304]. However, there are genera such as *Agelena* for example, that contain social species (*Agelena consociata* and *Agelena republicana*) but no maternal species. Conversely, many genera contain maternal species but no

social species. We can, therefore, assume abrupt transitions, which can only be explained if socialization requires only minor ethological modifications.

The ethological study of the mechanisms that govern the functioning of juvenile groups and parent/juvenile groups can answer these questions [219, 226, 305]. The young of all species of spiders stay grouped for a few days after emergence from the egg sac. They often weave a common three-dimensional web. The construction of this structure has never been studied, even though it is the result of cooperation between individuals. After their emergence from the egg sac, young spiders therefore have the potential required to cooperate in building a common network. Only after their dispersal do the young orb-weaving spiders build their individual geometric webs. The mere fact that they remain together also implies the involvement of a mechanism for group cohesion. Finally, perfectly capable of eating prey they are offered, cannibalism is very rare. The three characteristics of social spiders are present at birth, so fleeting, in all spiders, but disappear with the dispersal of young.

The disappearance of ethological mechanisms responsible for tolerance, group cohesion and cooperation is not strictly programmed in all species. Juvenile *Coelotes terrestris* (Amaurobiidae) normally stay in their mother's web for a month and then disperse to weave individual irregular webs. During this period, they are fed by the mother and can even devour their own mother if intake is insufficient [227, 303]. The interattraction and tolerance between juveniles still persists if we remove them experimentally from the nest of the mother [306]. Siblings of *Amaurobius ferox* will remain together for several weeks in the natal nest after the death of the mother [307]. The factor responsible for the cohesion of the group was not identified. However, we can imagine that this is an attraction for silk. In *Coelotes terrestris*, the gregarious phase can be experimentally extended to several weeks if the mother is endowed with abundant prey. The young grow faster but disperse later, indicating that the disappearance of the tendency to group and tolerate congeners is not related to a genetically programmed age [220]. If we prevent the dispersal of juveniles by keeping them in an enclosed space and providing abundant food, they grow to adulthood, reproduce, and build a structure very similar to the common silky webs of the social spider *Agelena consociata*, thus forming an artificial society [218]. They appear to develop cooperation in construction. Cannibalism is extremely limited, giving evidence to the persistence of tolerance, and sometimes two or three individuals even participate in capturing prey. The plasticity of tolerance opened the path to socialization. Presumably, under favourable ecological conditions and an abundance of prey there was persistence of tolerance and the emergence of social structures and behaviours.

There is a point to remember however. All species that exhibit maternal behaviour belong to groups that spin irregular webs, or at least build a silky shelter. The only exception is *Lycosides vagabondes* which carries its young on its back for several days without feeding them. No cases of maternal behaviour have been reported in orb-weaving spiders. We can, therefore, consider that the presence of a nongeometric web was essential to the emergence of maternal behaviour. All social spiders belong to species that spin webs or at least settle in a common silky shelter. Silk seems to be a key factor in the socialization of spiders.

The comparison between social orb-weaving spiders that are territorial and nonterritorial species also deserves consideration. The only form of cooperation in territorial social spiders, apart from occasional cooperation in the capture of large prey by *Eriophora bistriata* [236], is the building of a common framework for fixing the threads of their individual geometric webs. That is to say that the part built in common corresponds to an irregular network, and does not correspond to the geometric webs that are then built on a strictly individual basis. Moreover, the only orb-weaving spider that displays cooperation in the construction of webs and the capture of prey is a species from the genus *Philoponella* that weaves irregularly shaped webs [287]. We can therefore consider that the architecture of the geometric webs of orb-weaving spiders has been an obstacle to perfect socialization, both in regards to web construction and collective predation.

But the persistence of tolerance and cohesion in a group does not explain the coordination of individual activities or cooperation. Should there be specific coordination mechanisms as in social insects? The results concerning cooperation in construction and collective prey capture suggests not. The silken web directly provides spiders with the information they need. The structures in place drive the building behaviour of individuals, and information transmitted by vibrations in threads allows the coordination of their predatory activities. Cooperation is therefore a product that emerges from the operation of a common web and requires no modification to the ethological programming of solitary spiders. Even in solitary spiders there is an attraction for silk from congeners that would potentially allow group cohesion and also cooperative social organization if tolerance between individuals persisted. As no specific form of communication is fundamentally necessary, even spiders that ignore each other but exploit a communal silky structure could in effect cooperate. It is therefore possible to imagine an abrupt change of status from solitary to social living among spiders [308]. This suggests that silk is the main determinant of cooperation in social spiders and thus their socialization, as may also be true for caterpillars, mites, and social Embioptera. Some authors have argued the contrary based on individual cases such as *Diaea socialis* [211] and *Delena cancerides* [212]. But these spiders do not use webs when hunting, live in a communal retirement, and capture prey individually outside, which only confirms the importance of a common irregular web to elicit social cooperation.

Unlike social insects that have developed communication signals that are more or less complex and different to those of solitary insects, spiders are merely exploiting the social aspects of a pre-existing material found in solitary species, silk. Ants became social well before "learning" to use the silk of their larvae, so developed a complex chemical language, while spiders have become social because of their use of silk. "If one can consider that "the road to insect sociality is paved with pheromones" [309], one must grant that the society of the spider hangs by a thread" [119].

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Research Article

A Population Genetic Model of Evolution of Host-Mate Attraction and Nonhost Repulsion in a Bark Beetle *Pityogenes bidentatus*

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Studies have shown that the bark beetle *Pityogenes bidentatus* (Coleoptera, Curculionidae, Scolytinae) avoids volatiles of nonhost trees (Norway Spruce, birch, and oak) and healthy host Scotch Pine when orienting to aggregation pheromone. A population genetic model of two behavioral genes was hypothesized where AA, Aa, and aa were allele combinations regulating orientation to host tree and pheromone odors, and BB, Bb, and bb were combinations allowing avoidance of nonhost and unsuitable host odors. The nine possible genotypes were assigned different survival factors that remained constant during simulation. The initial proportion of aabb genotype (little aggregation/host response and little avoidance of nonhosts) was ~1.0 when a mutation was hypothesized that caused better orientation to host/beetle odors (Aabb) and another mutation causing more efficient avoidance of nonhosts (aaBb). After these initial mutations, the model used indiscriminate mating of genotypic proportions and subsequent survival as input for each successive generation. The results indicate that AABB eventually fixates in the populations in some scenarios, while AABB and other genotypes reach stable equilibriums in other models depending on genotypic survival values supported by ecologically sound assumptions. The models indicate how development of insecticide resistance in pest insects may proceed.

1. Introduction

Individuals of *Pityogenes bidentatus* (Herbst) (Coleoptera, Scolytinae) are fairly small (2–3 mm long) bark beetles that only colonize Scotch Pine, *Pinus sylvestris* L. during a yearly mating flight that occurs in April-May depending on the latitude [1]. These beetles are common in mixed deciduous and conifer forests of northern Europe where they prefer to feed on smaller diameter trunks and limbs of weakened hosts [1]. By 1989, the beetle had become established in the northeastern United States [2]. The males in flight appear to find weakened hosts by a combination of attraction to aggregation pheromone, (*S*)-*cis*-verbenol and grandisol [3–7], and by avoiding odors from nonhost deciduous trees [such as birch, *Betula pendula* Roth; Rowan (mountain ash), *Sorbus aucuparia* L.; English oak, *Quercus robur* L.; alder buckthorn (glossy buckthorn), *Frangula alnus* Mill.], and conifers Norway Spruce, *Picea abies* L., and fresh/healthy host Scotch Pine [5, 6]. In these studies, aggregation components, (*S*)-*cis*-verbenol and grandisol, were placed inside each of a pair of barrier traps separated 6 m apart at 1.2 m height and revolved slowly at 2 rph to even out any trap position effects [5–8]. One of the traps in the pair also had an inhibitory source, either monoterpenes (1 mg/h) or other synthetic plant volatiles, or a fine screen cage containing freshly cut bark chips or twigs with leaves/needles (80 to 200 g) of hosts or nonhosts.

Odors from unsuitable hosts and nonhosts have not been tested alone without aggregation pheromone, so it is not certain that the plant odors can act alone during the beetle's dispersal and search for hosts. However, it was observed that the plant volatiles did repel the beetles in flight as they approached to within 1 m from a source of aggregation pheromone [5]. Many individual monoterpenes and blends released at rates comparable to that released from physical wounds of trees also inhibited attraction to their aggregation pheromone [5–7]. Earlier, a body of evidence had accumulated that attraction responses of conifer-infesting bark beetles in several genera are reduced by volatiles from nonhost angiosperm trees (e.g., *Betula*, *Populus*, *Acer*) [9–20]. Conifers such as pines and spruce usually produce resin, consisting of about 80% of mildly toxic monoterpenes, in order to

defend against the penetrations of the attacking bark beetles [21, 22].

Once a male finds suitable host pine bark, he releases an aggregation pheromone that probably assists most individuals in finding suitable host and breeding habitat [23, 24]. The avoidance of nonhost volatiles may aid the pioneer males in finding suitable hosts during extensive searches as well as aid individuals while landing on colonized bark to avoid nearby nonhosts. On the other hand, little or nothing is known about the behavioral responses needed to select the appropriate host substrate, but it can be hypothesized that there is some attraction to host volatiles that might occur at close range after landing. Interestingly, host pine monoterpenes were only repellent to *P. bidentatus* during flight when responding to aggregation pheromone [5–7] and not when walking (Byers unpublished). Bolts cut from standing Scotch Pine placed in the forest were not colonized by *P. bidentatus* for several weeks during the same time that the beetles were caught in the hundreds on pheromone-baited traps (personal observations). However, several weeks later these bolts became infested, suggesting either a random landing after avoiding nonhosts or a weak attraction to fermenting host volatiles. Various monoterpene blends could indicate to arriving beetles that the trunk was the appropriate host since different tree species have different sets of monoterpenes [25, 26]. In a few cases, bark beetles in the genus *Tomicus* are significantly attracted to Scotch Pine and to its monoterpenes, especially α-pinene (both enantiomers), 3-carene, and terpinolene [27–30]. Several studies have found that certain monoterpenes enhance the attraction to pheromone components in some of the more "aggressive" bark beetles that kill standing trees [31–34].

The objective was to construct a population genetic computer model of evolution with selection of hypothetical genotypes of *P. bidentatus* with two genes each with two alleles, one gene for attraction to host/beetle semiochemicals (A and a) and the second for repulsion by nonhost semiochemicals (B and b). This means that there would be nine possible genotypes conferring special survival or reproductive benefits for each genotype that remained constant throughout the simulation of a specified number of generations. Throughout the population and in every generation, mating was assumed indiscriminate and proportional to each genotype currently present [35, 36]. The nine-by-nine pairings of genotypes gives 81 possible pairings resulting in certain proportions of the nine genotypes, each generation based on the preceding population's proportions of each genotype. The initial proportion of aabb (little or no repulsion by nonhosts and little attraction to hosts) was the prevailing genotype except that one individual would have a mutation of a $\rightarrow$ A, and a second individual would have a mutation of b $\rightarrow$ B to begin the simulations. At each generation the proportions of each genotype were calculated and used as input for the next generation. Survival factors were set initially for the nine genotypes based on logical assumptions. For example, genotype aabb would have a low survival compared to AABB since the latter's individuals would avoid toxic nonhosts (BB) and be attracted to hosts (AA); heterozygous (Aa or Bb) would be intermediate in survival. The results of the models following an evolutionary mutational event would reveal the dramatic to gradual genotypic changes that might be expected during a number of generations resulting in gene fixation or gene equilibrium depending on the survival benefits of the mutated alleles. The same processes illustrated by the models help in understanding the population dynamics of pest insects that overcome crop plant resistance or develop resistance to insecticides [36–38].

2. Materials and Methods

A genetic model of evolution with two alleles, A and a, for attraction to hosts and two alleles, B and b, for repulsion from nonhosts was developed. This two-gene model has nine possible combinations of alleles AABB, AABb, AAbb, AaBB, AaBb, Aabb, aaBB, aaBb, and aabb that can be found in male and female beetles. Mating proceeds according to the proportion of each genotype (pan mixing) giving 81 possible pairings as shown in Table 1. However, the genotype offspring in the table's boxes in the lower left of the diagonal line of the outlined boxes are replicated in the upper diagonal half. Thus, the number of unique pairings is reduced to $9 + 32/2 = 45$ as shown in Algorithm 1. This algorithm takes the proportion (P_1 to P_9) of each of the male and female mated genotypes (progeny are equally female and male) and multiplies it by the indicated proportions (1, 2, 4, 8, or 16) times 2 for those not in the diagonal line of the boxes or as indicated if in the diagonal line. Each such value is multiplied by the survival factor (S_1 to S_9) for the appropriate genotype. For example, reasonable survival factors that are relative to each other might be $S_1 = 1$ for AABB, $S_2 = 0.9$ for AABb, $S_3 = 0.5$ for AAbb, $S_4 = 0.8$ for AaBb, $S_5 = 0.6$ for AaBb, $S_6 = 0.3$ for Aabb, $S_7 = 0.4$ for aaBB, $S_8 = 0.3$ for aaBb, and $S_9 = 0.2$ for aabb. These survival factors can just as well be any values as long as they are relative in magnitude (e.g., 10, 9, 5, 8, 6, 3, 4, 3, and 2) since the sums of all multiplications for each of the nine genotypes (G_1 to G_9) are then expressed as a proportion of the total sum of the nine genotypes (P_1 to P_9) according to the following:

$$P_1 = \frac{G_1}{\sum_{k=1}^{9} G_k}. \quad (1)$$

The updated P_1 to P_9 values then serve as the mating proportions of the genotypes for the next generation, iterating until the last generation is attained to obtain the ending genotypic frequencies.

The initial population number based on the initial frequencies would be 10^7. The population would have an initial proportion of almost all aabb ($P_9 = 0.9999998$), except one individual would mutate to Aabb ($P_6 = 0.0000001$) and another would mutate to aaBb ($P_8 = 0.0000001$), and then the model would proceed as described above for at least 100 generations. The model can accommodate any population size by adjusting the initial proportions of the genotypes. The survival factor of each genotype determines the ultimate proportion of each genotype, and as such the possibilities appear unlimited. However, the relative survival of the nine genotypes is constrained as will be evident in four examples

Table 1: Nine genotypes of each sex and the 81 possible crossings and their proportions.

	AABB	AABb	AAbb	AaBB	AaBb	Aabb	aaBB	aaBb	aabb
AABB[1]	**16AABB**	8AABB 8AABb	16AABb	8AABB 8AaBB	4AABB 4AABb 4AaBB 4AaBb	8AABb 8AaBb	16AaBB	8AaBB 8AaBb	16AaBb
AABb	8AABB 8AABb	**4AABB** **8AABb** **4AAbb**	8AABb 8AAbb	4AABB 4AABb 4AaBB 4AaBb	2AABB 4AABb 2AAbb 2AaBB 4AaBb 2Aabb	4AABb 4AAbb 4AaBb 4Aabb	8AaBB 8AaBb	4AaBB 8AaBb 4Aabb	8AaBb 8Aabb
Aabb	16AABb	8AABb 8AAbb	**16AAbb**	8AABb 8AaBb	4AABb 4AAbb 4AaBb 4Aabb	8AAbb 8Aabb	16AaBb	8AaBb 8Aabb	16Aabb
AaBB	8AABB 8AaBB	4AABB 4AABb 4AaBB 4AaBb	8AABb 8AaBb	**4AABB** **8AaBB** **4aaBB**	2AABB 4AaBB 4AaBb 2aaBB 2aaBb	4AABb 8AaBb 4aaBb	8AaBB 8aaBB	4AaBB 4AaBb 4aaBB 4aaBb	8AaBb 8aaBb
AaBb	4AABB 4AABb 4AaBB 4AaBb	2AABB 4AABb 2AAbb 2AaBB 4AaBb 2Aabb	4AABb 4AAbb 4AaBb 4Aabb	2AABB 2AABb 4AaBB 4AaBb 2aaBB 2aaBb	**1AABB** **2AABb** **1AAbb** **2AaBB** **4AaBb** **2Aabb** **1aaBB** **2aaBb** **1aabb**	2AABb 2AAbb 4AaBb 4Aabb 2aaBb 2aabb	4AaBB 4AaBb 4aaBB 4aaBb	2AaBB 4AaBb 2Aabb 2aaBB 4aaBb 2aabb	4AaBb 4Aabb 4aaBb 4aabb
Aabb	8AABb 8AaBb	4AABb 4AAbb 4AaBb 4Aabb	8AAbb 8Aabb	4AABb 8AaBb 4aaBb	2AABb 2AAbb 4AaBb 4Aabb 2aaBb 2aabb	**4AAbb** **8Aabb** **4aabb**	8AaBb 8aaBb	4AaBb 4Aabb 4aaBb 4aabb	8Aabb 8aabb
aaBB	16AaBB	8AaBB 8AaBb	16AaBb	8AaBB 8aaBB	4AaBB 4AaBb 4aaBB 4aaBb	8AaBb 8aaBb	**16aaBB**	8aaBB 8aaBb	16aaBb
aaBb	8AaBB 8AaBb	4AaBB 8AaBb 4Aabb	8AaBb 8Aabb	4AaBB 4AaBb 4aaBB 4aaBb	2AaBB 4AaBb 2Aabb 2aaBB 4aaBb 2aabb	4AaBb 4Aabb 4aaBb 4aabb	8aaBB 8aaBb	**4aaBB** **8aaBb** **4aabb**	8aaBb 8aabb
aabb	16AaBb	8AaBb 8Aabb	16Aabb	8AaBb 8aaBb	4AaBb 4Aabb 4aaBb 4aabb	8Aabb 8aabb	16aaBb	8aaBb 8aabb	**16aabb**

explored here. In the first, it is hypothesized that AABB survives best ($S_1 = 1$) since AA confers a strong attraction to the host, while BB allows the beetle to avoid feeding in the nonhost that would kill the individual. AABb survives well ($S_2 = 0.8$) for the same reasons although Bb, being intermediate, causes some attacks on nonhosts and mortality. AAbb has considerably lower survival ($S_3 = 0.5$) due to bb causing nonhost feeding and mortality, but it does allow many AA to find hosts. AaBB ($S_4 = 0.9$) can be given higher survival than AABb because individuals of the former avoid nonhosts that is slightly more important than a specific attraction to hosts. Heterozygous AaBb ($S_5 = 0.6$) has intermediate survival, while Aabb ($S_6 = 0.3$) and aaBb ($S_8 = 0.3$) are of equally low survival. The aaBB ($S_7 = 0.4$) has slightly more survival due

AABB: $P_1 = 0$: $S_1 = 1$: AABb: $P_2 = 0$: $S_2 = 0.8$: AAbb: $P_3 = 0$: $S_3 = 0.5$
AaBB: $P_4 = 0$: $S_4 = 0.9$: AaBb: $P_5 = 0$: $S_5 = 0.6$: Aabb: $P_6 = 0.0000001$: $S_6 = 0.3$
aaBB: $P_7 = 0$: $S_7 = 0.4$: aaBb: $P_8 = 0.0000001$: $S_8 = 0.3$: aabb: $P_9 = 0.9999998$: $S_9 = 0.2$

For generation = 1 to 100
Row 1: AABB: $G_1=16*P_1*P_1+16*P_1*P_2+16*P_1*P_4+8*P_1*P_5$
AABb: $G_2=16*P_1*P_2+32*P_1*P_3+8*P_1*P_5+16*P_1*P_6$
AaBB: $G_4=16*P_1*P_4+8*P_1*P_5+32*P_1*P_7+16*P_1*P_8$
AaBb: $G_5=8*P_1*P_5+16*P_1*P_6+16*P_1*P_8+32*P_1*P_9$
Row 2: AABB: $G_1=G_1+4*P_2*P_2+8*P_2*P_4+4*P_2*P_5$
AABb: $G_2=G_2+8*P_2*P_2+16*P_2^*P_3+8*P_2*P_4+8*P_2*P_5+8*P_2*P_6$
AAbb: $G_3=4*P_2*P_2+16*P_2*P_3+4*P_2*P_5+8*P_2*P_6$
AaBB: $G_4=G_4+8*P_2*P_4+4*P_2*P_5+16*P_2*P_7+8*P_2*P_8$
AaBb: $G_5=G_5+8*P_2*P_4+8*P_2*P_5+8*P_2*P_6+16*P_2*P_7+16*P_2*P_8+16*P_2*P_9$
Aabb: $G_6=4*P_2*P_5+8*P_2*P_6+8*P_2*P_8+16*P_2*P_9$
Row 3: AAbb: $G_3=G_3+16*P_3*P_3+8*P_3^*P_5+16*P3*P_6$
AABb: $G_2=G_2+16*P_3*P_4+8*P_3*P_5$
AaBb: $G_5=G_5+16*P_3*P_4+8*P_3*P_5+32*P_3*P_7+16*P_3*P_8$
Aabb: $G_6=G_6+8*P_3*P_5+16*P_3*P_6+16*P_3*P_8+32*P_3*P_9$
Row 4: AABB: $G_1=G_1+4*P_4*P_4+4*P_4*P_5$
AaBB: $G_4=G_4+8*P_4*P_4+8*P_4*P_5+16*P_4*P_7+8*P_4*P_8$
aaBB: $G_7=4*P_4*P_4+4*P_4*P_5+16*P_4*P_7+8*P_4*P_8$
AABb: $G_2=G_2+4*P_4*P_5+8*P_4*P_6$
AaBb: $G_5=G_5+8*P_4*P_5+16*P_4*P_6+8*P_4*P_8+16*P_4*P_9$
aaBb: $G_8=4*P_4*P_5+8*P_4*P_6+8*P_4*P_8+16*P_4*P_9$
Row 5: AABB: $G_1=G_1+P_5*P_5$: AABb: $G_2=G_2+2*P_5*P_5+4*P_5*P_6$
AAbb: $G_3=G_3+P_5*P_5+4*P_5*P_6$: AaBB: $G_4=G_4+2*P_5*P_5+8*P_5*P_7+4*P_5*P_8$
AaBb: $G_5=G_5+4*P_5*P_5+8*P_5*P_6+8*P_5*P_7+8*P_5*P_8+8*P_5*P_9$
Aabb: $G_6=G_6+2*P_5*P_5+8*P_5*P_6+4*P_5*P_8+8*P_5*P_9$
aaBB: $G_7=G_7+P_5*P_5+8*P_5*P_7+4*P_5*P_8$
aaBb: $G_8=G_8+2*P_5*P_5+4*P_5*P_6+8*P_5*P_7+8*P_5*P_8+8*P_5*P_9$
aabb: $G_9=P_5*P_5+4*P_5*P_6+4*P_5*P_8+8*P_5*P_9$
Row 6: AAbb: $G_3=G_3+4*P_6*P_6$: Aabb: $G_6=G_6+8*P_6*P_6+8*P_6*P_8+16*P_6*P_9$
aabb: $G_9=G_9+4*P_6*P_6+8*P_6*P_8+16*P_6*P_9$
AaBb: $G_5=G_5+16*P_6*P_7+8*P_6*P_8$ aaBb: $G_8=G_8+16*P_6*P_7+8*P_6*P_8$
Row 7: aaBB: $G_7=G_7+16*P_7*P_7+16*P_7*P_8$: aaBb: $G_8=G_8+16*P_7*P_8+32*P_7*P_9$
Row 8: aaBB: $G_7=G_7+4*P_8*P_8$: aaBb: $G_8=G_8+8*P_8*P_8+16*P_8*P_9$
aabb: $G_9=G_9+4*P_8*P_8+16*P_8*P_9$
Row 9: aabb: $G_9=G_9+16*P_9*P_9$

total = $G_1*S_1+G_2*S_2+G_3*S_3+G_4*S_4+G_5*S_5+G_6*S_6+G_7*S_7+G_8*S_8+G_9*S_9$
$P_1 = G_1*S_1$/total: $P_2 = G_2*S_2$/total: $P_3 = G_3*S_3$/total: $P_4=G_4*S_4$/total
$P_5 = G_5*S_5$/total: $P_6 = G_6*S_6$/total: $P_7 = G_7*S_7$/total: $P_8 = G_8*S_7$/total: $P_9 = G_9*S_9$/total
Next generation

Algorithm 1: General code for algorithms to calculate the proportions P_1 to P_9 of the nine mated genotypes (AABB to aabb, see Table 1) for 200 generations based on initial proportions and nine constant survival factors S_1 to S_9 for individuals of these genotypes (an asterisk denotes multiplication).

to the importance of avoiding toxic nonhosts, while the genotype with the least survival would be aabb ($S_9 = 0.2$). Thus, the order of survival was $S_1 > S_4 > S_2 > S_5 > S_3 > S_7 > S_6 = S_8 > S_9$ (Table 2).

In the second example, the initial proportions were the same, and the survival factors were similar: S_1 (1) > S_4 (0.8) > S_7 (0.7) > S_2 (0.6) > S_5 (0.5) > S_8 (0.4) > S_3 (0.3) > S_6 (0.2) > S_9 (0.1). However, S_7 had significantly higher survival, as did S_8, than in the first example (Table 2). The justification was that the BB of aaBB would allow avoidance of toxic nonhosts better than Bb of AABb, and this advantage outweighs the benefits of a better attraction to hosts (AA versus aa). In the third example, the survival factors were S_2 (1) > S_1 (0.9) > S_5 (0.8) > S_4 (0.7) > S_8 (0.5) > S_7 (0.4) = S_3 (0.4) > S_6 (0.3) > S_9 (0.2). The rationale for this order was that the BB gene caused these bark beetles to be somewhat repelled from forests with nonhost trees [39] so these beetles found hosts less often than Bb, thus Bb > BB > bb and AA > Aa > aa in survival. In the fourth model (Table 2), the order was affected by the preceding rationale for BB, but in addition AA caused too much attraction and competition, while Aa allowed less attraction to crowded hosts and increased survival [40–43].

Table 2: Survival factors (S_1 to S_9) of the nine genotypes used in the five example models.

Example	S_1 AABB	S_2 AABb	S_3 AAbb	S_4 AaBB	S_5 AaBb	S_6 Aabb	S_7 aaBB	S_8 aaBb	S_9 aabb
1	1	0.8	0.5	0.9	0.6	0.3	0.4	0.3	0.2
2	1	0.6	0.3	0.8	0.5	0.2	0.7	0.4	0.1
3	0.9	1	0.4	0.7	0.8	0.3	0.4	0.5	0.2
4	0.8	0.9	0.4	0.4	1	0.5	0.1	0.5	0.2
5	1	0.98	0.95	0.99	0.96	0.93	0.94	0.93	0.92

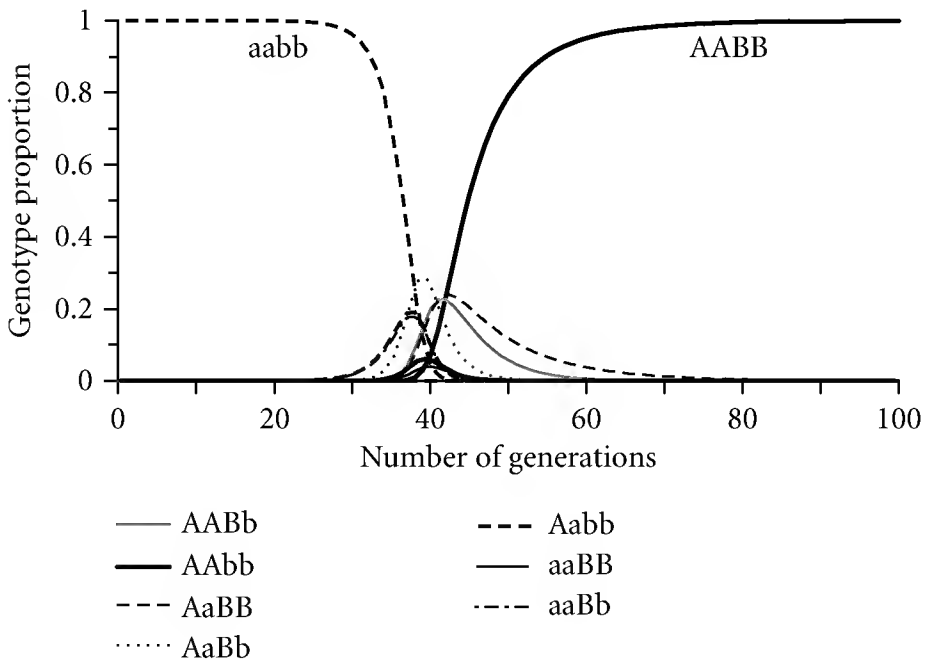

Figure 1: Change in proportions of the nine genotypes during 100 generations (example 1) with indiscriminate mating in which there were initially about 10^7 (0.9999998 aabb) individuals and initially one mutation of a to A (0.0000001 Aabb) and one of b to B (0.0000001 aaBb) resulting in 0.9994339 AABB after 100 generations. Relative survival of each genotype was $S_1 = 1$ (AABB), $S_2 = 0.8$ (AABb), $S_3 = 0.5$ (AAbb), $S_4 = 0.9$ (AaBB), $S_5 = 0.6$ (AaBb), $S_6 = 0.3$ (Aabb), $S_7 = 0.4$ (aaBB), $S_8 = 0.3$ (aaBb), and $S_9 = 0.2$ (aabb).

Thus, Aa > AA > aa, giving an order of S_5 (1) > S_2 (0.9) = S_4 (0.9) > S_1 (0.8) > S_8 (0.5) = S_6 (0.5) > S_3 (0.4) > S_9 (0.2) > S_7 (0.1).

The effect of smaller relative differences in the survival factors was tested in a fifth example where the first model's factors (Table 2) were altered S_1 (1) > S_4 (0.99) > S_2 (0.98) > S_5 (0.96) > S_3 (0.95) > S_7 (0.94) > S_6 (0.93) = S_8 (0.93) > S_9 (0.92), and the number of generations was noted at which P_1 (AABB) > 0.01 or P_9 (aabb) < 0.99. The model (Algorithm 1) was programmed in QuickBASIC 4.5 (Microsoft Corp., Redmond, WA, USA) with results graphed using PostScript 2.0 language (Adobe Systems Inc., San Jose, CA, USA). The model was also implemented in Java 6.0 code (Oracle, Redwood City, CA, USA) for general demonstration on the Internet with a web browser (http://www.chemical-ecology.net/java2/aabb.htm).

3. Results

The predominate initial genotype aabb, with no significant attraction to host volatiles and no avoidance of nonhost volatiles, appears stable for almost 30 generations before plummeting rapidly to near zero by generation 42 (Figure 1).

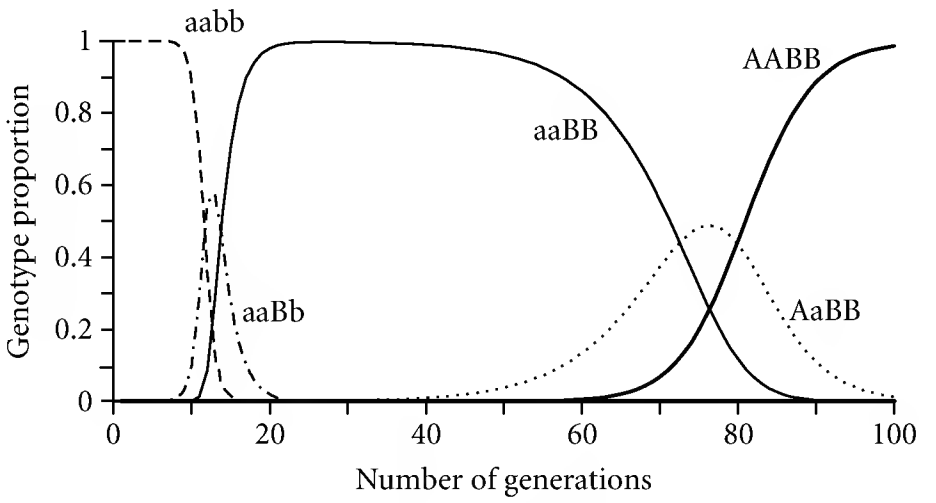

Figure 2: Change in proportions of the nine genotypes during 100 generations (example 2) with indiscriminate mating in which there were initially about 10^7 (0.9999998 aabb) individuals and initially one mutation of a to A (0.0000001 Aabb) and one of b to B (0.0000001 aaBb) resulting in 0.986431 AABB (four genotypes never achieved any significant proportion). Relative survival of each genotype was $S_1 = 1$ (AABB), $S_2 = 0.6$ (AABb), $S_3 = 0.3$ (AAbb), $S_4 = 0.8$ (AaBB), $S_5 = 0.5$ (AaBb), $S_6 = 0.2$ (Aabb), $S_7 = 0.7$ (aaBB), $S_8 = 0.4$ (aaBb), and $S_9 = 0.1$ (aabb).

Concomitantly, the dominant genotype AABB logistically grows to 1.0 from generations 38 to 60 and reaches 0.9994 by generation 100. The other seven genotypes rise and fall in approximate normal curves with some skews during generations 25 to 80 (Figure 1). It is apparent that AABB will fixate to 100% eventually.

In the second example, the initial aabb genotype also declines precipitously after near constancy for about 10 generations and approaches zero by generation 15 (Figure 2). However, AABB does not increase above zero for a considerable time until about generation 60 whereupon AABB rises logistically to 0.9864 by 100 generations. It is again clear that AABB fixates. For a number of generations aaBB rises after generation 10 and approaches fixation by generation 22 but then declines gradually until about generation 60 when the genotype then falls to zero (the same period when AABB increases). Only aaBb and AaBB genotypes rise and fall (as Gaussian-like curves) substantially during the fall of aabb and rise of AABB, respectively (Figure 2). The fixation occurs on a time scale that is similar to that found for pesticide resistance in insects and nematodes [37, 38, 44] and indicates that the survival factors chosen here are reasonable for strong selection.

In example 3 (Table 2), the initial aabb is again stable for about 12 generations and then falls when aaBb begins to increase (Figure 3). Other genotypes, aaBB, AaBb, and Aabb

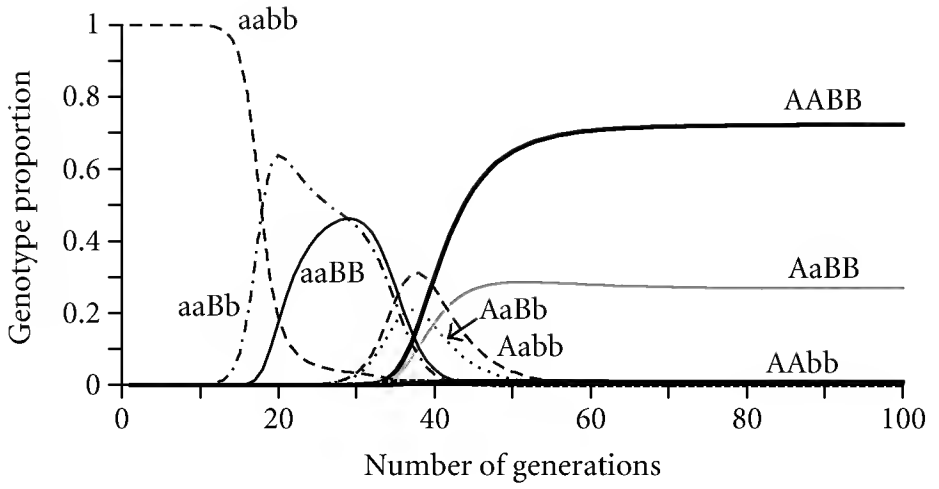

Figure 3: Change in proportions of the nine genotypes during 100 generations (example 3) with indiscriminate mating in which there were initially about 10^7 (0.9999998 aabb) individuals and initially one mutation of a to A (0.0000001 Aabb) and one of b to B (0.0000001 aaBb) resulting in 0.7230 AABB, 0.2681 AABb, and 0.09 AAbb (AABb never achieved any significant proportion). Relative survival of each genotype was $S_1 = 0.9$ (AABB), $S_2 = 1$ (AABb), $S_3 = 0.4$ (AAbb), $S_4 = 0.7$ (AaBB), $S_5 = 0.8$ (AaBb), $S_6 = 0.3$ (Aabb), $S_7 = 0.4$ (aaBB), $S_8 = 0.5$ (aaBb), and $S_9 = 0.2$ (aabb).

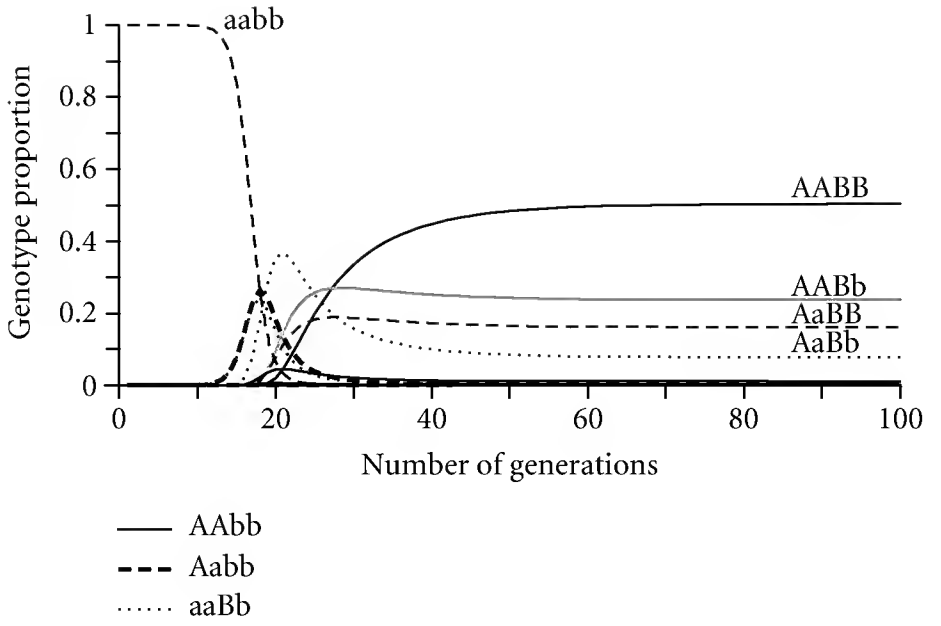

Figure 4: Change in proportions of the nine genotypes during 100 generations (example 4) with indiscriminate mating in which there were initially about 10^7 (0.9999998 aabb) individuals and initially one mutation of a to A (0.0000001 Aabb) and one of b to B (0.0000001 aaBb) resulting in 0.5037 AABB, 0.2376 AABb, and 0.1614 AaBB. Relative survival of each genotype was $S_1 = 0.8$ (AABB), $S_2 = 0.9$ (AABb), $S_3 = 0.4$ (AAbb), $S_4 = 0.9$ (AaBB), $S_5 = 1$ (AaBb), $S_6 = 0.5$ (Aabb), $S_7 = 0.1$ (aaBB), $S_8 = 0.5$ (aaBb), and $S_9 = 0.2$ (aabb).

rise, and fall during the rise of AABB and AaBB around generation 33. It is remarkable that AABB appears to reach equilibrium at 0.7230, as does AABb at 0.2681, accounting for most of the population's genotypes at 100 generations. AAbb reaches a low level of equilibrium at 0.0895 (9 percent). Running the model to 1000 generations did not appreciably change these results (AABB = 0.7232, AABb = 0.2679, and AAbb = 0.0893).

In example 4 (Table 2), the initial aabb genotype is stable until about generation 12 and falls rapidly to near 0 by generation 22 while three genotypes (Aabb, aaBb, and AAbb) rise after generation 10 and then decline around generation 20, reaching near 0 levels asymptotically (Figure 4). A few generations before 20, four genotypes rise, with AaBb falling gradually but then reaching a constant equilibrium of about 0.0779. The dominant genotype AABB appears to rise logarithmically to a stable equilibrium that was 0.5037 by generation 100. Similarly, AABb and AaBB rose and then fell slightly to stable equilibriums at 0.2374 and 0.1614, respectively (Figure 4). Running the model to 1000 generations did not change the results (AABB = 0.5039, AABb = 0.2373, AaBB = 0.1614, AaBb = 0.0779, AAbb = 0.0110). The other genotypes, Aabb, aaBB, aaBb, and aabb, also became stable but below 0.0050 proportion. In example 5 (Table 2), the survival parameters were compressed but related to example 1. In this case, the genotypic frequencies were identical but spread out over more generations (not shown). In example 1, the initial population of aabb began to decline significantly when the proportion fell below 0.99 on generation 27 while the AABB proportion began to significantly increase above 0.01 on generation 39. Using the compressed survival factors that caused less selection in example 5, aabb fell below 0.99 on generation 1001, and AABB rose above 0.01 on generation 1394.

4. Discussion

Bark beetles that are termed "aggressive" are among the tree-killing pest species thought to find trees in either of two ways. The first is through a nondirected flight and landing on trees at random whereupon the beetle, a male if in tribe Ipini (e.g., *Pityogenes* or *Ips*) or a female if in tribe Tomicini (e.g., *Dendroctonus* or *Tomicus*), must determine whether the tree is its host and probably whether the tree is acceptable [24, 45]. A beetle that lands on a tree and attempts to enter by boring through the outer bark is termed a "pioneer," especially if there are few others present. Pioneers were presumed to encounter significant host resistance and resin when attacking compared to later arrivals ("joiners") when the tree has succumbed [21, 22, 24, 46]. The hypothesis was that since pioneers must attack the tree and survive to produce pheromone before the rest of the population can exploit the resource, pioneers must be the largest and most vigorous of the population. Byers [24] questioned this paradigm since an individual would undertake a pioneer strategy only if no pheromone was encountered during the dispersal, or after leaving unsuitable colonization areas [41, 43], so that eventually its fat reserves became low [47]. In this "desperate" state, the beetle attempts to bore into any tree and may fortuitously find a tree of low resistance. Thus, smaller beetles that have suffered severe larval competition, or beetles regardless of size that have used up their fat reserves in flight, are hypothesized to be the pioneers.

The second way a beetle finds a host is by orienting to aggregation pheromone. It is evident from host finding models using EAR (effective attraction radius), representing trees and hosts under colonization, that the vast majority of beetles find hosts by orientation to aggregation pheromone [23]. This still means that many beetles perish as pioneers or

simply in the search for hosts; however, most find their host by means of aggregation pheromone. As mentioned earlier, some species in the genus *Tomicus* (e.g., *T. piniperda*) appear not to use a long-range aggregation pheromone but rely instead on volatile monoterpenes predominant in the hosts of their region (in Sweden: α-pinene, 3-carene, and terpinolene) [27–29]. *P. bidentatus* has a strong aggregation pheromone, and thus most individuals would seem to find hosts by the use of these semiochemicals. However, fresh hosts, even with synthetic aggregation pheromone, are repellent, while aged logs in the field may be attractive or located through a random landing process. Avoidance of nonhost volatiles has evolved in a number of bark beetles as stated earlier including *P. bidentatus*. All bark beetles colonize a thin layer (often only 2-3 mm) of cambium/phloem that causes both intraspecific and interspecific competition for food resources [48]. Thus, bark beetles have evolved avoidance of verbenone and aggregation pheromone in order to reduce competition [21, 22, 28, 29, 40, 43, 49].

The attraction to host odors, aggregation pheromone, and avoidance of volatiles indicative of crowding can be implemented in the survival factors of the hypothetical (A, a) gene. The avoidance of nonhost odors, both at the tree and forest stand level, and semiochemicals from unsuitable hosts was incorporated in the assumptions about the survival factors conferred by the (B, b) gene. The relative magnitudes of the survival factors used in the five examples were ecologically reasonable, but many other relative rankings are possible. The speed of evolution can be greatly affected based on the survival factors that represent selection pressures. In example 1 (Figure 1), 39 generations transpired before the first sign of an increase in AABB. It took until generation 45 for 50% of the populations to become AABB. In example 5 with the same order but less difference in relative survival factors, it took 1394 generations before AABB began to increase, and this genotype did not reach 50% until generation 1498 (about 104 generations to increase to 50%).

These results demonstrate that eventually there is a relatively rapid change in the genotype frequencies with fixation of the dominant alleles that are the most beneficial. This evolution is analogous to a mutation for resistance to an insecticide [36–38, 44, 50–52]. It supports why resistance may remain hidden phenotypically for many years (1 generation per year) before suddenly appearing to become widespread. Resistance to insecticides from a mutation that commonly shows up in several to tens of years would have survival factors similar to those used in the present study. According to example 5, resistance in a pest insect could remain hidden for hundreds of years before becoming established. In examples 3 and 4, in which the beneficial genes are heterozygous (Figures 3 and 4), it is evident that phenotypic changes can also take many generations before intermediate gene frequencies result that are stable thereafter. On the other hand, if the population already has an allele that is common that confers insecticide resistance, then there can be an immediate and rapid change favoring this gene, which fixates or reaches equilibriums as in the examples. The population genetic models show how only two loci with two alleles can result in complex genotypic frequencies. More genes are probably involved in both choosing a host and avoiding nonhosts in *P. bidentatus*, which makes the models, undoubtedly, exceedingly complex.

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Research Article

Host-Tree Monoterpenes and Biosynthesis of Aggregation Pheromones in the Bark Beetle *Ips paraconfusus*

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A paradigm developed in the 1970s that *Ips* bark beetles biosynthesize their aggregation pheromone components ipsenol and ipsdienol by hydroxylating myrcene, a host tree monoterpene. Similarly, host α-pinene was hydroxylated to a third pheromone component *cis*-verbenol. In 1990, however, we reported that amounts of ipsenol and ipsdienol produced by male *Ips paraconfusus* (Coleoptera: Scolytinae) feeding in five host pine species were nearly the same, even though no detectable myrcene precursor was detected in one of these pines (*Pinus sabiniana*). Subsequent research showed ipsenol and ipsdienol are also biosynthesized from smaller precursors such as acetate and mevalonate, and this *de novo* pathway is the major one, while host tree myrcene conversion by the beetle is the minor one. We report concentrations of myrcene, α-pinene and other major monoterpenes in five pine hosts (*Pinus ponderosa*, *P. lambertiana*, *P. jeffreyi*, *P. sabiniana*, and *P. contorta*) of *I. paraconfusus*. A scheme for biosynthesis of ipsdienol and ipsenol from myrcene and possible metabolites such as ipsenone is presented. Mass spectra and quantities of ipsenone are reported and its possible role in biosynthesis of aggregation pheromone. Coevolution of bark beetles and host trees is discussed in relation to pheromone biosynthesis, host plant selection/suitability, and plant resistance.

1. Introduction

The California five-spined engraver, *Ips paraconfusus* (Lanier) (Coleoptera: Scolytinae), is an important pest of young pine forests in California and Oregon. Struble and Hall [1] state that "all pine species within the range of this beetle are attacked", although the beetle occurs most frequently on ponderosa pine (*Pinus ponderosa* Laws) at elevations from 600 to 1,400 m in California. Due to the pest status of this insect, extensive studies have been conducted to elucidate the pheromone signal concerning the biosynthetic, behavioral, and ecological aspects [2–4].

The aggregation pheromone produced by males has been identified as a synergistic blend of three components, (*S*)-(−)-ipsenol, (*S*)-(+)-ipsdienol, and (4*S*)-*cis*-verbenol [5–7]. Ipsenol and ipsdienol are produced only in males when exposed to vapors of the host plant monoterpene, myrcene [8], and the quantitative relationships between precursor vapor concentration and pheromone products have been reported [9]. Hendry et al. [10] used D_2-labelled myrcene to demonstrate that it can be converted in the male to ipsenol and ipsdienol under vapor exposure conditions. Unexposed control males contained no pheromone components, nor did females, even when exposed to myrcene vapors [9]. Another host monoterpene, (−)-α-pinene, in the vapor phase is converted to *cis*-verbenol in both sexes [11], and the relationship between increasing (−)-α-pinene vapor concentration and increasing *cis*-verbenol production in both sexes has been quantified [12]. Based on the above studies and others, a paradigm was established that *I. paraconfusus*, and probably most other *Ips* species, use myrcene and α-pinene in their host tree as precursors to ipsenol and ipsdienol and to *cis*-verbenol, respectively.

However, this paradigm began to be questioned when Byers and Birgersson [13] reported that males of *I. paraconfusus* that had fed in five different host pine species produced almost identical amounts of the pheromone components ipsenol and ipsdienol, regardless of the concentration of

myrcene in the host species fed upon. In fact, gray pine, *Pinus sabiniana*, had so little myrcene that it could not be detected by gas chromatography and mass spectrometry (GC-MS). Thus, a beetle would need to eat at least eight times its weight in oleoresin in order to have any chance of obtaining the required amounts of myrcene [13]. This appears unrealistic since males were observed to ingest phloem alone. Therefore, coevolution of host myrcene and bark beetle pheromone production in regard to host selection and suitability appears unlikely. On the other hand, the conversion of host α-pinene to *cis*-verbenol appears to be the major pathway, and so in this case coevolution could occur. Here, we present a more complete analysis of the host pine monoterpenes in phloem and oleoresin from pines in 1985 presented in part in Byers and Birgersson [13], as well as additional data from 1986, and the mass spectra of ipsenone. We will discuss in more detail our previous findings in relation to knowledge about the biosynthesis of aggregation pheromone components in relation to behavior, physiology, and coevolution of host tree monoterpenes and bark beetle ecology.

2. Materials and Methods

Ips paraconfusus were reared from ponderosa pine (*P. ponderosa*) and introduced into five host species of pine: ponderosa, sugar (*P. lambertiana*), Jeffrey (*P. jeffreyi*), gray (*P. sabiniana*), and lodgepole (*P. contorta*) as reported earlier [13]. The latter species, however, is not listed as a primary host probably because it generally occurs at elevations above the range of *I. paraconfusus* [1]. Males were dissected from their nuptial chambers after five days, and the posterior two thirds of the alimentary canal was extracted in groups of eight in 150 μL diethyl ether with 10 ng heptyl acetate per μL as an internal standard. Three samples of phloem (dry weight of each about 22 $\pm$ 7 mg, $\pm$SD, $n = 15$) not affected by beetle galleries (not oxidized) from each of the infested pine species were each extracted in 250 μL diethyl ether with internal standard. Pheromone components and ipsenone in the hindgut extracts were identified and quantified by gas chromatography (GC) on a Hewlett-Packard model 5880 and by GC-MS on a Finnigan model 4021. GC analysis used a fused silica column (0.2 mm i.d. $\times$ 12.5 m) coated with SE-54 CL (General Electric, 1% vinyl-, 5% phenyl-, 94% methylpolysiloxane) on a temperature program of 60°C for 3 min, rising to 220°C at 5°C/min, and isothermal for 15 min. Nitrogen, 20 cm/s, was used as carrier gas. GC-MS used a column of fused silica (0.15 mm i.d. $\times$ 25 m, df = 0.3 μm) coated with Superox FA (Alltech, TPA-treated PEG, df = 0.3 μm) on a temperature program of 50°C for 4 min, rising to 200°C at 8°C/min and isothermal for 10 min and helium carrier gas at 35 cm/s. Synthetic chemical standards of ipsenol, ipsdienol, and *cis*-verbenol were obtained from Borregaard (Norway). Ipsenone was prepared by oxidation of ipsenol in Jones reagent [14].

The phloem extracts described above were analyzed by GC on the fused silica column of SE-54 above. GC-MS used the SE-54 column on a program of 50°C for 1 min, rising to 220°C at 5°C/min and isothermal for 10 min. Carrier gas was as described above. Synthetic monoterpenes used for reference spectra were obtained from Sigma-Aldrich. There was some question as to the species and chemical identification for Jeffrey and/or gray pine. This was because Jeffrey pine phloem contained large quantities of α-pinene and myrcene relative to some of the other pines while Jeffrey pine was expected to contain mostly n-heptane [15, 16]. Also, gray pine had virtually none of the monoterpene hydrocarbons. Therefore, phloem samples were collected Oct. 17, 1986, from four trees of each of the five species. Also one tree each of sugar pine and ponderosa pine were sampled in four cardinal directions to determine the variation in monoterpene hydrocarbon content between samples. Oleoresin was collected from each of the species except sugar pine in which resin flow was insufficient for collection. Chemical analyses were as described above.

3. Results

Extracts of the hindguts of the male *I. paraconfusus* that had fed on the five host pines contained only a few major components, with ipsenol and ipsdienol dominating (Figure 1). Ipsenone, the ketone of ipsenol, was observed (Figure 1) in *I. paraconfusus* males fed in ponderosa pine, sugar pine, Jeffrey pine, gray pine, and lodgepole pine at 161 $\pm$ 124 (ng/male $\pm$ SD), 115 $\pm$ 83, 111 $\pm$ 85, 86 $\pm$ 41, and 87 $\pm$ 36, respectively.

The quantities of ipsenol and ipsdienol in fed males in each of the pine species were reported previously [13]. The quantities of these two pheromone components were similar and not significantly different; while it appeared that males from Jeffrey and lodgepole pines had more *cis*-verbenol than those from the other species where it could not be detected [13]. Here, we report that correlations between ipsenol and ipsdienol were consistently high within host species (R^2 from 0.64 to 0.97), and an overall $R^2 = 0.85$ ($N = 25$) for all species. However, correlations between ipsenone and ipsenol ($R^2 = 0, N = 25$) or ipsdienol ($R^2 = 0.14, N = 25$) were low.

The monoterpene hydrocarbons, myrcene and α-pinene, in the infested logs, were found in the largest amounts in phloem of Jeffrey pine, with significant amounts in lodgepole pine, lower amounts in ponderosa and sugar pine, and undetectable levels in gray pine. These phloem samples showed a large variation (within tree) in monoterpene hydrocarbons (Table 1). Phloem samples from several trees of each of the five species taken in October 17, 1986, showed an even larger variation (between tree) in monoterpene hydrocarbons (Table 2), but the relative amounts were consistent with those of the previous year (Table 1). These results are in agreement with field observations of the phloem during the dissection of the logs where Jeffrey pine was observed to contain "many 1 mm diam. resin pockets", lodgepole as "resinous," gray as "not resinous," and sugar and ponderosa as "slightly resinous." The relative amounts of the major monoterpenes in oleoresin of four of the pine species (sugar pine oleoresin could not be obtained) were found in percentages similar to those for the phloem

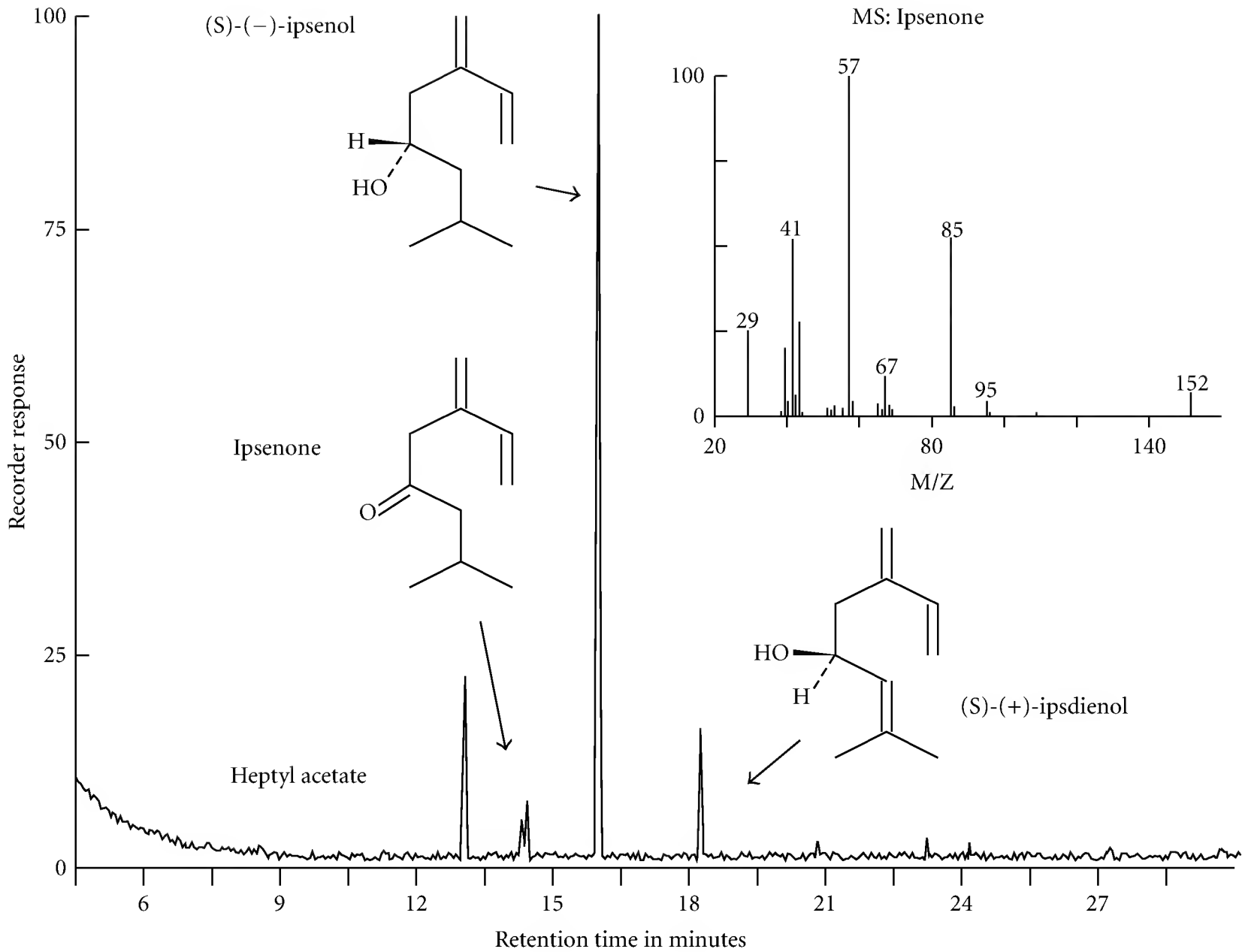

Figure 1: Gas chromatogram (Superox FA) of extract of hindguts of eight male *Ips paraconfusus* that had fed in Jeffrey pine. Heptyl acetate was used as an internal standard to quantify the pheromone components ipsenol and ipsdienol. Ipsenone, a related analog, eluted immediately before diacetone alcohol in a doublet peak. The mass spectrum of ipsenone is shown in the inset.

Table 1: Amounts of monoterpene hydrocarbons in phloem samples (15–25 mg dry weight) from five species of pine that were fed on by *Ips paraconfusus* (near Bass Lake, California, USA, September 3, 1985).

	Monoterpene hydrocarbons (μg) per g phloem (dry weight)				
Pine species	α-pinene mean ± SD (range)	β-pinene mean ± SD (range)	Myrcene mean ± SD (range)	3-Carene Mean ± SD (range)	Limonene mean ± SD (range)
Ponderosa	6.0 ± 9.0	1.1 ± 0.5	3.53 ± 2.69	21.7 ± 32.7	13.5 ± 21.0
$N = 3$[1]	(<1.3–16.4)	(<1.3–1.7)	(1.6–6.6)	(<1.3–59.3)	(<1.9–37.7)
Sugar	5.4 ± 2.2	<1.5	2.5 ± 0.1	<1.9	<1.9
$N = 3$	(3.8–7.9)	(<1.5)	(2.4–2.6)	(<1.9)	(<1.9)
Jeffrey	335.7 ± 160	116.9 ± 52.2	36.0 ± 18.7	246.3 ± 107	16.0 ± 7.4
$N = 3$	(152–445)	(56.6–147)	(15.8–52.6)	(129–338)	(7.5–20.8)
Gray	<1.0	<1.0	<1.0	<1.0	<1.0
$N = 3$	(<1.0)	(<1.0)	(<1.0)	(<1.0)	(<1.0)
Lodgepole	50.5 ± 22.5	8.5 ± 3.4	26.4 ± 11.0	18.6 ± 8.4	695.3 ± 297.8
$N = 3$	(35.0–76.3)	(6.0–12.4)	(18.6–39.0)	(12.5–28.2)	(479–1035)

[1] Number of samples from each tree.

(Tables 1–3). However, the percentage of the oleoresin that consisted of monoterpene hydrocarbons was much higher in ponderosa (83.5%) and lodgepole pine (89.9%) than in Jeffrey pine (2.9%); and gray pine oleoresin was only 0.08% monoterpene hydrocarbons (of those in Table 3).

4. Discussion

The similarity of chemical structure between the major host monoterpene, myrcene, and ipsenol and ipsdienol led Hughes [8] to propose that the tree's myrcene was a

Table 2: Amounts of monoterpene hydrocarbons in phloem samples (15–25 mg dry weight) from five species of pine (near Bass Lake, California, USA, 17 October 1986).

	Monoterpene hydrocarbons (μg) per g phloem (dry weight)				
Pine species	α-pinene mean ± SD (range)	β-pinene mean ± SD (range)	Myrcene mean ± SD (range)	3-Carene mean ± SD (range)	Limonene mean ± SD (range)
Ponderosa	1076 ± 1904	1179 ± 1921	428 ± 338	2747 ± 2483	2345 ± 3997
$N = 4$[1]	(<20–3930)	(<50–4060)	(<50–1015)	(237–6750)	(<50–8335)
Sugar	395 ± 378	180 ± 224	54 ± 55	121 ± 145	<15
$N = 4$	(97–1015)	(425–577)	(<15–131)	(<15–307)	(<15)
Jeffrey	1665 ± 1612	1216 ± 1692	272 ± 291	1924 ± 1563	4410 ± 3673
$N = 4$	(601–3520)	(124–3165)	(75–606)	(283–3395)	(1105–8365)
Gray	<20	<20	<20	<20	<20
$N = 4$	(<20)	(<20)	(<20)	(<20)	(<20)
Lodgepole	1400 ± 1811	3261 ± 4595	780 ± 1034	2413 ± 3096	14284 ± 14566
$N = 4$	(<25–4060)	(<25–10075)	(<25–2300)	(98–6965)	(1095–35000)

[1] Number of trees.

Table 3: Amounts of monoterpene hydrocarbons in oleoresin samples from four species of pine (near Bass Lake, California, USA, 17 October 1986).

	Monoterpene hydrocarbons (μg) per μL oleoresin					
Pine species	α-pinene	β-pinene	Myrcene	3-Carene	Limonene	Monoterpene Percent of Oleoresin
Ponderosa[1]	43.5 ± 6.4	102.6 ± 14.3	120.5 ± 19.6	498.5 ± 79.2	70.0 ± 12.7	83.5
Jeffrey	1.32	1.00	3.37	16.35	6.93	2.9
Gray	0.68	<0.06	<0.06	<0.06	<0.06	0.08
Lodgepole	43.2	39.7	23.7	69.5	723.0	89.9

[1] Four samples from cardinal directions of one tree, mean ± SD.

precursor of these pheromone components in *Ips*. Evidence for this theory was based on exposure of *Ips paraconfusus* males to myrcene vapor and the subsequent production of compounds with GC retention times identical to ipsenol and ipsdienol [8]. Byers et al. [9] confirmed the identifications using GC-MS and behavioral assays and reported a male-specific increasing relationship between precursor vapor concentration and pheromone products. Hendry et al. [10] labeled myrcene with deuterium and established the direct conversion of myrcene vapor to the pheromone components. Hughes [8] suggested that ipsdienol was directly converted to ipsenol since topical application of ipsdienol on males resulted in ipsenol production. Fish et al. [17] supported this by using deuterium-labeled ipsdienol (64% D) that was converted in males to labeled ipsenol (25% D). Some deuterium at carbon 4 was lost suggesting that an alternate pathway to ipsdienone (ketone at carbon 4) and back again to ipsdienol was occurring before conversion to ipsenol. However, *I. paraconfusus* contained no detectable ipsdienone, although it may occur in small proportions accounting for the loss of deuterium on the recovered ipsdienol (59% D). Fish et al. [17] showed that males could convert synthetic ipsdienone to ipsdienol, which was then converted to ipsenol.

In the present study, we did not find ipsdienone but instead ipsenone (also ketone at carbon 4) in feeding males (Figure 2) and this compound could explain the loss of deuterium in ipsenol (25% D) by a reversible pathway. Ipsenone can also explain the observed loss of deuterium in the recovered ipsdienol since it would be expected that a reversible pathway exists between ipsdienol and ipsenol. In fact, until ipsdienone is found in beetles naturally, it is more logical to assume that ipsenone rather than ipsdienone is involved in the deuterium loss observed earlier by Fish et al. [17]. Later work by Ivarsson et al. [18] found that when ^{3}H-ipsdienone was injected into males, radiolabel was incorporated into both ipsenol and ipsdienol, found mainly in the metathorax, while incubation of male tissues with ^{3}H-ipsdienone did not produce radiolabel in these components. *In vitro* incubation of tissues from *I. paraconfusus* with ^{14}C-acetate gave radiolabeled ipsenone/ipsdienone, but these were not chromatographically separated.

Hughes [8, 20] hypothesized that aggregating pheromones in *Dendroctonus* and *Ips* are "waste products from the metabolism of terpenes that have secondarily been utilized as chemical messengers." According to this hypothesis one would expect no differences between the *cis*-verbenol and ipsenol/ipsdienol production in regard to vapor exposure and feeding conditions or between the sexes—but great differences are evident [12, 21]. The *cis*-verbenol system appears to be a detoxification process in part, although males produce about twice as much *cis*-verbenol, and the ratios with other metabolites are different than in females [12]. The ipsenol/ipsdienol system has clearly evolved beyond that of a simple detoxification process

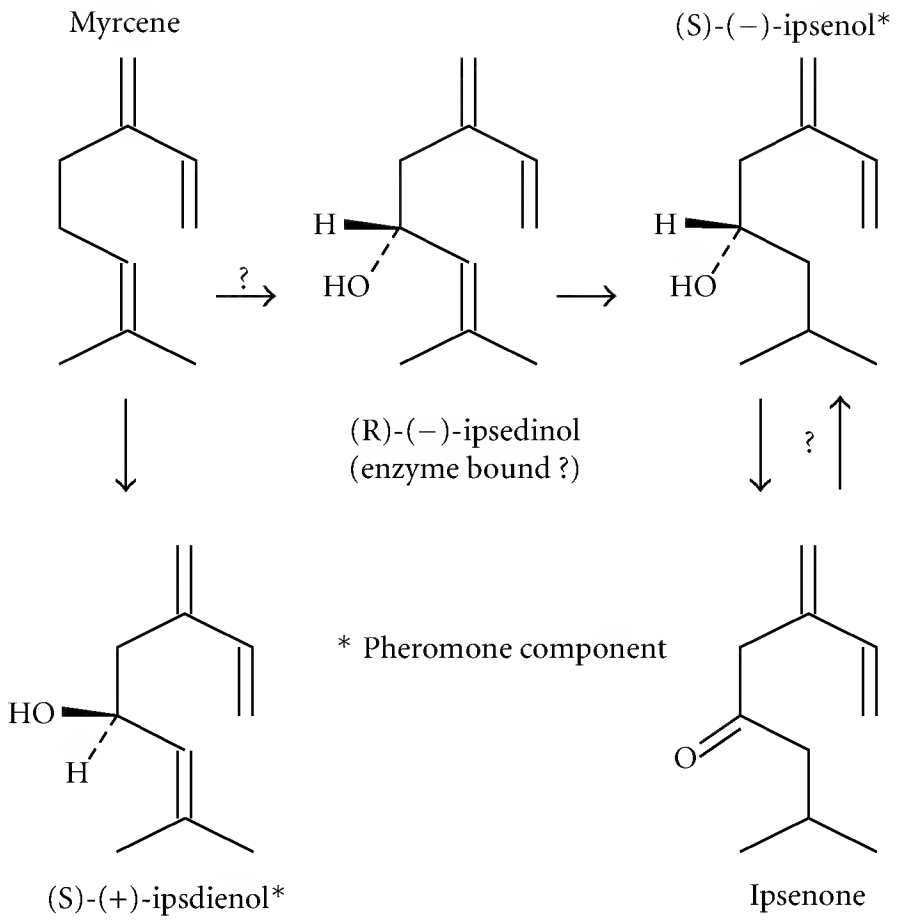

FIGURE 2: Proposed scheme for the conversion of the host tree compound, myrcene, to the pheromone components (*S*)-(−)-ipsenol and (*S*)-(+)-ipsdienol in *Ips paraconfusus* based on radiolabelling experiments and enantiomers found in the male [5, 7–12, 17, 19]. Conversion arrows with question marks have not been proven. (*R*)-(−)-ipsdienol does not accumulate in the hindgut but may occur as an enzyme-bound intermediate. However, contrary to the scheme, the amounts of ipsenone, (*S*)-(−)-ipsenol and (*S*)-(+)-ipsdienol in males were not correlated with myrcene titres in the host trees.

since this system (a) is sex-specific [9], (b) specifically influenced by juvenile hormone (JH) [22, 23], and (c) selectively inhibited by the antibiotic streptomycin [19]. The *cis*-verbenol system, on the other hand, is not affected by JH or streptomycin, and *cis*-verbenol is produced in both sexes, although females produced about half the amounts as males. Another difference between the ipsenol/ipsdienol and the *cis*-verbenol systems is that the male reduces his production of ipsenol and ipsdienol while feeding if he is joined by several females in his nuptial chamber [24]. The inhibition of pheromone production (and release) is physiological since males with females produced very little ipsenol and ipsdienol even when exposed to myrcene vapors, compared to males alone. In contrast, the production of *cis*-verbenol from α-pinene vapors in males was not affected by females [24].

Earlier work provided intriguing suggestions that symbiotic microorganisms may convert myrcene to pheromone components. Byers and Wood [19] fed males in a diet of powdered cellulose and ground phloem (22%) with and without streptomycin antibiotic. The males were removed from both diets and exposed to vapors of myrcene and α-pinene whereupon only those in diets without streptomycin-produced ipsenol and ipsdienol (there was no affect of antibiotic on *cis*-verbenol production). However, Conn et al. [25] reported that axenically reared *I. paraconfusus* can produce their aggregation pheromones "completely in the absence of the normal, extracellular complement of symbiotic microorganisms." Their data show that five axenic beetles produced half as much ipsenol as five feral (wild) beetles when feeding in logs and that axenic beetles without yeast as adults produced only about 10% the normal amounts. Hunt and Borden [26] repeated these tests and also found no significant statistical differences between axenic and control males, but again the axenic males produced only 36% as much ipsenol and ipsdienol. They also fed streptomycin to males and then introduced them into a ponderosa pine log. The ipsenol production in these males was reduced to only 2% indicating that both the feeding and aeration pathways are inhibited by the antibiotic [19, 26]. No further work has implicated microorganisms, but in any case, it seems that the ipsenol/ipsdienol system is peculiarly sensitive to streptomycin.

According to the paradigm when our experiments were conducted (1985-1986), catches of *I. paraconfusus* on five species of host pines infested with conspecific males should be correlated with quantities of aggregation pheromone components ipsenol, ipsdienol, and *cis*-verbenol that were converted directly from myrcene and α-pinene in the host trees. The attractions of *I. paraconfusus* to each of the five pine species of infested logs were similar except for an approximate doubling of catch on the Jeffrey pine log, as reported previously [13]. The sex ratios of catch (females per male) on four of the species were also similar (2.5 to 3.9) with more females than males, but the catch on Jeffrey pine was the most female biased (15.6) and this ratio was significantly different from the others [13]. The generally similar attraction to each of the pine species agrees with the similar amounts of the pheromone components, ipsenol and ipsdienol, found in the hindguts of the feeding males. However, there was no correlation between the widely varying amounts of myrcene in the host pines and the uniform amounts of ipsenol and ipsdienol in the males.

The increased catch on Jeffrey, and to a lesser extent on lodgepole, can be explained by the higher amounts of α-pinene in the phloem that was converted to the third pheromone component, *cis*-verbenol [13]. Detection of *cis*-verbenol in hindguts of feeding males is difficult [12], and Silverstein et al. [27] found that *cis*-verbenol occurred in quantities of only 2.5% the amount of ipsdienol in male frass. We found *cis*-verbenol to be 1% or 0.05% the amount of ipsdienol in hindguts of males feeding in Jeffrey and lodgepole pines, respectively. *cis*-Verbenol was presumably present in sufficient quantities in the males feeding in the other pines (although we could not quantify the amounts) as to be synergistically active with ipsenol and ipsdienol, since the latter two components have low activity without *cis*-verbenol in the field [28].

The content of ipsenol and ipsdienol in groups of eight males (within or between species) was rather consistent [13] with a total ($n = 25$) coefficient of variation (CV) of 26% for both ipsenol and ipsdienol. In comparison, the variations of the precursors α-pinene and myrcene in phloem were much larger (Tables 1–3), and the total CV for α-pinene was 185% and for myrcene 126%. Even within a tree the variation in α-pinene and myrcene in phloem could be large (ranges

in Table 1), which was probably the result of the rather small sample units (15–25 mg dry weight). Resin pockets are probably not evenly distributed in phloem so smaller samples would tend to vary more in the numbers of pockets. However, the sample unit was equivalent to about 80% of a nuptial chamber and thus indicates that beetles could ingest large differences between individuals in monoterpene hydrocarbons (calculation based on [12, 29]). The amounts of myrcene and α-pinene reported earlier [13] in the pine species as well as the other three major monoterpenes (Table 1) were considerably lower in phloem sampled in 1985 than they were in 1986 (Table 2). We are not sure why this was apparently the case unless the log's phloem had lost monoterpenes during the week-long behavioral tests in the field (1985) compared to immediate extraction of phloem cut from trees in 1986. Byers [12] showed that monoterpene vapors in male nuptial chambers in logs remain constant for about a week before declining rapidly in concentration.

Could males obtain enough myrcene in host phloem or oleoresin to account for the quantities of ipsenol and ipsdienol found in the hindguts? The male does not eat the entire contents of the nuptial chamber (fecal pellets appear to be a minor component of the frass), and it is doubtful that he selectively eats the "toxic" oleoresin [30–33]. The headspace concentration of myrcene in a nuptial chamber of ponderosa pine (2.8×10^{-8} g/mL) [12] is expected to account for only 1.6% at most of the ipsenol in feeding males (by linear interpolation between lowest value and 0, Figure 1 in Byers et al. [9], note: equations should be $Y = 2.72 + 1.05 \ln X$ and $Y = 0.62 + 0.26 \ln X$). Also, a feeding beetle must produce and release several times over the amounts found in hindguts at the end of the feeding period. The gut turnover rate (pheromone content of gut release per time) can be estimated from the airborne collection of components and gut contents. Studies with *D. brevicomis* [34, 35] can be used to calculate that females release *exo*-brevicomin at 16 gut contents per day at the peak of mass attack. *P. chalcographus* males release chalcogran at 18 gut contents per day [36, 37], and *I. typographus* males release 2-methyl-3-buten-2-ol at about 240 gut turnovers/day and *cis*-verbenol at 48 turnovers/day [38, 39].

Assuming conservatively that gut turnover rates above are just 10 per day, then based on the quantities of myrcene in ponderosa pine phloem (fresh weight is 3.87 $\times$ dry weight) [29] or oleoresin (Tables 1–3), a male would need to eat a minimum of from 99 to 413 nuptial chambers in the 1985 experiment (Table 1), or from 0.6 to 13 chambers in the 1986 samples (Table 2, 111 μL at 0.895 g/mL) to account for pheromone amounts [12]. However, only 0.14 μL oleoresin is needed (1986 samples) to produce the estimated amounts of ipsenol and ipsdienol released over two days. Thus, *I. paraconfusus* would need to eat some oleoresin to account for pheromone production based on the myrcene precursor theory, as suggested earlier [12]. However, assuming amounts of myrcene in gray pine oleoresin of at most 0.06 μg/μL (our quantification limit), then at least 280 μL of oleoresin from gray pine would be required (again assuming 100% conversion). Thus, a beetle would need to eat more than 28 times its weight in oleoresin to have any possibility of producing the observed amounts of ipsenol and ipsdienol from eating gray pine. Even higher amounts of oleoresin would be required to replace pheromone released. Therefore, another biosynthetic pathway (*de novo*) is indicated since beetle's guts contain mostly phloem, and oleoresin is toxic to bark beetles (*I. paraconfusus* and *D. brevicomis*) [12, 30–33]. Because small quantities of *cis*-verbenol are produced and required for attraction, it is probable that sufficient α-pinene precursor is available from the host.

It is apparent that all five species of pine are about equally suitable as hosts, at least in terms of adult survival, nuptial chamber construction, pheromone production, and attraction [13]. Sugar pine is a soft pine (subgenus *Haploxylon*) while the others are hard pines (*Diploxylon*). However, sugar pine had monoterpene hydrocarbon characteristics more similar to ponderosa pine than these two species had with Jeffrey and gray pines. The Jeffrey pine with a low titer of α-pinene and myrcene in the oleoresin is consistent with earlier reports [16] but the large amounts of oleoresin in its phloem were unexpected.

I. *paraconfusus* feeding in *P. monticola* and *P. monophylla* also appear to produce at least some of their pheromone components since *I. montanus* and *I. confusus* were significantly attracted [40]. *I. paraconfusus* can also produce attractant (pheromone) when boring in nonhosts Douglas fir in the laboratory [41] and white fir in the field [42]. In the latter species, however, it was shown that the amounts of ipsenol and ipsdienol were only one or two percent of the amounts produced in beetles feeding in ponderosa pine [42]. Differences in attractiveness of *I. pini* boring in two host species have also been observed [43], but it is not known which semiochemicals were responsible.

Elkinton et al. [42] proposed that evolution of host selection behavior by *Ips* bark beetles could have been influenced by the amounts of α-pinene and myrcene in the tree needed for pheromone biosynthesis. Since α-pinene in the tree appears to be converted to *cis*-verbenol, beetles may select trees high in this monoterpene. A related hypothesis is that tree genotypes lower in pheromone precursor monoterpenes may have evolved through natural selection [2]. This is doubtful since in the case of the ipsenol/ipsdienol system there does not appear to be any limitation in pheromone production when feeding in the wide variation of myrcene-containing trees [13]. Thus coevolution of host selection and insect resistance does not seem to be occurring, except possibly with respect to α-pinene. There does seem to be coevolution of detoxification genes for monoterpenes and tree genotypes, which has a major impact on host selection by Ips [44].

Assuming the detoxification theory was the first evolutionary stage of pheromone biosynthesis as proposed [8], then why was myrcene selected as the pheromone precursor instead of another monoterpene like limonene or 3-carene? Our results for ponderosa pine in 1985 show that myrcene and α-pinene were found in four of the five pine species while sugar pine did not have detectable amounts of β-pinene, 3-carene, and limonene (Table 1). Myrcene had the least variation among the five monoterpenes in ponderosa pine in 1986 (Table 2). These data are limited, but Smith

[45] sampled 74 areas across California and western USA and Canada and found that most areas had lower variation for myrcene and α-pinene, while variation in 3-carene, limonene, and β-pinene was higher (his Figure 7). In another study of 64 ponderosa pines, he reported that myrcene in oleoresin varied from 4.6 to 27.5% and α-pinene from 1.5 to 13.3%, while variation of limonene, β-pinene, and 3-carene varied from 0 to 31, 57, and 82%, respectively [46].

After the initial use of myrcene vapor as a precursor to ipsenol/ipsdienol in an Ips species, later speciation events appear to have evolved a *de novo* biosynthesis that now predominates in *Ips* species (at least in *I. pini*, *I. paraconfusus*, *I. typographus*, and *I. duplicatus*). Seybold et al. [47] state the benefits of "redundancy" would result by adding *de novo* biosynthesis and thus provide "assurance" of producing pheromone. Byers [2] argued that a *de novo* system would be advantageous to an individual since he could control the quantity of pheromone for optimal benefit and not be dependent on the host tree for precursor. The *de novo* system would be especially beneficial when a species radiates to use other host pines or when a particular host tree had unusually low amounts of precursor such as to limit pheromone production and fitness. In *I. paraconfusus*, a *de novo* system seems especially beneficial when colonizing host pines of species with little or no myrcene such as in gray pine as reported here and earlier [13]. The *de novo* systems of *I. pini* and *I. paraconfusus* could have become different with evolutionary time as the two species are moderately separated phylogenetically [48]. This is indicated by findings of Tillman et al. [49, 50], who showed that JH III from the corpora allata and by injection induced pheromone production in *I. pini*, but not as much in *I. paraconfusus*, compared to amounts in both species after feeding in host logs.

The aggregation pheromone components are essential to reproductive success, and thus, it may be too "risky" to rely on either levels of precursor in the tree or on generally available microorganisms —but rather generate the components *de novo* from acetate or mevalonate using the beetle's enzymatic systems [51]. As early as 1969, studies had shown that *I. paraconfusus* produced ipsenol and ipsdienol after application of JH analogues without feeding in hosts or exposure to myrcene [22, 23]. This indicated that the corpora allata released JH due to feeding, which then stimulated *de novo* biosynthesis of the two aggregation pheromone components from energy reserves. Lanne et al. [52] showed that *I. typographus* can convert radiolabelled mevalonate to one of its two aggregation pheromone components, 2-methyl-3-buten-2-ol, indicating that *de novo* pheromone biosynthetic pathways exist in *Ips*. Following this, Ivarsson et al. [53] injected an inhibitor of mevalonate biosynthesis into *I. duplicatus* and then allowed the beetles to feed in host Norway spruce. The accumulation of aggregation pheromone components *E*-mrycenol and ipsdienol were reduced 40 to 70%, indicating these components are synthesized *de novo* when feeding via mevalonate. Although ipsdienol and *E*-mrycenol were found "to be produced *de novo* and not from myrcene" [51], exposure to myrcene did cause more of these two components to accumulate than controls, but only about 10 to 20% as much as application of JH analog or feeding in host alone.

Seybold et al. [54] provided further evidence that *I. paraconfusus* produces ^{14}C-labeled ipsenol and ipsdienol (and traces of amitinol) *de novo* from injected ^{14}C-labelled acetate prior to feeding in host logs. Similarly, in 1995, the same group showed that *I. pini* synthesized ^{14}C-labeled ipsdienol (and large amounts of amitinol) from labeled acetate [54]. Interestingly, amitinol has not been reported as a major constituent of *I. pini* or *I. paraconfusus* aggregation pheromones, although its presence was noted in *I. paraconfusus* frass extracts [6]. JH III induced expression of regulatory enzymes (probably 3-hydroxy-3-methylglutaryl-CoA reductase = HMG-R) in *I. paraconfusus* metathoraxes to begin *de novo* isoprenoid pathways resulting in ipsenol and ipsdienol [18, 55]. Hall et al. [56] localized the pheromone biosynthesis in *I. pini* to the anterior midgut (region just after the proventriculus). The HMG-R expression was in the anterior midgut, and when these and other tissues were incubated *in vitro* with radiolabeled acetate, then only the anterior midgut produced radiolabeled ipsdienol. The involvement of microbial symbionts was discounted since anterior midgut tissues when cut open and washed still incorporated radioactivity in ipsdienol. However, internal cell symbionts are still possible, if unlikely. Byers [21] found most ipsenol and ipsdienol in the rectum of *I. paraconfusus*; however, he dissected and extracted only the alimentary canal that is posterior to the anterior midgut. It is likely that although these components are produced in the anterior midgut epithelia, they migrate with the alimentary flow and accumulate in the rectum. Nardi et al. [57] provided electron micrographic evidence that the digestive secretory cells are interspersed with the pheromone-secreting cells in the anterior midgut. The pheromone-secreting cells are distinguished by many highly ordered arrays of smooth endoplasmic reticula. There was no evidence of internal symbiotic bacteria in this region [57].

Seybold and Tittiger [4] point out that JH III stimulated HGM-R enzyme activity in male *I. pini*, but not in male *I. paraconfusus*. Feeding in both species, however, induces HMG-R and pheromone production. It was found earlier that decapitated *I. paraconfusus* treated with JH were inhibited from producing pheromone, possibly due to a brain hormone from corpora cardiaca [23] that is not important in *I. pini* [4]. Mature (emerged) and callow (pre-emerged) adults of both sexes of *I. paraconfusus* do not contain detectable aggregation pheromone components, but after exposure to myrcene and α-pinene vapors only the mature males produced ipsenol and ipsdienol, indicating certain "detoxification" enzyme systems become functional after maturity in males [21]. It is not known if HMG-R can be induced by JH in *Ips*, but in *D. jeffreyi* there is a weak activity compared to mature adults [4]. HMG-R is involved in the early (upstream) steps of isoprenoid biosynthesis that then diverges at isopentenyl diphosphate and geranyl diphosphate in scolytids [4]. Somehow, it seems that these diphosphate precursors are converted to myrcene, which is then hydroxylated by novel enzymes of each *Ips* species [4]. The question remains whether myrcene vapors play any role

in pheromone biosynthesis or are merely artifacts of the manipulated near-saturation concentrations, since these can be about 70 times higher than in nuptial chambers [12]. Dietary myrcene could play a role, but in the case of *I. paraconfusus* feeding in gray pine with undetectable myrcene, the amounts of ipsenol and ipsdienol were similar to that produced when males fed in other host pines [13]. Seybold et al. [54] showed that the enantiomeric composition of ipsenol and ipsdienol is racemic when exposed to myrcene vapor, but specific enantiomers result when feeding. This shows that the *de novo* system is by far the major pathway.

Sandstrom et al. [58] isolated an NADPH-cytochrome P450 reductase that converted myrcene to the appropriate natural enantiomer (4*R*)-(−)-ipsdienol in male *I. pini*. They concluded that this was a myrcene hydroxylase functioning near the end (downstream) of the pheromone biosynthetic pathway. A second report found that *I. confusus* in pinyon pine also had a cytochrome P450 enzyme that hydroxylated myrcene in males to about 85% (−)-ipsdienol, similar to that in *I. pini* [59]. However, since *I. confusus* has a natural ipsdienol of >90% (4*S*)-(+)-ipsdienol, they state there are still additional enantio-specific enzymes that regulate the end product that have yet to be identified [59]. Since various species of *Ips* have different ratios of enantiomers of ipsenol and ipsdienol, then there are likely species-specific enzymes in the different species [59].

Further work is needed to determine the importance of the host tree monoterpene pathways that appear quantitatively minor (and more primitive?) compared to the major *de novo* pathways (derived?). It would also be interesting to determine when the biosynthetic pathways evolved in the various *Ips* species by using molecular clocks [60] and phylogenetic relationships of the biosynthetic genes (as done for other genes in *Ips* [61]).

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Research Article

A Preliminary Report on the Use of Bench-Top X-Ray Micro-Computerised Tomography to Study the Malpighian Tubules of the Overwintering Seven Spotted Ladybird *Coccinella septempunctata* L. (Coleoptera: Coccinellidae)

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The application of micro-CT scanning techniques on a small sample of "Seven-spot ladybirds" *Coccinella septempunctata*, collected in December 2009, identified an accumulation of material with a very high, relative X-ray attenuation value in the malpighian tubules of most but not all of the individuals sampled. The passage of metals such as cadmium in soil through a food chain to finally accumulate in high concentrations in ladybirds and lacewings has been previously reported. The identification of the dense material found in our sample of ladybirds, its origin, and the process by which it accumulates in, and is processed by, the malpighian tubules is the challenge ahead. The authors speculate that a straightforward means of monitoring levels of metallic pollutants in the environment might emerge.

1. Introduction

X-ray Computerised Tomography (X-ray CT) as well as X-ray micro-Computerised Tomography (X-ray micro-CT) are being increasingly used to study both insect colony behaviour [1–6] and also anatomy of individual insects [7–11]. One of us (MKG) has coined the phrase "Diagnostic Radioentomology" (DR) to describe such studies [7].

We set up and then used a small, relatively inexpensive Skyscan Bench-top micro-XT scanner (Skyscan 1174 micro-CT) from 22nd December 2009 to 5th January 2010 to study a small number of overwintering ladybird beetles, *Coccinella septempunctata* L. (Coleoptera: Coccinellidae). The micro-CT images showed that the lining of the malpighian tubules of several of the *C. septempunctata* appeared to contain a dense, radio-opaque material.

As nicely summarised by Wigglesworth in his excellent monograph of Insect Physiology in the chapter on excretion [12], "The malpighian tubules still remain unquestionably the chief excretory organs. The malpighian tubules are relatively simple tubular glands which open at the junction of the mid-gut and the hind-gut. They are exceedingly variable in form: sometimes being numerous (e.g., 100) and short, sometimes few in number (e.g., 2) and long; sometimes simple and sometimes branched; occasionally anastomosing to form closed loops; while sometimes more than one type may be present. Their histological structure is no less variable. Usually their epithelial cells bear a striated border,

but this may be wanting. Many histological changes have been described in the active cells of the malpighian tubes: the discharge of vesicles, the eruption of vacuoles, and so forth. It is not improbable that more than one cytological mechanism of excretion may exist; but certainly many of the recorded observations are artefacts."

Of direct relevance to the present study (please see below) was the observation of Marcus, that many beetles "phytophagous, carnivorous and omnivorous species as well as those that feed on dry substances have the distal part of their malpighian tubules closely investing the rectum, being surrounded to it by a delicate membrane" [13] and for illustration see [12, page 66]. Further work on this topic in the 1960s and 1970s, particularly that of Maddrell and colleagues, was particularly key [14]. The nature of the so-called "cryptonephridial" condition was further discussed and illustrated in Imms' General Textbook of Entomology in 1977 [15, page 249]. About the same time, one of us (DMH), as part of her PhD thesis [16], and in subsequent publications [17, 18] summarised the then known anatomy and physiology of insect malpighian tubules with particular emphasis on those of *Locusta migratoria* L. (Orthoptera: Acridoidea). For relevant recent references up to the present time on studies of insect malpighian tubules, see [19, 20] and the reference they contain in their "Excretory System" Chapters.

This paper reports on the X-ray micro-CT appearances of 10 overwintering [21] *C. septempunctata* with particular reference to their malpighian tubules and also includes a dissecting light microscopy examination of a further 5 *C. septempunctata* to confirm that the structures highlighted by micro-CT were indeed the insects' malpighian tubules. Also, the possible nature of the dense, radio-opaque material found in some of the ladybirds' malpighian tubules is discussed.

2. Materials and Methods

2.1. The Insects and Traditional Dissection Techniques. Ten specimens of overwintering *C. septempunctata* were euthanised on the 23rd of December 2009 by placing in a deep freezer (−20°C) for 12 hours and then stored in 70% ethanol until scanned using the Skyscan 1174 X-ray micro-CT (see below). Before euthanising, the overwintering *C. septempunctata* were situated on the South-facing side of the inside of a poorly fitting wooden window frame of one of our houses (GDB). The house is set in a rural village in Suffolk, East Anglia, UK, surrounded by agricultural land (52°N : 1°35′E).

An additional five non-overwintering *C. septempunctata* were collected in September 2011 by (LW) from his garden near Bury St. Edmunds in Suffolk (52°15′N : 00°4′E) and were subjected to dissecting light microscopy examination after euthanising using ethyl acetate.

2.2. Examination of C. septempunctata by Traditional Dissection Techniques. One of us (LW) aided by a dissecting light microscope as described elsewhere [22] performed abdominal dissections of the malpighian tubules of five *C. septempunctata* in the non-overwintering state that had been collected in September 2011. There were 2 female and 3 males.

2.3. X-Ray Micro-CT Technique. A Skyscan 1174 Desktop X-ray micro-CT scanner was set up by using the sample scanning, reconstruction, analysis, and visualization (2D and 3D) methodology and protocols according to Tarplee and Corps [23], which also contains useful guidelines, notes, and selected references on micro-CT scanning.

The ladybird samples were contained in plastic tubes and scanned at several different energy levels varying from 30 to 50 kV with and without the addition of a topically applied iodinated radiographic contrast agent (Omnipaque). 180 degree scans with an angular rotation step of 0.5 degrees were used. The results of using dual energy algorithms and the effect of applying such radio-dense material to the exterior of the insect's exoskeleton are beyond the scope of this paper and will be reported in a separate paper. However, for the present study, tomographic reconstruction of the transmission X-ray images from the 50 kV dataset using the Skyscan NRecon software produced 184 transverse/axial slices of 1024 × 1024 pixels, which were saved as 16 bit TIFFs. The pixel resolution in X, Y, and Z (inter-slice) directions was 9.86 μm.

2.4. Software for Viewing the 3D Micro-CT Data. We used both the Skyscan in house software, see [23], as well as the "Disect" viewing software (http://www.disectsystems.com/) for viewing the 2- and 3D data as described and illustrated with various training videos on the "Disect website." The Malpighian tubules were masked, segmented, and cropped using the commercially available software, TomoMask (http://www.tomomask.com/).

3. Results

3.1. Traditional Dissection. The first two dissections turned out to be female *C. septempunctata* with their abdomens containing eggs. In each case, removal of the abdominal section of the alimentary tract revealed the presence of what appeared to be two sets of malpighian tubules with the proximal ends of each set attached to the gut. Neither of these two sets of tubules had the form of gastric caecae found in some insects (which tend to originate near the distal portion of the crop, are relatively short, are of greater diameter than a malpighian tubule, and essentially resemble a fold in the gut wall [24]).

The next dissection was a male specimen. Removal of the elytra, posterior wings, and dorsal tergites revealed well-developed fat bodies (Figure 1). By carefully teasing away of the surrounding soft tissue, the complete lengths of two of the malpighian tubules were revealed (Figure 2). It is clear from the two photomicrographs shown (Figures 1 and 2) that each malpighian tubule forms a complete loop; attachment at the anterior end is around the junction of the mid gut and ileum (i.e., hind gut), whilst the posterior end appears closely attached to the lower alimentary tract.

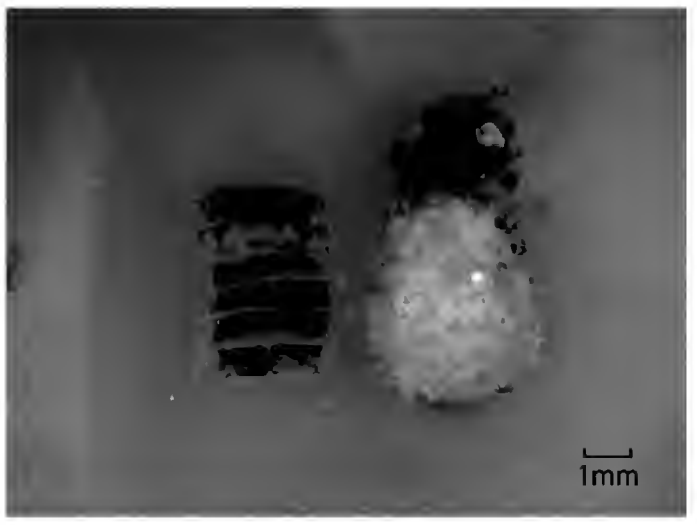

FIGURE 1: Photomicrograph of "stage 1" dissection of *C. septempunctata* with elytra and tergites removed which enabled *in situ* visualization of the dorsal aspect of gross internal anatomy.

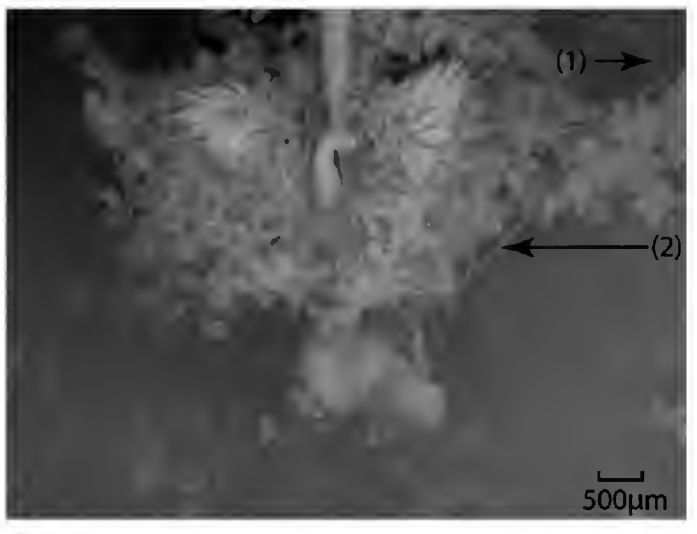

FIGURE 2: Photomicrograph of "stage 2" dissection of *C. septempunctata* detailing the abdominal portion of the alimentary tract. Two complete malpighian tubules (1) and (2) were physically unfolded for improved examination.

Figures 3 and 4 show a further photomicrograph and diagram of specimen 4 in which the thoracic and abdominal section of the alimentary tract have been removed. In total, there appear to be six individual malpighian tubules in *C. septempunctata*.

3.2. Micro-CT Image Appearances and Their Software Manipulation. Figures 5, 6, 7, and 8 show various views of some of the male and female overwintering *C. septempunctata* specimens scanned, while Figure 8 shows just the malpighian tubules after the use of Tomomask to segment out the radio-opaque malpighian tubules.

A rough indication as to the relative density of the material in the malpighian tubules when compared with (say) the ladybird's exoskeleton or internal organs can be illustrated by the "Advance" feature of the 'window and levelling tool of "Disect", where higher gray scale values indicate more X-ray absorption by a denser material. When using the 16 bit TIFF data loaded into "Disect", the degree of radio-opacity can be viewed on a histogram on a scale of 0–65,536. By making the "window size" just 1 unit wide and "levelling" up and down (i.e., effectively binarising the data), one can see when a particular structure either first begins to appear or conversely when it starts to disappear

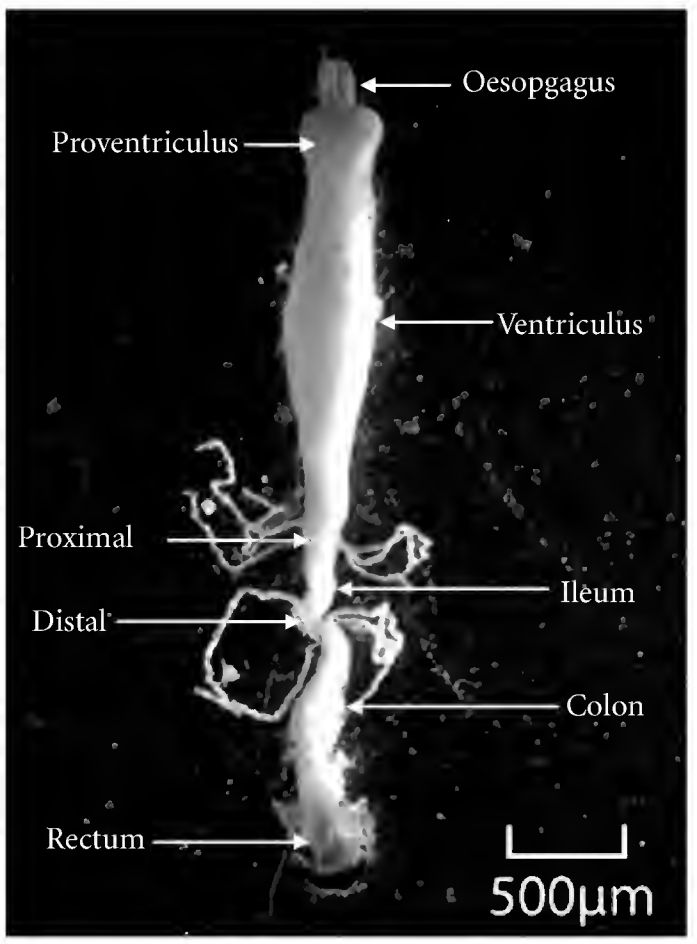

FIGURE 3: Photomicrograph of the resected thoracic and abdominal sections of the alimentary tract of *C. septempunctata*, with *proximal* and *distal* ends of the malpighian tubules attached.

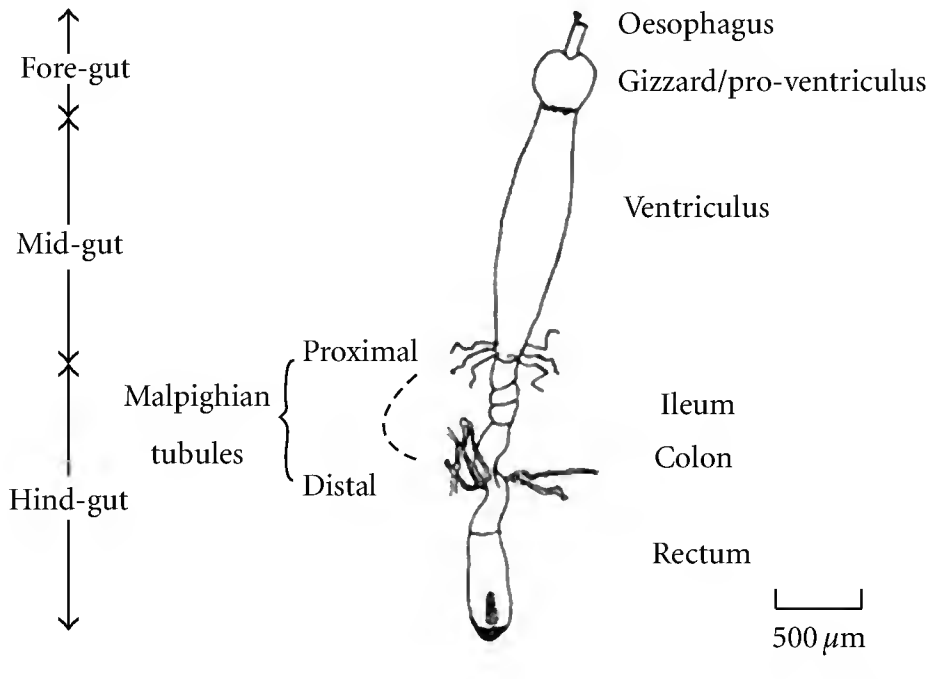

FIGURE 4: Schematic diagram of thoracic and abdominal sections of the alimentary tract of *C. septempunctata*.

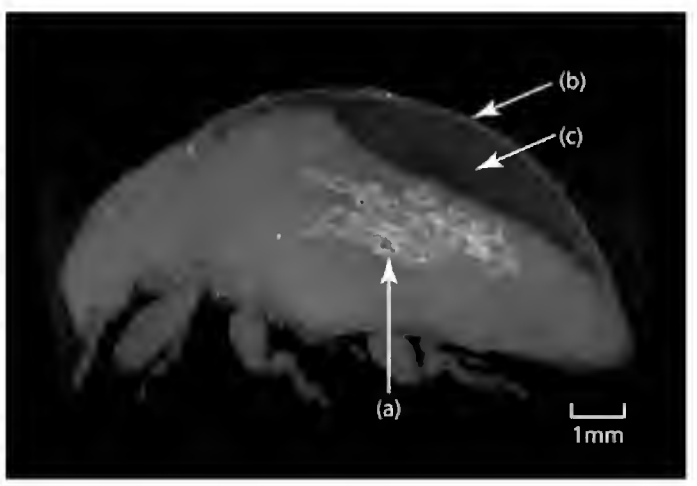

FIGURE 5: A 3D, volume rendered, micro-CT image of a male *C. septempunctata* using Maximum Intensity Projection (MIP) showing (a) radio opaque malpighian tubules, (b) elytra, and (c) folded wings in the air space between elytra and tergites.

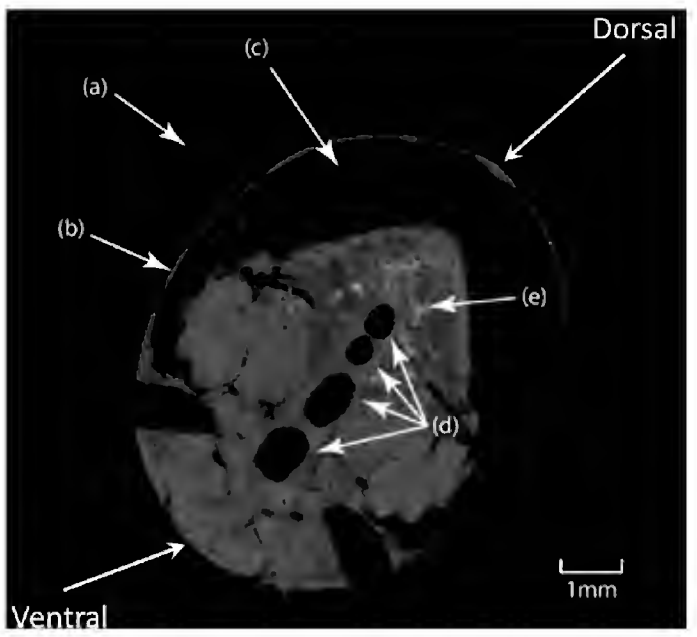

Figure 6: A 2D micro-CT image showing a transverse section through a *C. septempunctata* abdomen with details of (a) wall of plastic tube-mount, (b) elytra, (c) air space containing folded wings between elytra and dorsal tergites, (d) air sacs, and (e) malpighian tubules.

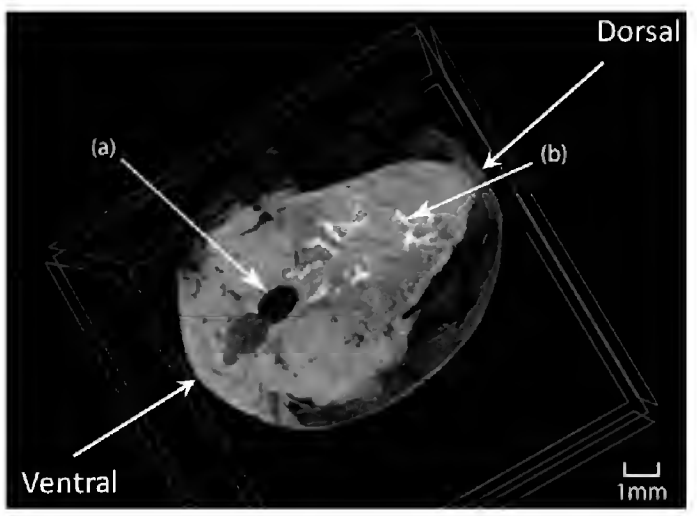

Figure 7: A 2D micro-CT image showing a transverse section through a *C. septempunctata* abdomen showing (a) nondense "black" radio-lucent air-sacs and (b) dense "white" radio-opaque malpighian tubules. Blue lines indicate the selectable cutting planes.

as one levels up from 0 upwards: thereby obtaining a range estimate. Using the data illustrated (Figure 6), the range of values were 6,930–14,000 for the plastic tube, 12,770–21,760 for the elytra, 13,860–30,400 for the internal organs, and from 34,000 to 44,870 for the radio-opaque material in the malpighian tubules. These values demonstrate that micro-CT gray scale (density) data can be useful in diagnosing, segmenting, and studying insect malpighian tubules.

In agreement with the dissection and photomicrograph results, the micro-CT data suggest that there are 6 malpighian tubules and that they are of the cryptonephridial in type.

4. Discussion

The micro-CT results in the overwintering seven spotted ladybird; *C. septempunctata* clearly showed that the malpighian tubules contain an unknown extremely radio-opaque material. The fact that there were six such structures with the distal ends in close contact with the rectum is

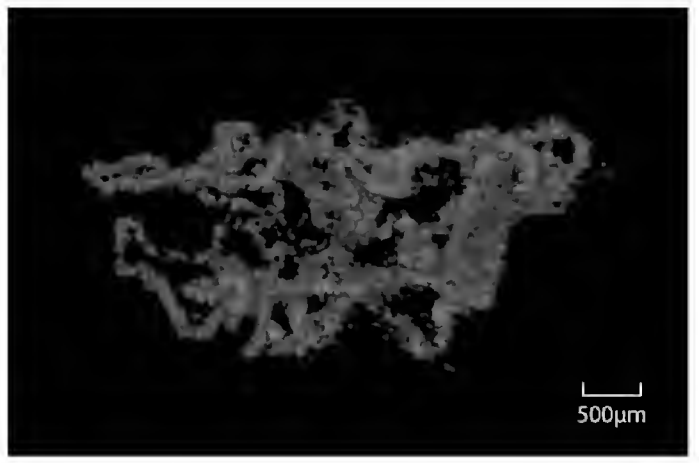

Figure 8: A 3D, volume-rendered and segmented image of the radio-opaque malpighian tubules in *C. septempunctata*.

in close agreement (a) with our finding at dissecting light microscopy and (b) the available literature that suggests that most polyphagic coleopterans such as ladybirds have cryptonephridial malpighian tubules [12–20, 24].

Various authors stress how highly variable are both the number of malpighian tubules and the histological appearance of the tubular lining cells in different regions of the tubule. That the proximal ends of the tubules enter the gut between the mid and hind gut is not in doubt as shown elegantly (for instance) by scanning and transmission microscopy [16, 17]. Sometimes the proximal end of each individual malpighian tubule enters the gut separately while in some several malpighian tubules will join a common ureter before entering the gut.

The received wisdom is that the distal ends of the malpighian tubules are "blind-ended" even when the distal ends are in close association with the rectum [12–20, 24]. Having said this, Imms shows in one of his diagrams of the malpighian tubules of a Bloody-nosed beetle, *Timarcha tenebricosa* Latrielle (Coleoptera: Chrysomelidae), a cryptonephridial looping distal end but with the lumen of each tubule clearly being continuous with that of the gut at both ends [15]. More recent studies [20, pages 437–439] have suggested this may be an artifact and that in fact in all insects with a cryptonephridial system in which the distal ends of the tubules are enveloped within a membrane and held close to the surface of the rectum, but that the tubules do not penetrate the lumen of the rectum but rather lie on the outer surface of the rectum within a perinephric chamber bounded by the perinephric membrane. In our opinion, further studies are clearly required in the case of *C. septempunctata,* which will require a combination of micro-CT, light microscopy, and electron microscopy.

Further, more detailed studies are also required to elucidate (a) the nature of the radio-opaque material we have demonstrated in the overwintering *C. septempunctata* malpighian tubules and (b) clarify if this phenomenon is confined to just this species of ladybird or whether it is a common phenomenon in other beetle species. At the present time, we can only speculate as to what the radio-opaque material might be. It is so radio-opaque that it must contain a significant amount of some metal compound such as calcium, magnesium, or (as in the case of the locusts' mandibles) zinc [8].

On reviewing the literature on ladybird malpighian tubules, we came across a reference to the detection of cadmium-containing compounds [25] in these insects. The authors postulate that possibly cadmium salts were somehow in the soil as a result of fertilising the fields with unprocessed or poorly processed waste material. They further postulated that the cadmium in the soil was then taken up by plants, which in turn could get into phytophagous sap-sucking insects such as aphids, which in turn could then be ingested by ladybirds. If so then the ladybird could be at the top of a food chain for cadmium accumulation and thus monitoring its presence and concentration in ladybirds might be potentially of value. The same authors have subsequently reported a similar phenomenon when the lacewing rather than the ladybird is the insect consuming the aphids [26].

In this study, we have shown that with a relatively inexpensive X-ray bench-top micro-CT scanner, excellent images with high resolution can be obtained [23]. We found that "Disect" viewing software was easy to learn [27, 28] and also had the added benefit of sharing micro-CT data across the world if ever required [29–34].

The use of the Tomomask software greatly speeded up and simplified the process of masking, segmenting, and cropping data CT data stacks whether they are in the form of DICOMs or (as is more normal with micro-CT data) stacks of TIFFs, JPEGs, JEG 2000, BMPs or PNGs.

5. Conclusion

Marcello Malpighi, the Italian physician and anatomist (1628–1694), is regarded by many as the founder of microscopic anatomy. His treatise on the internal organs of the silkworm was the first monograph on an invertebrate. He described insect air sacs and the tracheal system as well as the tubules that now bear his name [35, 36]. Malpighi would be interested that over 300 years on there is still at lot to learn about the insect excretory tubules named after him.

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Research Article

Addition to the Distributional Record of *Ageniella* (*Neotumagenia*) *amazonica* Fernández, 1998 (Hymenoptera: Pompilidae) and Establishment of a Neotype

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Ageniella is a diverse and poorly studied genus in Ageniellini (Pompilidae: Pepsinae). It is composed of nine subgenera with four being endemic to the Neotropical region. Herein, the second record in the literature for the subgenus *Neotumagenia* is documented, the distribution range is extended, and a neotype is established. This is the first record of this subgenus in Brazil.

1. Introduction

Ageniella Banks is the second most diverse genus in Ageniellini with about 110 valid species and nine subgenera. The genus is found throughout the New World [1], from Canada to Argentina, but it is most diverse in the Neotropical region [2].

Ageniella wasps are highly variable morphologically. Body size in males, for example, varies from some having a body length of <2.0 mm to longer than 15.0 mm [3]. Coloration of the species is also variable. Species can have all of the integument or certain parts areas red, orange, black, or blue metallic, with or without long setae. Taxonomists working on *Ageniella* struggle to determine diagnostic characters for the genus and its subgenera. Shimizu [4] performed a phylogenetic analysis of the genus using morphological characters but did not recover any synapomorphic characters, or a monophyletic *Ageniella*. The variation in the genus is not only remarkable within subgenera, but also within species as well.

The last subgenus added to *Ageniella* was *Neotumagenia* Fernández [5]. This subgenus is monotypic and is restricted to the Neotropics. *Ageniella* (*Neotumagenia*) *amazonica* Fernández was described from Colombia based on a single female exemplar. This species is unique within *Ageniella* by having a large, grooved, polished swelling on the front, just above each torulus, and stout legs. The biology of *A. amazonica* is unknown, and the subgenus and species are based only on the female. The holotype of *Ageniella* (*Neotumagenia*) *amazonica* is currently lost (F. Fernández pers. com.).

Herein, we establish a Neotype for the species, provide illustrations, and extend the distributional range of the taxon to Brazil.

2. Material and Methods

The studied material was derived from a loan from the Museu Paranaense Emílio Goeldi (MPEG), Belém, Pará, Brazil. Besides MPEG, two specimens will be deposited in different collections, as designated in text: Entomological Museum of Utah State University (EMUS), Logan, Utah, USA; Instituto de Ciencias Naturales-Museo de Historia Naturales (UNCB), Universidad Nacional de Colombia, Bogotá, Colombia.

Abbreviations used in the descriptions are the same as those used by Fernández [5] and Wasbauer and Kimsey [6]. They are defined as follows: FD = facial distance; LA3 = length of third antennal segment; MID = middle interocular distance; OOL = ocellocular length; POL = postocellar length; TFD = transfacial distance; UID = upper interocular

distance; WA3 = width of third antennal segment. Measurements of the clypeus are as follows: WC, width of clypeus, measured from the widest points; LC, highest length of clypeus.

Collection locality of the specimens was obtained from the literature (holotype) and from the specimen labels (new records). These data were used to obtain the geographical coordinates with Google Earth to construct the distribution map with ArcView version 9 software by ESRI [7].

3. Results and Discussion

3.1. Taxonomy and New Records. Subgenus *Neotumagenia* Fernández.

Type-Species. Ageniella (*Neotumagenia*) *amazonica* Fernández, 1998. Caldasia 20:1-4 [f#, COLOMBIA (lost)]. Original designation.

Ageniella (*Neotumagenia*) *amazonica* Fernández, 1998 (Figures 1(a) and 1(b)).

Neotype. 1 female. Brasil, Pará, Serra Norte, N1-Mata, 28-31.x.1985, Armadilha 1.6 m suspensa, 6°00′25″S 50°18′32″W, N. Bittencourt [col.] (MPEG no. HYM 11005855).

Diagnosis. This species can be recognized from other *Ageniella* by the following combination of characters: the integument is black on head and metasoma, orange with black ventrally on mesosoma (Figure 1(a)); the face has two large protuberances (Figure 1(b)), and it is covered by silver scale-setae, except by the protuberances, which are shining; the clypeus is convex, with apical margin slightly prolonged; the fore wing is hyaline with a dark band.

Measurements (mm) and Ratios. Body length: 7.72; forewing 6.59; maximum wing width 1.80. FD: 1.69; MID: 1.18; UID: 0.78; LID: 0.94; TFD: 2.00; OOL: 0.19; POL: 0.08; WC: 0.98; LC: 0.47; WA3 0.33X LA3.

3.2. Examined Material

1 Female. Brasil, Pará, Serra Norte, Serraria, 20-23.vi.1986, armadilha 1.6 m suspensa, 5°58′37″S 50°19′43″W, J. Dias [col.] (MPEG no. HYM 11006147: deposited in UNCB);

1 Female. Brasil, Pará, Juruti, Beneficiamento, Malaise 4, 15-25.vi.2009, 2°31′29″S 56°11′13″W (MPGE);

1 Female. Brasil, Amazonas, Marãa, R. Japurá, Maguari, 11-17.x.1988, Armadilha 1.6 m suspensa, 1°49′28″S 65°21′28″W, J. Dias [col.] (MPGE # HYM 11090903: deposited in EMUS).

The Neotype (Figures 1(a) and 1(b)) does not vary from the original description, and there is no notable variation between studied specimens.

(a)

(b)

Figure 1: Neotype of *Ageniella* (*Neotumagenia*) *amazonica*. (a) Habitus, lateral view. Scale = 2.4 mm. (b) Head, frontal view. Scale = 1.29 mm.

The frontal processes (Figure 1(b)) present in this species are intriguing, but other examples of this feature exist in the family (e.g., *Auplopus iris* (Banks)) and even in the genus *Ageniella*. In *Ageniella*, these processes are present also in *Ageniella* (*Cyrtagenia*) *fallax* Arlé, but in this taxon, they are not as large or as prominent as the shiny protuberances found in *A. amazonica*. Other genera of Pompilidae, such as some African *Cryptocheilus* reported by Arnold [8], have frontal protuberance as well. *Auplopus iris* (Banks) has very similar head morphology, differing only by having silver scale setae in small areas of inner face of the eyes, and the clypeus is more sharpened medially and convex; while *A. amazonica* has setae covering most of the face, but the protuberances and the apical margin of clypeus are less prolonged. *Ageniella amazonica* has the frontal processes acute, invaginated, and asetose, which has an obvious groove for housing the antenna. Perhaps the frontal processes in *A. amazonica* are related to antennal support while female is hunting for a spider or building a nest, but this is conjecture, and the function of these processes remains a mystery.

Ageniella amazonica also differs from the other species of *Ageniella* by having stout front legs. Many species of *Ageniella* dig nests on ground using the front legs [9]. It is possible that these females use the head to close the burrow

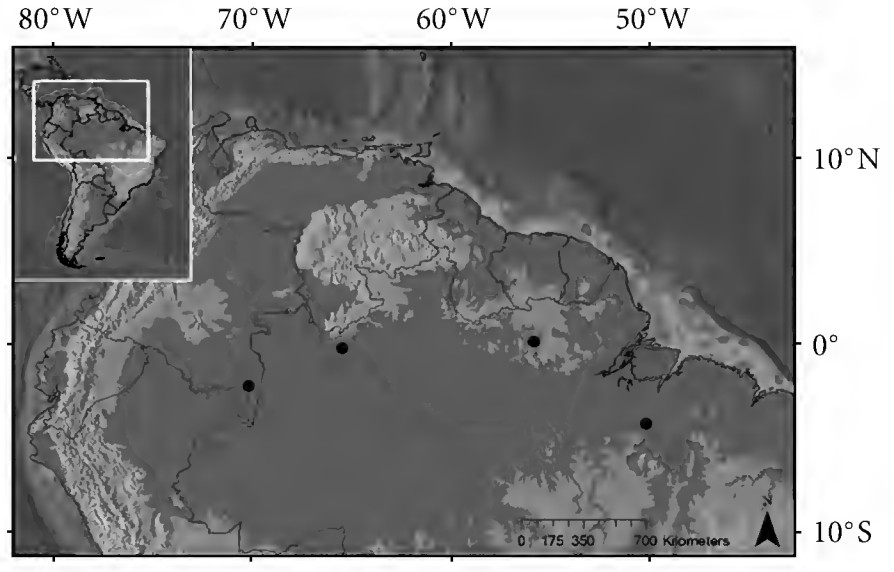

FIGURE 2: Distributional map of *Ageniella* (*Neotumagenia*) *amazonica*.

dug and pack the soil. In this case, the stout femora of the front legs, the polished head with scale-like setae, and frontal protuberances may be adaptations to this behavior.

Unfortunately, the Neotropical fauna of *Ageniella* is taxonomically unknown and poorly studied. The species described for the Neotropics lack comprehensible descriptions, keys, and illustration. Moreover, the current outdated taxonomy of the group impedes ecological, behavioral, and biogeographic studies. The last Neotropical survey was made by Evans [10], who studied the Ageniellini of the Neotropics, provided a key for females to subgenera, and corrected taxonomic problems created by Haupt.

This study represents the first record of *A. amazonica* for Brazil. It was previously only known from the type locality [5, 11]. Our study indicates that this species is restricted to the Amazon Forest (Figure 2), and it is probably widespread through it. Yearly precipitation in the occurrence area varies from 1600 mm to 3600 mm; regional variation in climate is homogenous and is the least variable in Brazil [12].

Finally, the designation of a neotype and the record of new specimens are significant to the taxonomy of *Ageniella*, contributing to enrich distributional and morphologic information regarding this New World taxon.

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Editorial

Ants and Their Parasites

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Ants accumulate and protect collective resources and, with the exception of nomadic species, live in a nest which is considered to be one of the bases of the evolution of eusociality. Resources and/or protective services provided by ant colonies are exploited in manifold ways by an amazing diversity of other organisms acting as guests and/or parasites: viruses, bacteria, fungi, sporozoa, amoeba, ciliates, nematodes, trematodes, cestodes, mollusks, diplopods, crustaceans, mites, spiders, and a large variety of parasitic or parasitoid insects. Such associations can be obligatory or facultative, permanent or temporary, harmful or beneficial for the host. Due to the diversity of interactions, an understanding of the nature of these relationships and the mechanisms of integration used by parasites as well as the defense strategies developed by their potential host remains a challenge. Parasites certainly increase specific selection pressures on colony phenotype, and they may also shape the composition and dynamics of ant communities. Over the past two decades there has been a growing interest in the impact of parasites on colony phenotype, and their role in the ecology and evolution of their hosts. Despite the apparent importance of ant parasites, detailed knowledge is lacking, for example, about their diversity and abundance or selection pressures imposed through parasitism on host reproductive strategies.

Parasitism in ants has attracted the attention of numerous scientists in the last two centuries (see the numerous publications on this topic by authors like E. W. Janson, A. Forel, C. Janet, E. Wasmann, H. Viehmeyer, J.-J. Kieffer, K. Escherich, W. M. Wheeler, H. St. J. K. Donisthorpe, W. M. Mann, C. Rettenmeyer, E. O. Wilson, B. Hölldobler, D. Kistner, U. Maschwitz, and P. Schmid-Hempel among others). Since the synthesis of Kistner in 1982 [1] and of Schmid-Hempel in 1998 [2], only one complete review has been published [3] (but see also [4] for social parasites) and, in spite of the accumulation of much information in the last decades, this meaningful topic has not been brought together in a specific issue for a long time. This special issue, of course, cannot cover all possible ant parasites, but it examines a wide range of species: viruses, bacteria, fungi, nematodes, silverfishes, flies, butterflies, beetles, spiders, wasps, and ants themselves. It is divided into two main sections: (1) behavioral and ecological aspects of parasitism, in which existing associations are reviewed and discussed, some new associations are described, and some concepts are reanalyzed in a more up-to-date integrative vision; (2) infection, impact on ants and biological control, in which particular effort has been made to provide both an analytical review of the experimental material actually available and a proposal of guidelines for future research on the topic.

Behavioral and Ecological Aspects of Parasitism. Numerous species take advantage of the supplies of other organisms and cleptobiosis, a quite common phenomenon among animals, also occurs at ants' expenses. M. D. Breed et al. review concepts linked to food stealing in social insects, distinguishing cleptobiosis from some related phenomena, and place this knowledge in ecological and evolutionary contexts. In most cases, success in parasitizing ants depends largely on the degree of resemblance to the host, which varies considerably among the diverse mimetic parasites found in

ant societies. Different morphological, behavioral, and/or chemical adaptations allow cleptoparasites to deceive ant defense mechanisms. Reviewing more especially the use of the terms that describe the chemical resemblance between the cuticular chemical profiles of parasites and that of their hosts, C. von Beeren et al. give an overview of cases concerning adaptive chemical resemblance and propose a terminology more consistent with that generally used in biology: "chemical crypsis" when the operator (the host) does not detect the mimic as a discrete entity and "chemical masquerade" when the operator detects the mimic but misidentifies it as an uninteresting entity.

The reports of adaptive resemblance (visual, morphological, and/or chemical) are amazingly numerous. Spider-ant associations, for example, involving either myrmecomorphy, myrmecophily or myrmecophagy, or a combination of the three, are very common, and P. E. Cushing provides an excellent update of her important 1997 review [5] with a welcome summary of recent work testing the adaptational significance of these associations. Among spiders, myrmecomorphy is supposed to usually involve Batesian mimicry but such an assumption has not been demonstrated experimentally. X. J. Nelson provides the first evidence that salticid ant mimicry is truly Batesian mimicry. She shows experimentally both that ant mimicry is perceived by the predator and has a protective effect for the mimic, and how a predatory spider is affected by the degree of visual resemblance of ant mimics to ants.

Among the myrmecophiles, the Coleoptera are probably the most diverse group. In some cases, associations have been known for a long time but the behavioral adaptations allowing the integration of the parasite remain poorly studied. Through a quantitative analysis, E. Maurizi et al. show that the rewarding behavior, during which the ground beetle *Paussus favieri* provides attractive chemical substances to its host *Pheidole pallidula*, is one of the key factors for acceptance and full integration in the ant society. Another way of deceiving a host is through innate chemical mimicry (*sensu* C. von Beeren et al.), involving a change in the parasite's chemical profile in accordance with the host nest odor. This is what A. Lenoir et al. report for a histerid and a staphylinid beetle, both of which parasitize *Aphaenogaster senilis*, whereas a silverfish that shares the same host shows only low quantities of host hydrocarbons, which are probably acquired passively. After isolation, the histerid beetles *Sternocoelis hispanus* keep their hydrocarbon quantity, showing that they are able to synthesize them and adjust their profile to the host colony via direct contacts. This is the first such demonstration in a myrmecophile beetle. In all the other cases studied, the hydrocarbons are provided by the host as occurs, for example, in *Malayatelura ponerophila*, a kleptoparasitic silverfish of *Leptogenys distinguenda* [6]. Various species, like many coccinellids, indirectly affect ants through their predation on trophobiotic Hemiptera. Possibly, frequent interactions with ants led some species to become myrmecophilous and to use chemical mimicry to get close to their prey. A. Vantaux et al. provide an overview of the evolution of myrmecophilous traits in ladybirds and discuss from an evolutionary perspective both costs and benefits of myrmecophily and even dietary shifts to myrmecophagy, which occurred in a few species. By comparison, the myrmecophagy on leaf-cutter ant queens by dung beetles of the genus *Canthon* is a much better known phenomenon, even if behavioral studies are scarce. L. C. Forti et al. present in their thorough behavioral study many details on how *Canthon virens* attacks *Atta* queens.

Besides hemipterans, numerous butterflies species are associated mutualistically with ants, and species from the family Lycaenidae are among the best studied. Interestingly, associations also changed towards parasitic interactions several times. K. Fiedler presents a comprehensive review of the host ants of parasitic lycaenids and analyzes the macroecological patterns that could be related with the use of particular ant genera as hosts. His large-scale survey reveals that those ant genera in which associations with lycaenids are particularly numerous are also more likely to serve as hosts for parasitic species. Among lycaenids, associations between the genus *Maculinea* and the ant genus *Myrmica* are certainly the most investigated. F. Barbero et al. present an overview of the adaptations used by *Maculinea* butterflies to infiltrate and live as parasites in *Myrmica* ant colonies, and more particularly, based on a synthesis of recent research, how they mimic the acoustic communication of their hosts. Some other parasitic butterflies are less well known, and L. A. Kaminski and F. S. Carvalho-Filho describe and illustrate for the first time the immature stages of *Aricoris propitia* and uncover the diversity of life cycles in the still enigmatic butterfly family Riodinidae.

A large number of parasitoid species have been reported in association with ants but, most often, the true nature of their relationships is poorly known and few species are really highly specialized on ants in general. High specialization on ants is however the case in various genera of phorid flies and in numerous species of wasps from three superfamilies: Chalcidoidea, Ichneumonoidea, and Diaprioidea. B. V. Brown and S. M. Philpott describe three new species of *Pseudacteon* flies with some details on the natural history of the ant-fly interaction and provide a useful taxonomic key to the species. Successful parasitism by phorids involves utilization of multimodal cues to locate and recognize the host. K. A. Mathis and S. M. Philpott review some important components of phorid biology, and the variety of strategies and cues used by the three most common phorid genera attacking ants (*Apocephalus*, *Pseudacteon*, and *Neodohrniphora*). Apart from their direct parasitic effects on ants, phorid flies also affect their behavior, and H.-Y. Hsieh and I. Perfecto review the impact of parasitoid phorids on ants and other organisms that interact with ants. They focus both on the variety of mechanisms used by ants to cope with phorid parasitism and on the complexity of these interactions through trait-mediated indirect effects on other trophic levels. Finally, in an attempt to understand how parasitoids affect their host ants' foraging success in a community framework involving species of different body size and behavioral dominance, E. B. Wilkinson and D. H. Feener Jr. examine how habitat structural complexity affects the foraging behavior of two species of the genus *Pheidole* by interacting with parasitoids of the genus *Apocephalus*. Hymenopterous parasitoids of ants also exhibit a wide

array of adaptations to attack such potentially dangerous hosts. Reports of parasitoid wasps associated with ants are numerous but real primary parasitoidism has rarely been proven. J.-P. Lachaud and G. Pérez-Lachaud review all of the cases for which such primary parasitoidism has reliably been established, providing an updated list of at least 138 species from 9 families. They report some new associations and focus both on the diversity of these parasitoid wasps and the diversity of the types of interactions they have formed with their ant hosts. Among these hymenopteran parasitoids of ants, the eucharitid family has been particularly under scrutiny for the last three decades, especially the genus *Kapala*, the most common in the Neotropics [7]. A. A. Vásquez-Ordóñez et al. present useful natural history information on the interactions between a *Kapala* species and the host ant *Ectatomma ruidum* in Colombia. Evaluating the effect of habitat type on eucharitid parasitism, they report a significantly higher prevalence of parasitism in host ant colonies in woodland compared to grassland habitat.

Infection, Impact on Ants and Biological Control. In numerous applied studies on ant parasitism, specific attention has been given to the ways in which parasite pressure may affect patterns of life history in ant hosts. For example, generalist entomopathogenic fungi could be used in biological control of pest ants as discussed by M. M. R. Ribeiro et al. in the case of *Beauveria bassiana* and *Aspergillus ochraceus* against the grass-cutting ant *Atta bisphaerica*, one of the most important pests of pastures and crops in Brazil. It is the first time that *A. ochraceus* is reported to infect *Atta* with a high prevalence. However, field experiments are necessary to test for their effect as biological control. Pathogens are difficult to identify because sick or dying ants are promptly removed from the nest or leave the nest themselves (see the recent review by Shorter and Rueppell [8]). Some entomopathogenic fungi are ant specific and X. Espadaler and S. Santamaria review what is known concerning the taxonomy, natural history, and/or ecology of ecto- and endoparasitic fungi specialized on ants throughout the Holarctic region. The fungi considered in this paper show a gradient of negative effects on the host, and their specificity does not seem to be always very strict since various fungi are known from a range of hosts (e.g., *Laboulbienna; formicarum* is hosted by 24 ant species belonging to 3 formicine tribes). Specificity is apparently higher both in the mutualistic basidiomycetous fungi cultivated for food by neotropical fungus-growing ants of the tribe Attini, and the specialized microfungal parasites which coevolved with these associations and have a negative impact on the fungus gardens. Recent research on this issue has provided novel insights into coevolution, antibiotic defense mechanisms, and behavioral interactions within symbiotic systems. F. C. Pagnocca et al. pulled together diverse literature and present a review of the microfungi associated with leaf-cutting ant gardens, while S. H. Yek et al. synthesize our current understanding on the evolution of specialized parasites of the attine fungus gardening system. Using a modified version of Tinbergen's four categories of evolutionary questions to structure their review, they focus on development, mechanism, adaptation, and evolutionary history and suggest further directions for investigations of this symbiosis.

Various other organisms, in addition to entomopathogenic fungi, can affect the biology of their hosts. Bacteria of the genus *Wolbachia*, for example, are known to alter the reproductive capabilities of their hosts significantly, showing complex interactions with them, which, in some cases, have evolved to symbiotic associations. K. K. Ingram et al. examine possible parameters affecting the spread of *Wolbachia* infections in a newly established population of *Formica fusca*. Their results show that horizontal transmission of *Wolbachia* is apparently uncommon and that there are no marked fitness differences between infected and noninfected colonies. This is an additional illustration of the complex role of *Wolbachia* in ants which is not yet explained (see [9, 10]). Ants can also serve as hosts of a variety of internal or external parasitic nematodes from several families with more or less complex life cycles. Different entomopathogenic nematodes like *Steinernema* and *Heterorhabditis* have been suggested to control ants through inundative applications. The current state of knowledge regarding the occurrence, systematics, life history, and pathology of all described nematodes associated with formicids is summarized by G. Poinar Jr. through a richly illustrated review. Apart from including a simple key to the higher taxa of ant-infecting nematodes, he identifies the large gaps that exist in our understanding of this very interesting system.

The use of ant parasites as a means of biological control has been most heavily investigated in relation to one of the most important pests in the New World, the imported fire ants (*Solenopsis*). Focusing their review on research programs that have been carried out over 25 years in their laboratory, J. Briano et al. give a wide panorama of the natural enemies of fire ants (microsporidia, nematodes, viruses, phorid flies, eucharitid wasps, myrmecolacid strepsipteran, and social parasitic ants). They summarize published information and include many complementary unpublished observations. Among these natural parasites, a more special focus is given by S. M. Valles about the research on viruses through a compilation of the literature on fire ant viruses, and a review on the properties of three particular viruses infecting *S. invicta*. It is worth noting that viruses were unknown in any ant species before the first discover by Valles and colleagues in 2004 [11], and this topic will certainly be a central issue in fire ants control programs in the future. Finally, the evaluation of the use of *Pseudacteon* parasitoid flies as potential biological control agents of invasive *Solenopsis* fire ants is critically and exhaustively reviewed by L. W. Morrison. The sound conclusions of this review about the realities of biological control of fire ants by phorid flies contrast with most of the literature on that topic and emphasize the necessity of a battery of complementary natural enemies, in addition to the release of phorid flies, for potentially successful regulatory effects on fire ant populations. This points to a need for investing more effort into studies on other potential control agents.

More and more studies show a fascinating coevolution between parasites and their hosts. An accurate survey of this topic will provide useful information to refine our understanding of both the mechanisms involved and their

phylogenitical and evolutionary components. With the growing interest in biodiversity, we realize that we are far from concluding our assessment of existing forms of parasitism. Considering the increasing losses in biodiversity due to habitats restructuration and climatological changes, the urgent need for making such inventories is obvious. The world of the microorganisms is one of the most promising. For example, G. Poinar Jr. indicates that some 20,000 nematodes have been described, while their species diversity has been estimated to be as high as 10 million, and even if the proportion of known species associated with ants is low, their real number is surely much more impressive than actually suggested. A metagenomics approach will be useful in the future; it has already begun in honeybees [12] and in termites [13] and is just starting up in ants [14]. Parasites contribute to maintaining complex ecosystems and have a role in stabilizing mutualisms as observed in fungus-growing ants [15]. Their role as a "top-down" process, structuring ant communities and populations, is also considered to be important. Some authors, like Feener [16], suggest that the assembly of ant communities is mediated by parasitoids. Others [2, 17, 18] suspect that parasites and parasitoids may be involved in the emergence of alternative reproductive strategies such as polygyny and/or multiple mating (polyandry), by inducing an enhancement in the genetic diversity of the workers that would increase resistance to parasites and pathogens (but see [19]). Recent data on the evolution of elaborate mushroom bodies in the brains of hymenopteran insects even suggest that the neurobehavioral modifications linked to the capacity for associative and spatial learning during host-finding behavior in parasitoids may have served as preadaptations for central place foraging in social hymenopterans [20]. Ants (and, more generally, social insects) and their parasites are an exceptional model. In the next years, more studies examining their complex interactions from every possible angle, attempting to bring a more global vision of the functioning of such an evolutionary important relationship, will surely constitute a challenging and fascinating goal for us and many colleagues.

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Research Article

Nesting Activity and Behavior of *Osmia cornifrons* (Hymenoptera: Megachilidae) Elucidated Using Videography

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Osmia cornifrons Radoszkowski (Hymenoptera: Megachilidae) is utilized as an alternate pollinator to *Apis mellifera* L. (Hymenoptera: Apidae) in early-season fruit crops. This study was conducted to investigate nesting activities and associated behaviors of *O. cornifrons*. *Osmia cornifrons* nesting activity was recorded by using a digital video recorder with infrared cameras. Nesting behavior of ten female *O. cornifrons* was observed, and the number of nesting trips per hour was recorded. Trends in daily activity were determined with regression analysis, and chi square analysis was used to determine if *O. cornifrons* spent a greater amount of time performing certain activities. The percentage of time required to gather nesting resources and complete nest construction activities was recorded from the video footage. Results of this study showed that pollen gathering was the most time-consuming gathering activity, requiring 221.6 ± 28.69 min per cell and cell provisioning was the most time-consuming intranest activity, requiring 28.9 min ± 3.97 min. We also found that *O. cornifrons* activity was correlated with time of day, temperature, and precipitation. Various nesting behaviors, including cell provisioning and partitioning, oviposition, grooming, resting and sleeping, nest-searching, and repairing behaviors, are described in this paper.

1. Introduction

Pollination services are both economically valuable [1] and essential to many crop production systems [2]. With colony collapse disorder and various pests threatening the honeybee [3] and issues such as habitat fragmentation and pesticides threatening wild pollinators [4], considerations for alternate pollination strategies by effectively managing solitary bees have become more relevant to agricultural production [5].

The Japanese hornfaced bee, *Osmia cornifrons* Radoszkowski (Hymenoptera: Megachilidae), is an important pollinator of rosaceous fruit crops such as apple and pear. Historically, *O. cornifrons* has been managed in Japan for apple pollination since the 1940s and was introduced into the United States for pollination in 1977 [6]. Additionally, *O. cornifrons* is being used for orchard pollination in Korea and China [7, 8]. *Osmia cornifrons* has been shown to be up to 80 times more effective at pollinating apples than *A. mellifera* [9] and has several benefits over *A. mellifera* such as flower constancy and consistent anther contact [10]. Despite these benefits, *O. cornifrons* remains an underutilized pollinator in the United States.

Understanding the nesting biology of *O. cornifrons* is important for management of the bees for growers, population managers (i.e., those who sell the bees to growers), and researchers. For example, by understanding *O. cornifrons* nesting biology, one can select release sites where *O. cornifrons* has access to adequate resources. Understanding the limiting factors of *O. cornifrons* activity, such as temperature thresholds, allows one to predict if *O. cornifrons* will be pollinating on a given day of the blooming season. In addition, knowing the nesting biology of *O. cornifrons* provides growers and researchers with insights into the biology of other *Osmia* bees such as *O. lignaria*, a managed solitary bee pollinator in the United States.

Observing nesting behavior of solitary bees such as *O. cornifrons* can be challenging because it is difficult to observe bees inside their nests. Despite this challenge, several aspects of *O. cornifrons* nesting biology have been described previously. Yamada et al. [11] described nesting behaviors of

O. cornifrons including cell provisioning, mud wall partitioning, and the time required to gather pollen and mud by utilizing glass tubes wrapped in paper as artificial nests. The paper could be removed from these glass tubes after the bee entered, which permitted *O. cornifrons* nesting activities to be observed. A major disadvantage of using glass tubes is that *O. cornifrons* could be disturbed by a sudden and un-natural increase in light levels in the innermost portion of the tube when the paper is removed from the glass tube; Lee et al. [12] noted that luminance is an important factor affecting *O. cornifrons* activity. In addition, the presence of visible observers has been found to alter the frequency of activities in some insects, such as damselfly nymphs [13].

This study investigated the nesting biology of *O. cornifrons* and described in detail the behaviors associated with nesting activities. There were four objectives in this study: (1) developing an unobtrusive and novel method to observe the nesting behavior of solitary bees, (2) investigating the factors that affect *O. cornifrons* activity levels, (3) determining how much time is allocated to gathering nesting resources and constructing the nest, and (4) describing the behaviors that occur during nest construction.

2. Materials and Methods

2.1. Experimental Insects. *Osmia cornifrons* used in this experiment were acquired from a population that had been successfully established and managed for several years prior to the experiment on a blueberry farm in Independence, WV (N 39.46992, W 79.934651). In early November 2009, the bees were brought into the laboratory at West Virginia University (Monongalia County, WV) and placed into cold storage at 5°C for overwintering. On 9 May 2010, the bees were released in a residential area in Morgantown, WV, USA (N 39.666871, W 79.965523), where a power source for prolonged video recording of bee nest was readily available. Wilson et al. [14] stated that *O. cornifrons* could be successfully released and propagated on landscape plants in a city.

2.2. Developing a Protocol for Observing O. cornifrons In-Nest Activity. To effectively record in-nest activities of *O. cornifrons*, three camera housings were constructed from white pine and Masonite boards (Figure 1). An opening was cut into the front of the box to allow six observation nest blocks [15] to sit below the camera (Figure 2). A Masonite board roof with a 3.5 cm × 4 cm × 1.5 cm block of white pine attached to the center held an infrared camera (The Hawk Eye Nature Cam, West Linn, OR, USA) with the lens 44.3 cm from the bottom of the release box. The camera emits infrared light allowing for continuous observation without disturbing *O. cornifrons*. Cameras were connected to a 4-channel digital video recorder (DVR) (Falco Model LX-4PRO, Falco Pro Series, Taiwan) to record continuously for the entire duration of *O. cornifrons* nesting activity. The three camera housings were placed next to a building facing south and were covered with plastic to help shelter the nests from rain. A fourth camera was set outside the three camera housings facing the nest entrances. This camera was used

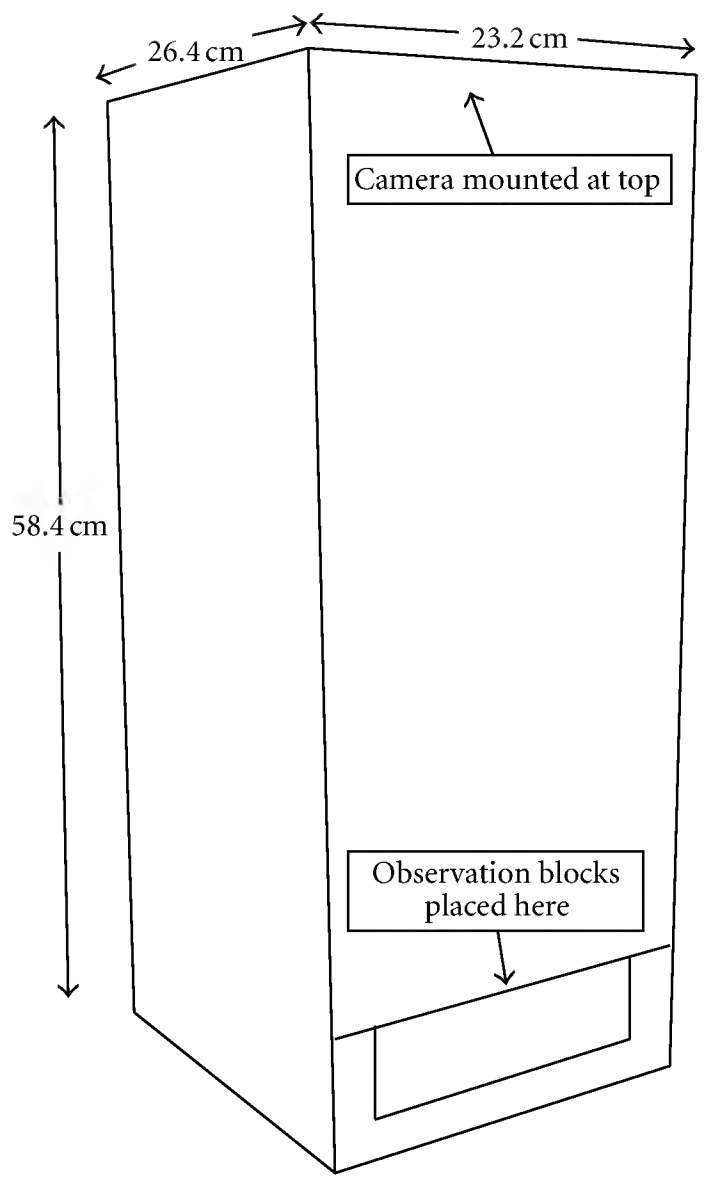

Figure 1: Diagram of camera housings for recording intranest behavior of *O. cornifrons*. The frame was made of white pine timber, and the walls were made from Masonite boards. The camera was mounted to the lid and faced down toward the observation blocks. A small groove was cut into the back of the frame to allow space for the camera cord.

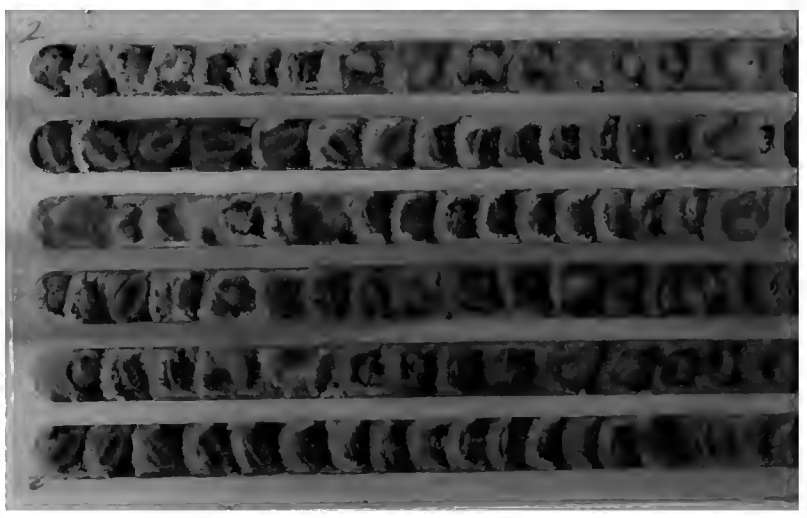

Figure 2: An example observation nest block with *O. cornifrons* cocoons. The nest block was designed for videotaping nesting behavior of *O. cornifrons* by covering the top of the block with transparent plastic film.

to observe *O. cornifrons* searching behaviors and to record the weather. Nesting activities of *O. cornifrons* were recorded from 9 May 2010–1 June 2010.

2.3. Determining Factors Affecting O. cornifrons Activity. To determine the daily activity pattern of *O. cornifrons*, the number of trips from observation nest blocks initiated per

hour was recorded for ten bees from 15 May 2010–21 May 2010. Data were taken from the start of nesting to six days later. Only those trips where *O. cornifrons* gathered nesting materials (i.e., pollen or mud) were used to determine daily activity levels. The relationship between the number of trips *O. cornifrons* initiated and the time of day was determined using nonlinear regression analysis (SigmaPlot 11, Systat Software, Inc., San Jose, CA, USA).

To determine the effect of temperature on *O. cornifrons* activity levels, hourly climate data were obtained from a National Climate Data Center (NCDC) weather station located ca. 3.3 km from the study site. The weather station (i.e., MGTN RGNL-W L B HART FD AP located at N 39.642867, W 79.919947) is an automated surface observing system weather station which reports NCDC version 3 climate data. Bee activity data (i.e., number of trips initiated per hour) from 18 May 2010 (sunny day) were used to correlate temperature with activity. Correlation between precipitation and bee activity from 16-17 May 2010 (rainy days) was analyzed to determine the effect of precipitation on *O. cornifrons* activity. Because the weather station reported trace precipitation (<0.25 mm rain) without a numerical value, hours of trace precipitation are considered to be 0.025 mm of rain. Correlations of bee activities with temperature and precipitation were analyzed with Pearson's product moment correlation using SigmaPlot 11.

2.4. Intranest Activity of O. cornifrons. To determine the amount of time spent by *O. cornifrons* on different in-nest activities, video data were logged for ten bees from 15 May 2010–21 May 2010: three bees from camera 1, three bees from camera 2, and four bees from camera 3. Intranest activities included nest scouting, construction of preliminary plugs, cell provisioning, oviposition, cell partitioning, resting, grooming, sleeping, fighting, and other activities. For each activity, duration was measured as follows: (1) the start time was taken from the point at which the bee reached the area of the nest being constructed, (2) the stop time was taken from the point work activity ceased, (3) if the bee stayed in the nest for >20 s after building activity ceased, this extra time was recorded along with noting after work activities. A chi-square test determined if the time requirements of intranest activity differed significantly using SigmaPlot 11.

2.5. Gathering Activity of O. cornifrons. To determine the amount of time *O. cornifrons* requires for gathering nesting materials, time away from the nest was recorded for ten bees during every trip made. Trip times were recorded from the start of nesting (15 May 2010) until six days later (21 May 2010). Only trips in which nesting materials were brought back to the nest were used in data analysis. A threshold of 1 h was set for pollen gathering trips and 30 min for mud gathering trips. Any trips exceeding these thresholds were excluded from the data used in calculating the time requirements of gathering activities. The thresholds were not used to calculate the number of trips that *O. cornifrons* took to complete one part of a cell. This was done to account for trips in which *O. cornifrons* engaged in both gathering and nongathering activity (e.g., resting), while still being able to report an accurate number of trips required to complete the provisioning and partitioning of a cell. A chi-square test determined if time requirements of gathering activity differed significantly using SigmaPlot 11.

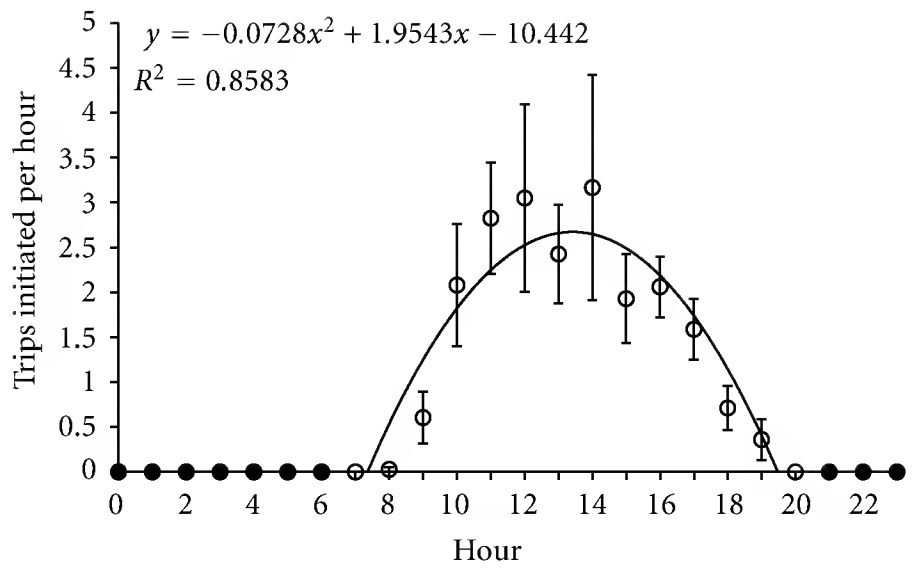

FIGURE 3: The average number of trips taken per hour by *O. cornifrons*. Hour 0 is 12:00 am and hour 23 is 11:00 pm. Error bars indicate standard error. Only the open circles were used to determine the regression equation.

2.6. Description of O. cornifrons Behaviors. To describe *O. cornifrons* behaviors, 30 *O. cornifrons* were observed from the time nesting was initiated (15 May 2010) until nesting ceased or six days later (21 May 2010), whichever came first. Behaviors were divided into nesting behaviors and nonnesting behaviors. Any behaviors performed during nest constructing activities were considered nesting behaviors and all other behaviors were considered nonnesting behaviors. Nesting behaviors included scouting behavior, preliminary plug behavior, cell provisioning behavior, oviposition behavior, and cell partitioning behavior. Nonnesting behaviors included grooming behavior, resting behavior, sleeping behavior, fighting behavior, nest-searching behavior, nest repair, and nest supersedure. Additionally, other behaviors that did not fall under any of the listed categories were also recorded and described.

3. Results

3.1. Factors Affecting O. cornifrons Activity. Data of daily nesting activity of *O. cornifrons* was fitted with a second-order polynomial trend (Figure 3): $y = -0.0728x^2 + 1.9543x - 10.442$ (d.f. = 2, 13; $F = 33.30$; $P < 0.0001$; $r^2 = 0.86$), where y is the number of trips initiated per hour and x is time of day. Daily activity was tested for normality using the Shapiro-Wilk normality test and was found to be normally distributed ($W = 0.9053$; $P = 0.1346$; $\alpha = 0.05$). Variance of daily activity data was constant when disregarding the time of day based on the constant variance test ($P = 0.3642$). All nesting activities of *O. cornifrons* occurred between 7:00 am and 8:00 pm, and the most trips initiated per hour occurred between 10:00 am and 6:00 pm. *O. cornifrons* was not active on days when it rained (Figure 4).

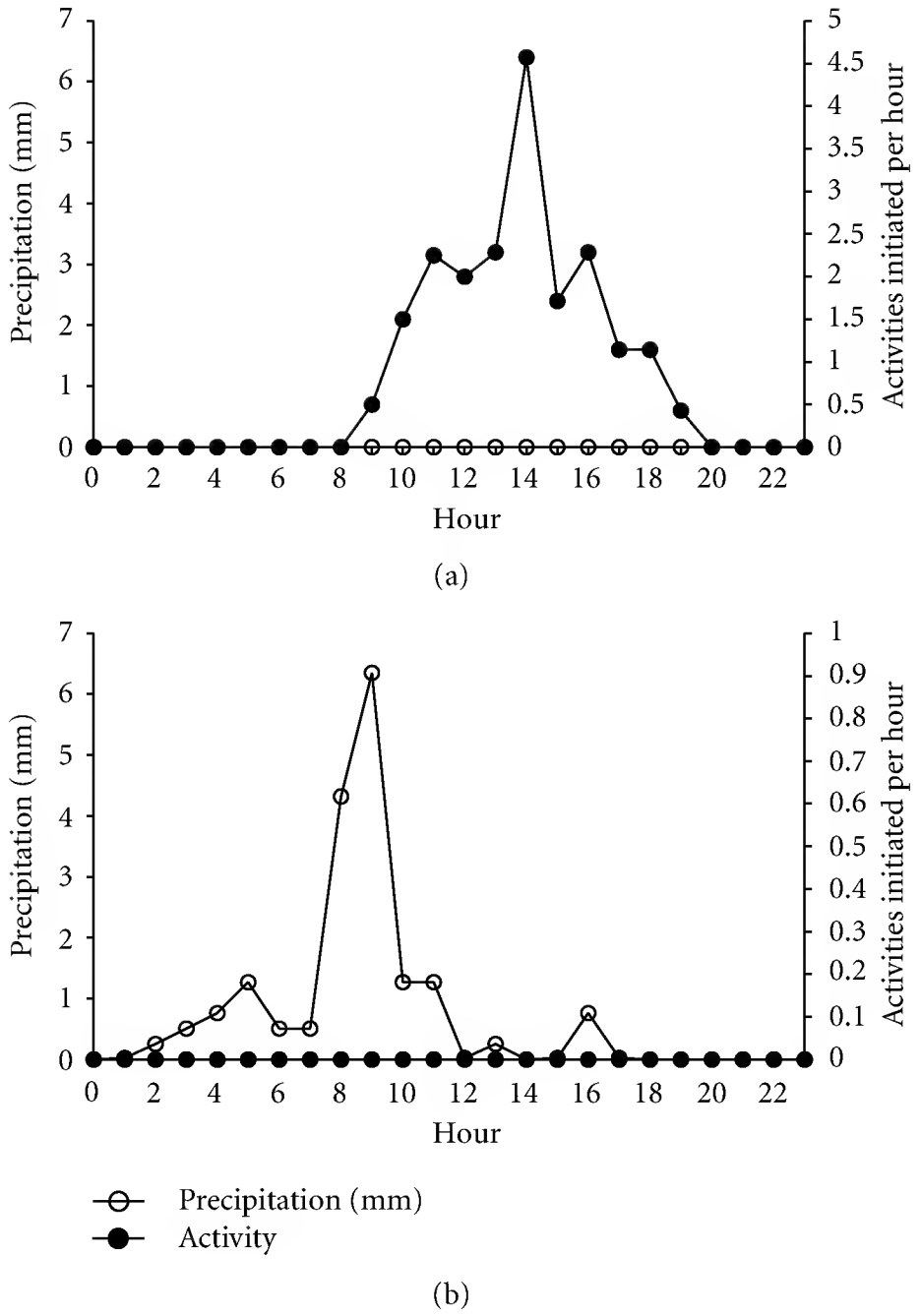

FIGURE 4: Relationship between *O. cornifrons* activity and precipitation on a day without rain (16 May 2010) (a) and a day with rain (17 May 2010) (b). Hour 0 is 12:00 am and hour 23 is 11:00 pm.

Individuals responded to rain by staying in their nests and occasionally walking to the nest entrance and looking out, but not exiting the nest.

Results of the Pearson product moment correlation test showed that activity and temperature were significantly correlated ($n = 24$; $\rho = 0.856$; $P < 0.0001$). The positive correlation coefficient indicates that *O. cornifrons* activity increased with temperature (Figure 5). *O. cornifrons* were not active below 13.9°C.

3.2. Intranest and Gathering Activities of O. cornifrons. The average total duration of labor required for cell completion (i.e., pollen provisioning, oviposition, and mud wall partitioning) was 51 min ± 6.5 min, and average time to complete the preliminary plug was 27 min ± 2.5 min. Provisioning the cell took most of the total time, requiring 29 ± 4.0 min (i.e., 57% of the total time to complete a cell). Building the mud-wall partition required 20 ± 1.8 min (i.e., 40% of the total time to complete a cell). Oviposition required only 3% of the total time to complete a cell, requiring 2 ± 0.7 min. Cell provisioning was the most time-consuming intranest activity, requiring 28.9 min ± 3.97 min.

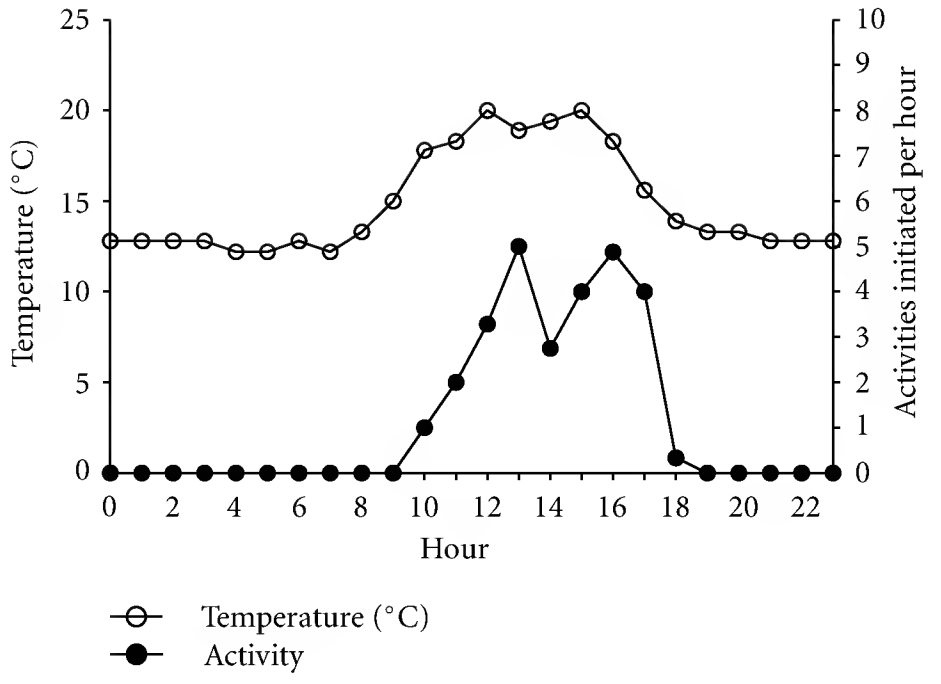

FIGURE 5: Relationship between *O. cornifrons* activity and temperature. Hour 0 is 12:00 am and hour 23 is 11:00 pm.

The total time required for *O. cornifrons* to gather pollen and mud for one cell was 255 ± 36.8 min, and gathering mud for a preliminary plug required 45 ± 13.7 min. Gathering pollen took 222 ± 28.7 min with an average of 19.8 trips. Gathering mud for the cell partition took 33 ± 8.1 min with an average of 11.5 trips.

3.3. Nesting Behaviors of O. cornifrons. Most nesting behaviors were distinct and consistent throughout the recorded video. Scouting behavior was the most variable behavior observed. When *O. cornifrons* searched for nests, they entered empty nests and moved to the back of the nest. Then they performed a series of forward and backward movements accompanied by turning upside down and left to right, inspecting the nest thoroughly. Finally, they turned and left the nests, occasionally coming back and performing these behaviors again.

During preliminary plug activity, *O. cornifrons* focused on plugging the upper edges of the nest, where the transparency film was attached to the observation block. During many of these trips, *O. cornifrons* moved back and forth repeatedly. This is likely a method used by the bees to measure distance [11]. They used their middle legs for support by holding them out perpendicular to their bodies and grasping the sides of the nest. Then, while holding mud with their mandibles, they bent their abdomen up until the apex of the abdomen was nearly in contact with the mandibles. Moving backwards, the mud ball was spread like a paste onto the nest surface. Use of the abdomen for nest building usually occurred during the preliminary plug activity and most often when the corner formed by the nest and the transparency film was being plugged. Although the use of abdomen during preliminary plug construction may be an artifact of the transparency film in this study, such behavior was observed in *O. lignaria* during mud gathering [16, 17] and mud wall construction [17].

Cell provisioning started when *Osmia cornifrons* females approached the rear of the nest where the pollen ball was being made. They then manipulated the pollen ball with their

mandibles by either pecking at the pollen ball or pushing the pollen ball with the mandibles, using them like a shovel. During this time nectar from the crop was added to the pollen ball. After mandibulating the pollen ball, they turned in the nest and backed up so that their abdomen was over the pollen ball. Then they scraped the pollen from their scopa with their hind legs.

Oviposition behavior looked deceptively similar to cell provisioning behavior. Before oviposition *O. cornifrons* mandibulated the pollen ball, and then turned to oviposit. The primary difference in behavior between oviposition and provisioning was that the abdomen moved vigorously during nest provisioning, but it was very still during oviposition.

Osmia cornifrons females started building a mud wall partition by creating a mud ring around the inner circumference of the nest. Building the ring usually took several trips, and the abdomen was occasionally used to spread mud around the ring in concentric circles. Once there was just a small opening left in the ring they placed mud in the hole and then rotated their entire body several times with their face seemingly directly in contact with the mud wall.

3.4. Nonnesting Behavior of O. cornifrons. Grooming behavior and resting behavior were the most commonly observed nonnesting behaviors. An *Osmia cornifrons* female often groomed itself right after provisioning a cell. Grooming entailed using the front legs to clean off the antennae as well as shaking the abdomen back and forth and rubbing it with the hind legs, seemingly to clean the scopa before the next pollen load was gathered. Frequently *O. cornifrons* would groom itself as it made a hasty exit from the nest. Usually the process did not take more than 20 s to complete and did not slow down nest building activity. When grooming took more than 20 s, it was often followed by resting activity. *Osmia cornifrons* was considered resting when it was in the nest but was not performing any noticeable activity.

Sleeping behavior was defined as all activity that occurred between the final trip of one day and the first trip made the following day. Most frequently, after the final activity of the day, *O. cornifrons* would move about the nest, seemingly giving the nest a thorough inspection. After inspection, activity would cease for several minutes at a time, and if *O. cornifrons* moved, it was only ca. 4 cm. Finally activity would cease for several hours at a time, and if the bee moved, it would most often simply turn sideways. The bees often slept sideways or upside down inside the nest. Many bees did not sleep in the nests at all but returned the next day. If it rained on the morning after a bee had been sleeping outside its nest, the bee did not return to its nest during the rainy day but did return the following day. Some *O. cornifrons* also slept in empty nests. In the morning, as light entered the nest entrances, *O. cornifrons* would begin to move again. Most often, *O. cornifrons* moved ca. 4 cm then ceased movement again for some time. Eventually *O. cornifrons* would go to the nest entrance and look outside. Sometimes they left immediately, but more frequently they moved back into the nest and waited. On a few occasions a bee took flight only to return a few minutes later and resume a resting state.

There was only one observation of an attempt to repair a damaged nest. The nest became damaged when one corner of the transparency film cover became detached from the nest, and when that occurred, one individual attempted to repair the uncovered area. First it spent a great deal of time inspecting the damaged area, then it began gathering mud and trying to patch the open area at the back of the nest. It made 13 trips and patched a large area of the opening but was unable to successfully close it. After the bee's unsuccessful attempt to repair the nest, it seemed to abandon the nest.

4. Discussion

Solitary bee activity levels might be affected by time of day, temperature, or precipitation. *Osmia cornifrons* has previously been observed foraging as early as 6:10 am and as late as 6:00 pm [18]. The earliest time *O. cornifrons* became active in our study was 8:00 am and activity continued until as late as 8:00 pm, a result similar to that recorded by Matsumoto and Maejima [18]. Lee et al. [12] reported that temperatures above 20°C caused an increase in *O. cornifrons* activity. Our study showed that the minimum temperature for *O. cornifrons* to be active was 13.9°C, and bee activity increased with temperature. Matsumoto and Maejima [18] observed *O. cornifrons* activity at temperatures as low as 10.7°C. The difference in the observed minimum temperature for *O. cornifrons* activity in our study and Matsumoto and Maejima's [18] observations may be due to temperature tolerance differences between populations of *O. cornifrons*. In our study, *O. cornifrons* did not fly on rainy days, though this might be attributed to low temperatures on those days; the maximum temperature on the rainy day analyzed in this study was 14.4°C which is 0.5°C above the determined minimum temperature threshold for activity. *O. cornifrons* was most active on warm, sunny days. Therefore, *O. cornifrons* can be expected to be most active from 10:00 am to 6:00 pm on warm days (>13.9°C) without precipitation.

The majority of time that *O. cornifrons* spent performing nesting activities was used for gathering pollen and provisioning the nest, which agrees with information reported by Lee et al. [12]. The average number of cells in *O. cornifrons* nests is 9.5 [15], and results of this study showed that the average number of trips to complete a cell was 31.3. This means that it takes an average of 297 trips for *O. cornifrons* to complete a nest, though this could vary as the nesting season progresses. Comparatively, *O. lignaria* was found to require an average of 32.4 trips to provision a cell and 6.9 trips to construct a mud wall [17] and was found to construct 3.64 cells per nest on average [19].

In addition to observing *O. cornifrons* behaviors described previously, two unusual behaviors were observed that have not been described in detail previously. First, one case of nest supersedure was observed in this study. An *O. cornifrons* female had oviposited in the back of its nest and began building a mud wall. For an unknown reason the bee seemed to abandon the nest but may have been a victim of predation. Two days later, another bee entered the nest, destroyed the original egg, laid a new egg, and finished the mud wall.

Second, *O. cornifrons* females sometimes seemed to have difficulty relocating their nests. Many times a female would enter a nest and immediately turn around and leave, then enter an adjacent nest. Sometimes a bee would enter two or three nearby nests before finally entering its own nest. When *O. cornifrons* entered a nest occupied by another female *O. cornifrons*, fighting took place. During fighting, *O. cornifrons* utilized its mandibles to fight off intruding *O. cornifrons*. Also during fighting, *O. cornifrons* would bend its abdomen forward putting its body in a C-shape. It was difficult to observe from the video footage if the abdominal behavior was being used for offensive or defensive purposes. The duration of fighting was usually several minutes, and most often the original bee displaced the intruder. On some occasions nest constructing activity was interrupted by intruding bees. When an interruption like this occurred, the bee failed to complete the activity it had been working on prior to the interruption and instead inspected the back of the nest and began the behavior all over again.

Osmia cornifrons behaviors described by Yamada et al. [11] were found to be similar to those observed with the video in our study. This indicates that *O. cornifrons* is likely not disturbed by using glass tubes to view their nesting behaviors. Still, the video method has advantages over using glass tubes which make it a valuable tool for studying solitary bees: (1) it does not require the physical presence of the researcher, (2) it can gather data on activities nonstop for weeks at a time which is nearly impossible to do otherwise, (3) video footage can be rewound, sped up, or slowed down as needed to analyze the data, and (4) video footage can be archived and used in other studies. The biggest disadvantages of using the video are the power requirements to run the equipment, the time-consuming nature of watching video footage, the cost of the equipment, and the possibility of technological failure.

This study showed that *O. cornifrons* was most active between 10:00 am and 6:00 pm, and they spent most of their active time gathering pollen and provisioning their nests. It also showed that temperature and precipitation have strong effects on the activity of *O. cornifrons*. This information is important as it can be used to avoid pesticide application during *O. cornifrons* peak activity. Our results indicate that pesticide application should be avoided between the hours of 11:00 am and 4:00 pm to reduce direct contact with foraging *O. cornifrons*.

Ideally, pesticide application should not occur between 7:00 am and 8:00 pm, but this is an impractical recommendation for most growers. Furthermore, observations of *O. cornifrons* sleep habits indicate that they frequently sleep outside the nest, which means that it may be impossible to completely avoid affecting *O. cornifrons* with pesticide sprays. Previous management practice has been to place *O. cornifrons* in the field seven to ten days before crop bloom [10]. From the data gathered it is recommended that growers wait for several days of temperatures above 13.9°C so that the bees can maintain activity after emergence. Releasing *O. cornifrons* in colder weather than this will hinder their ability to perform pollination duties and may cause the bees harm as they cannot forage in the cold temperatures. Additionally, this type of data would be useful in investigating seasonal age differences in the time to provision a brood cell and determining the effect of pesticides on *O. cornifrons* behavior by comparing video footage of *O. cornifrons* in treated and untreated fields.

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Review Article

Generalist Bee Species on Brazilian Bee-Plant Interaction Networks

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Determining bee and plant interactions has an important role on understanding general biology of bee species as well as the potential pollinating relationship between them. Bee surveys have been conducted in Brazil since the end of the 1960s. Most of them applied standardized methods and had identified the plant species where the bees were collected. To analyze the most generalist bees on Brazilian surveys, we built a matrix of bee-plant interactions. We estimated the most generalist bees determining the three bee species of each surveyed locality that presented the highest number of interactions. We found 47 localities and 39 species of bees. Most of them belong to Apidae (31 species) and Halictidae (6) families and to Meliponini (14) and Xylocopini (6) tribes. However, most of the surveys presented *Apis mellifera* and/or *Trigona spinipes* as the most generalist species. *Apis mellifera* is an exotic bee species and *Trigona spinipes*, a native species, is also widespread and presents broad diet breath and high number of individuals per colony.

1. Introduction

Bees are important keys to global diversity providing vital ecosystem services such as pollination [1]. For bees, plants species are basically their main food sources, where they collect pollen and nectar and eventually other resources, such as oil. Plants are those which have interest on bees' skills to achieve successful reproduction. They have to deal with different foraging techniques employed by bees [2–4] to reach their main reward, reproduction.

In Brazil, until the end of 1960s, information about bee-plant interactions came mostly from observations made by naturalists of the early 20th century. However, the study of [5] proposed a standardized methodology to perform bee surveys that was subsequently applied to most of them, allowing further comparisons between the different surveyed localities.

The studies of [6–8] made previous synthesis of Brazilian surveys. The first one only compared information about species richness found in different biomes. The other two studies used only data from eusocial Apidae found on the surveys. Until now, no attempt was made to determine the generalist bee species in interaction networks of different localities using all Apoidea species found on them.

Interaction networks are built as a matrix of interacting species and have been justified mainly because networks involving plants and pollinators are generalists and form complex systems bringing additional challenges to their study [9].

In plant-pollinator interactions, species are commonly seen as generalists when they interact with many species of different taxa, and specialists if they interact with one or a few closely related species [10]. Reference [11] showed that in pollination systems the most generalized species are usually network keystone species. Since they interact with most plant species, they play an important role to maintain the whole network.

The main goal of this study was to determine the most generalist bee species on bee surveys conducted on different localities in Brazil.

2. Material and Methods

We searched the academic literature for bee surveys on flowering plants on different localities of Brazil, aiming to

Figure 1: Brazilian localities where the bee surveys were conducted.

build a matrix of bee-plant interactions to each locality. We considered the surveys that used the standard procedure suggested by [5] and whose observations were made for at least one year. In this procedure a fixed amount of time is spent at each flowering plant (or patch) and the coverage of transects is randomized in time, order, and direction. On most of these surveys, the interactions were not detailed and could include effective pollination and/or nectar, pollen, or oil foraging.

Many survey datasets have been published only in M.S. or Ph.D. thesis and are only available to the public at their universities. When these works were subsequently published as a paper, both datasets were compared and both were cited on the reference list.

The bee taxonomic names were updated according to [12]. We discarded the observations that were taxonomically unresolved.

We used the bipartite package [13] for R 2.11.1 (The R Foundation for Statistical Computing) to analyze each matrix. Each cell of the matrix represents a single bee-plant interaction and can have a value of 0 if the interaction is not observed, or 1, if observed [14]. With this tool we determined the first three bee species with the highest number of interactions.

The declared coordinate point of each survey was also used to build a map with ArcGIS 10 software (Esri Inc.).

3. Results

We found 47 localities whose surveys fulfilled the requirements previously quoted on the methodology section. Most surveys were done on South, Southeast, and Northeast regions of Brazil, either on urban areas, on seasonally dry areas of Tropical Dry Forest (Brazilian Caatinga) and Tropical Shrublands (Brazilian Cerrado), or on Tropical Moist Forest (Brazilian Atlantic Forest) biomes (Figure 1, Table 1). We did not find any bee survey on the North region and only one on the Midwest region of Brazil.

The first, second, and third most interacting species on each surveyed locality are found on Table 1. We found a total number of 39 different species. Most of them belong to Apidae (31 species) and Halictidae (6) families, and to Meliponini (14) and Xylocopini (6) tribes (both from Apidae family). The genus with the highest number of interacting species was *Xylocopa* (5 species). The genera *Trigona*, *Exomalopsis*, and *Augochloropsis* presented each three interacting species.

Table 1: Number of times bee species were quoted as the first, second, or third most interacting species on the Brazilian bee surveys.

Family	Tribe	Species	1st	2nd	3rd	Biome (*sensu lato*)	References
Andrenidae	Oxaeini	*Oxaea flavescens*		1	1	Tropical Shrublands	[15, 16]
Apidae	Apini	*Apis mellifera*	20	15	2	Various	[15, 17–56]
	Bombini	*Bombus morio*		1		Tropical Shrublands	[26]
		Bombus pauloensis	2	4	3	Tropical Shrublands, Tropical Moist Forest and Urban Area	[26, 33, 36, 57–62]
	Centridini	*Centris klugii*			1	Tropical Shrublands	[26]
		Centris leprieuri			1	Tropical Shrublands and Tropical Moist Forest	[45, 46]
		Melissoptila cnecomala		1		Tropical Shrublands	[26]
	Euglossini	*Eulaema nigrita*	1			Tropical Moist Forest	[27]
	Exomalopsini	*Exomalopsis analis*			1	Dune	[22]
		Exomalopsis auropilosa	1			Urban Area	[62]
		Exomalopsis fulvofasciata	1		1	Tropical Shrublands	[41, 63, 64]
	Meliponini	*Cephalotrigona capitata*		1		Tropical Moist Forest	[65]
		Melipona scutellaris			1	Tropical Moist Forest	[51–53]
		Mourella caerulea	1			Tropical Shrublands	[61]
		Paratrigona lineata	2			Tropical Dry Forest and Tropical Shrublands	[32, 57]
		Paratrigona subnuda	1	1	1	Urban Area and Tropical Moist Forest	[34, 35, 42, 43, 55, 56]
		Plebeia droryana		1	1	Urban Area and Tropical Shrublands	[50, 66]
		Plebeia emerina		1	1	Tropical Moist Forest	[39]
		Scaptotrigona bipunctata			1	Tropical Shrublands	[66]
		Scaptotrigona tubiba			1	Tropical Moist Forest	[67]
		Tetragona clavipes			1	Tropical Shrublands	[38]
		Tetragonisca angustula		1	6	Various	[17, 27, 28, 40, 48, 50, 54, 63, 64]
		Trigona fulviventris			2	Tropical Moist Forest	[37, 39]
		Trigona pallens	1			Tropical Shrublands	[44]
		Trigona spinipes	16	14	11	Various	[15–21, 24–56, 61, 63–67]
	Tetrapediini	*Tetrapedia rugulosa*			2	Tropical Shrublands	[23–25, 30]
	Xylocopini	*Ceratina maculifrons*			2	Urban Area and Tropical Shrublands	[16, 62]
		Xylocopa carbonaria	1			Dune	[22]
		Xylocopa cearensis			1	Urban Area	[49]
		Xylocopa grisescens			1	Tropical Dry Forest	[29]
		Xylocopa ordinaria			1	Urban Area	[49]
		Xylocopa suspecta			1	Tropical Moist Forest	[47]
Halictidae	Augochlorini	*Augochlora esox*			1	Tropical Dry Forest	[57]
		Augochloropsis callichroa		1		Tropical Moist Forest	[67]
		Augochloropsis crassiceps			1	Tropical Moist Forest	[65]
		Augochloropsis illustris		1		Urban Area	[62]
		Ceratalictus theius		1		Tropical Shrublands	[58–60]
	Halictini	*Dialictus opacus*		3		Urban Area, Tropical Dry Forest and Tropical Shrublands	[18–21, 49, 58–60]
Megachilidae	Anthidiini	*Hypanthidium divaricatum*			1	Tropical Moist Forest	[39]

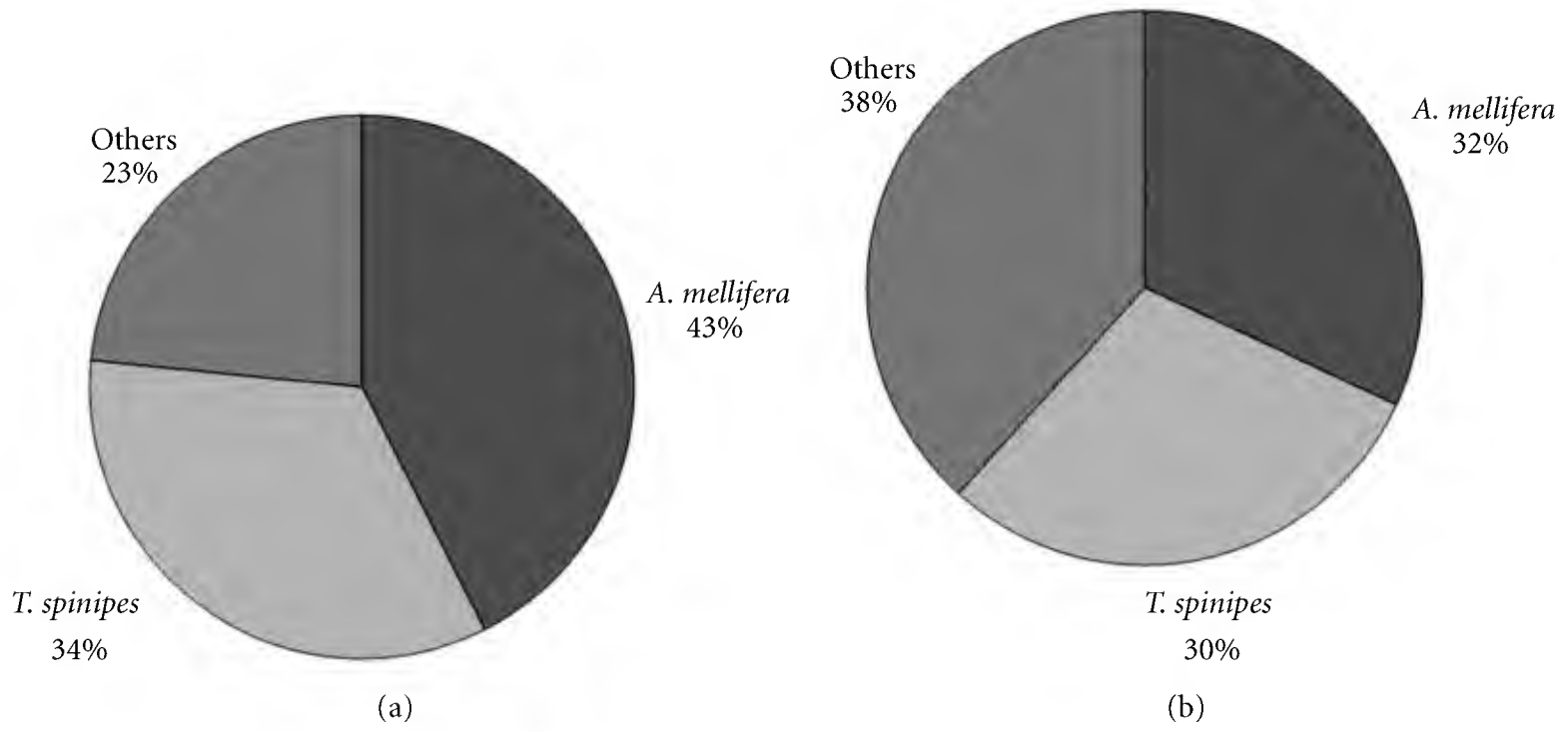

Figure 2: Percentage of Brazilian bee surveys presenting *Apis mellifera* or *Trigona spinipes* as (a) the first species with highest number of interactions and (b) the second species with highest number of interactions.

On most surveys *Apis mellifera* and/or *Trigona spinipes* were the most interacting species (Table 1). Considering the first and second species with the highest number of interactions, *A. mellifera* was present on 75% of the Brazilian surveys and *T. spinipes* on 64% (Figure 2).

Other important interacting species were *Bombus pauloensis* that was quoted nine times on the different localities and on different ranks (first, second, or third interacting species), *Tetragonisca angustula* (7 times), *Dialictus opacus,* and *Paratrigona subnuda* (3 times each) (Table 1).

Halictidae and Xylocopini species as well as *A. mellifera* and *T. spinipes* were found on different biomes (Table 1). Most of Meliponini species were found on Tropical Moist Forest and on Tropical Shrublands.

4. Discussion

Apidae is a large family of bees, whose species are mostly generalist foragers. It is widely distributed, occurring on different biomes under different environmental characteristics [68]. Bee species from the Meliponini tribe live in tropical and subtropical regions of the world and are considered to be important pollinators of plant species on different environments [69].

T. spinipes, one of the most generalist stingless bee species according to our results, presents colonies with a huge number of individuals and wide diet breath, and it shows widespread distribution over the Brazilian territory. Moreover, they build aerial nests, being independent of any kind of holes to nidify. Independence of holes and the great availability of workers may determine the degree of dispersion over the countryside and the generalist interacting behavior [70].

A. mellifera is an exotic bee species also widespread in different biomes. It is well adapted to different climatic conditions and presents a generalist foraging behavior. Despite the potential negative impact on native pollinator species [71], it was already recognized as the most important pollinator of natural environments and also of agricultural crops [72].

Although we are not aware of any study comparing *A. mellifera* and *T. spinipes* pollinating performance, they are probably important resource competitors, due to their similar colony size and widespread distribution. The efficient communication system exhibited by *Apis mellifera* and the aggressive behavior on flowers, already reported to *T. spinipes*, complete this scenario [73, 74].

Recent reports of the colony collapse disorder syndrome of *Apis mellifera* species arouse the awareness of the importance of this species [75], especially due to its importance to agriculture. At the same time, it also brings the attention to the native pollinators and their importance to local crops, and international initiatives have been suggested to protect them [76].

Far from the number of interactions found for the two main species, two other generalist bee species were *B. pauloensis* (9 interactions) and *T. angustula* (7 interactions). Both species were found in distinct biomes, including urban areas, thus suggesting a broad ability to survive at different environmental conditions. But unlike *T. spinipes* both depend on cavities to nidify and do not present an efficient communication system as *A. mellifera* and its ability to leave for other places when conditions become hard [77], or the aggressive behavior on flowers reported to *T. spinipes*. Besides, their colonies are much smaller than those of these two species. All these factors together are responsible for the lower number of interactions presented by them in comparison to the two main species.

In summary, we demonstrated the importance of a native bee species (*T. spinipes*) and of an exotic one (*A. mellifera*) to interaction networks on surveys conducted in Brazil. As already mentioned, their populous colonies, broad distribution, and aggressive behavior probably are the most important contributors to these results. Comparisons involving their pollinating performance and resource

partitioning are suggested as important lines for further research.

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Research Article

Intraspecific and Intracolonial Variation in the Profile of Venom Alkaloids and Cuticular Hydrocarbons of the Fire Ant *Solenopsis saevissima* Smith (Hymenoptera: Formicidae)

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Fire ants are aggressive Neotropical ants that are extensively similar in general biology and morphology, making species identification difficult. Some fire ant species are top-rated pests spreading throughout the world by trade vessels. Many researchers attempted to sort between invasive and native species by using chemical characters, including patterns of venom alkaloids. The present study is the first to report intraspecific variation in some chemical characters, namely, cuticular hydrocarbons and venom alkaloids, within the Brazilian fire ant species *Solenopsis saevissima* and also reports on within-nest variations among members of different castes. Two different haplotypes (cryptic species) of *S. saevissima* were clearly identified, one presenting a predominant combination of the venom alkaloids *cis*- and *trans*-2-methyl-6-undecylpiperidine with the cuticular hydrocarbons C_{23}, 3-Me-C_{23}, 10-$C_{25:1}$, C_{25}, and 3-Me-C_{25}, and the other a predominant combination of *cis*- and *trans*-2-methyl-6-tridecenylpiperidine with predominance of 12-$C_{25:1}$, C_{25}, 11-Me-C_{25}, 3-Me-C_{25}, 13-$C_{27:1}$, C_{27}, and 13-Me-C_{27}. Intranest variations revealed that the proportions among these compounds varied sensibly among workers of different sizes, gynes, and males (no alkaloids were detected in the latter). Larva contained vestiges of the same compounds. The recorded chemical profiles are quite different from previous reports with *S. saevissima* samples from São Paulo. The finds thus support other recent claims that *S. saevissima* includes cryptic species; the study, moreover, adds the find that they can occur in the same geographical location.

1. Introduction

The fire ants of the genus *Solenopsis* Westwood include species considered pests of worldwide importance, especially *Solenopsis invicta* Buren, which were accidentally transported to other countries outside their native range in South America. To date, fire ant invasion is a major concern in the USA. These ants, particularly those belonging to the *Solenopsis saevissima* species group, react aggressively when their earthen nests are disturbed. Their stings, in addition to pain, can cause serious anaphylactic reactions to sensitive subjects (e.g., [1]).

The species *Solenopsis saevissima* Smith is native to South America and common in Brazil [2], wherein it is potentially responsible for over 35% of the registered accidents with insects (personal communication of Mário Sérgio Palma).

This fire ant was not as extensively studied as other species officially considered pests, like *S. invicta* and *Solenopsis richteri* Forel.

The fire ants are unique among arthropods for their venom composition—alkaloids combined with trace amounts of protein [3, 4]—besides being of special interest to taxonomists because of the historical difficulty of distinguishing between the different species, particularly in South America [5–7]. Cuticular hydrocarbons proved useful in separating between similar species in other difficult groups of ants [8, 9], and the profiles of cuticular hydrocarbons of some *Solenopsis* species have already been determined [10–13]. The use of relative amounts of venom alkaloids—currently credited to be species specific—has also been proposed [8, 14]. In fact, these chemical characters were also used in attempts to build a solid phylogeny among fire ant species [4, 8, 15]. Yet, considering the great number of extant species of *Solenopsis*, there are few comparative or qualitative studies of cuticular hydrocarbons and venom alkaloids available so far [14, 16, 17].

The venom alkaloids and cuticular hydrocarbons of *S. saevissima* were determined by [18] and recently confirmed by [14]; both studies were based on field-obtained samples from São Paulo State, Southeastern Brazil. Yet, another study [19] from the French Guyana presented another chemical profile for *S. saevissima* completely different from these other studies. However, a recent study [7] challenged the present classification of the Brazilian fire ant *S. saevissima* as a single species, having observed that it apparently embraces several distinct genetic lineages with similar morphology (i.e., cryptic species); these authors, for example, detected a distinct lineage of *S. saevissima* that occurs along the littoral of Brazil that apparently hybridizes with *Solenopsis geminata* F. It thus remained to be investigated if *S. saevissima* from different geographical localities in Brazil would have the same venom alkaloid composition.

The present investigation described the cuticular hydrocarbons and venom alkaloids of *S. saevissima* from different regions in Brazil and the intraspecific variations from analyzing individuals of different castes and sex within a same locality. The results are compared with finds from other authors.

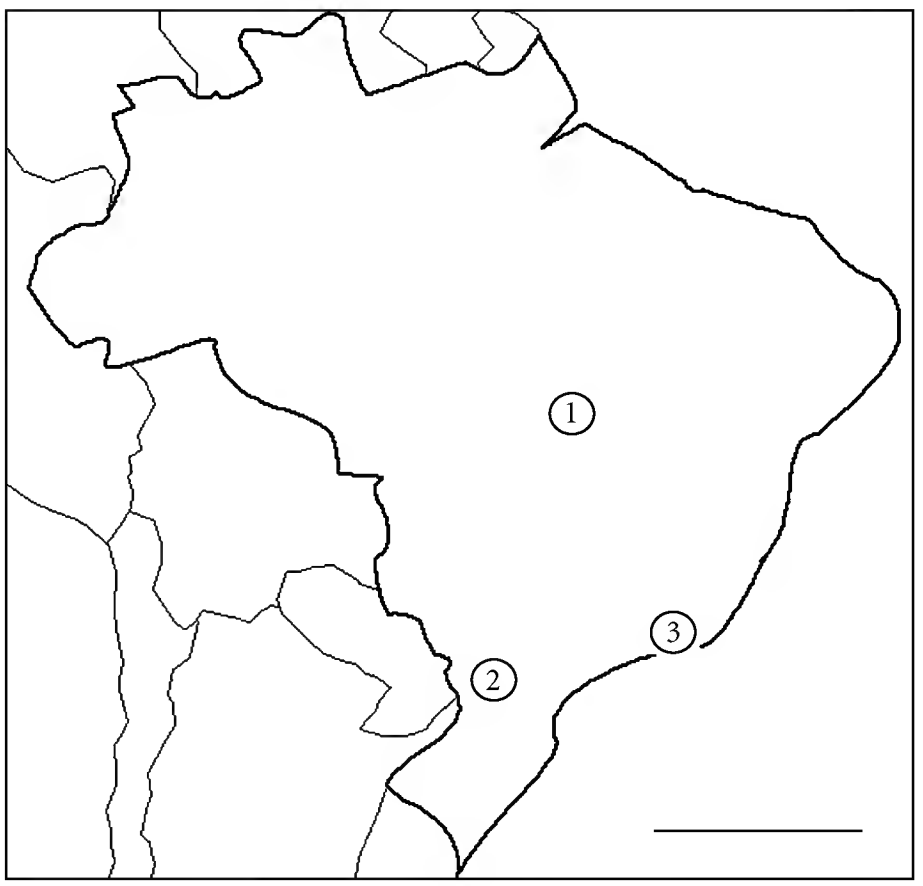

FIGURE 1: Sites of sample collections within Brazil. (1)-Brasilia, Distrito Federal; (2)-Palmas, Parana; (3)-Rio de Janeiro, RJ. These localities are quite different in terms of climate and vegetation. Scale bar = 800 km.

2. Materials and Methods

2.1. Chemicals. *n*-Alkane standards (range C_{16}–C_{31}) were purchased from Sigma Aldrich (Aldrich, Germany, 98%), and anhydrous sodium thiosulphate (Merck, Brazil, 99%) was used as provided. Distilled solvents used were hexane (98.5%), ethanol, and ethyl acetate (99.5%), all from Merck, Brazil.

2.2. Collection of Samples. Samples of workers of mixed sizes were obtained upon disturbing nests of *S. saevissima* in the field at Brasilia, Distrito Federal (15°48′00”S 47°51′50″W), Palmas, Paraná State (26°13′44″S 52°40′15″W), and Rio de Janeiro (22°51′45”S 43°13′26″W), Brazil (Figure 1). Additionally, samples of each individual caste were obtained from five whole fire ant nests collected from a house garden at the municipality of Pedro do Rio, Rio de Janeiro, Brazil (22°20′30″S 43°07′44″W) following the methods for handling and rearing these insects in the laboratory described in [20].

Species identification was based on the series of characters given in [5] and additional useful traits from [14]; the following diagnostic characters of major workers of *S. saevissima* were confirmed: no postpetiolar process, complete mandibular costulae, absence of a frontal medial streak or ocellus, and a poorly developed median clypeal tooth. Voucher specimens are deposited in the Adolph Hempel Entomological Collection of Instituto Biológico de São Paulo, SP, Brazil.

2.3. Sample Preparation. Random worker samples obtained in the field were directly immersed in 100 μL of hexane. Workers from the whole nests were separated in the laboratory into different size classes (size interval, mean weight ± SD) as follows: minor workers (1-2 mm; 0.40 ± 0.08 mg), medium workers (3-4 mm; 0.9 ± 0.18 mg), and major workers (5-6 mm; 2.0 ± 0.52 mg). This division was merely analytical, as most authors only recognize a continuous size range from minors to majors. Males (1.9 ± 0.4 mg) and gynes (unmated queens; 3.1 ± 0.2 mg) were separately analyzed.

Venom Alkaloids. Females of different size classes and castes were cold-anesthetized and had their venom sacs dissected with a fine forceps and macerated in bidistilled ethyl acetate. The venom extracts were adjusted to a final extract concentration of 1 mg/mL.

Cuticular hydrocarbons from body wash: the bodies without venom glands were washed with distilled water

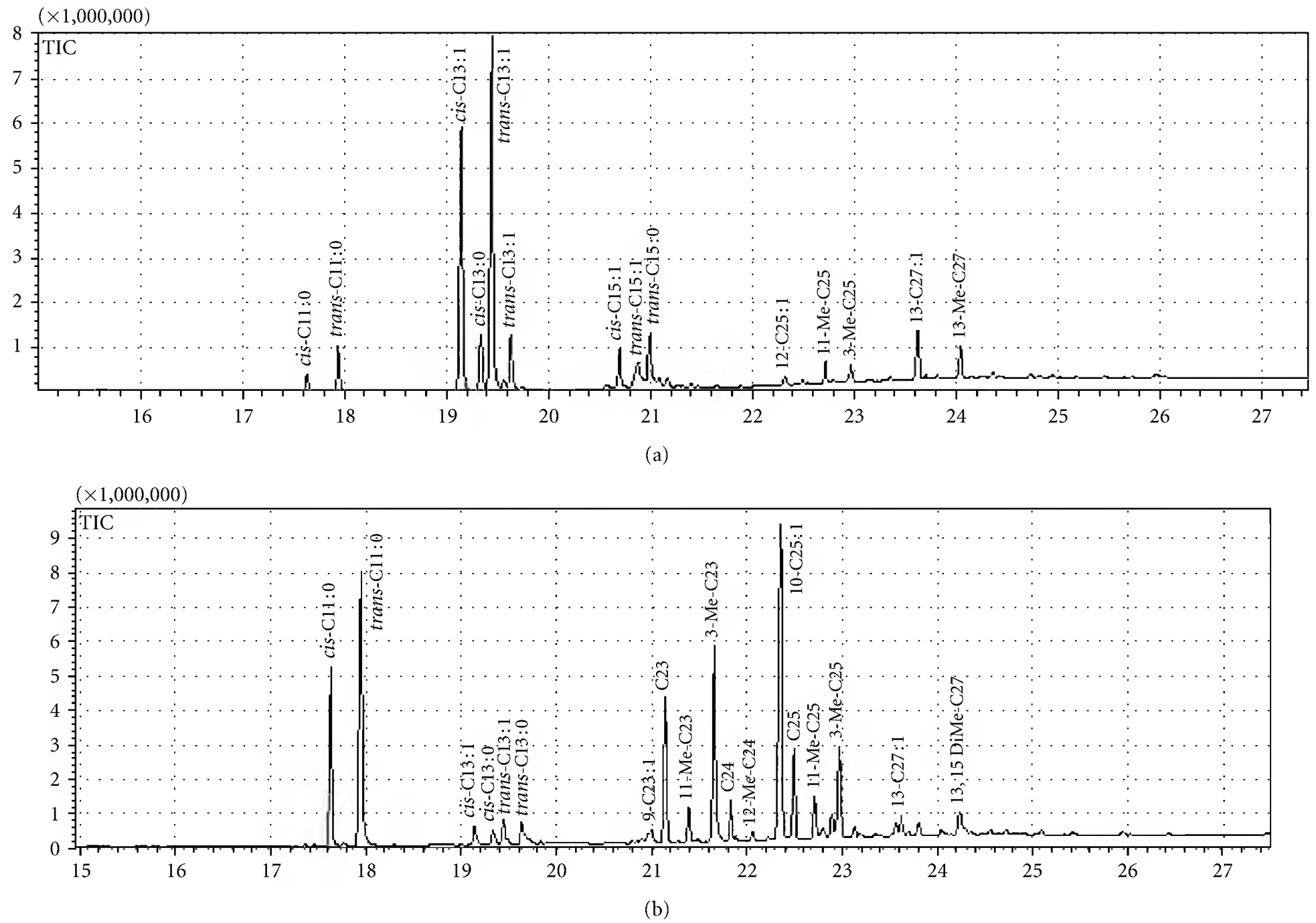

FIGURE 2: Total ion current chromatograms of (top) field worker sample of *Solenopsis saevissima* variety A from Brasília, Distrito Federal, Brazil; (below) field worker sample of *S. saevissima* variety B from Rio de Janeiro, Brazil. For further information on the chemical fingerprints, refer to Table 1 and narrative.

(in 5 mL for 10 min) three times, dried with a piece of filter paper, and then dipped into 2 mL of hexane for 5 min. The obtained body wash extracts were then adjusted to 1 mg/mL in hexane.

Cuticular Hydrocarbons from Crushed Heads. Several workers of different size classes, gynes, and males were cold-anesthetised and decapitated. The excised heads were crushed in 2 mL of bidistilled hexane, filtered, and dried, and the resulting wash was adjusted to 1 mg/mL in hexane.

For quantification of venom alkaloids, certain amounts of individuals (workers or gynes) were pooled, immersed in hexane for 10 min, and the final yield of dry extract weighed with an analytical scale, being divided by the number of individuals used. The same method for measuring cuticular hydrocarbons proved impracticable as the extracted amounts were too small to be accurately measured; thus, the relative amounts of cuticular hydrocarbons could be only estimated from comparing the peak area of *n*-alkane standards with obtained peaks from GC-MS chromatograms with known numbers of individuals and solvent volume.

2.4. GC-MS Analyses. The obtained extracts—venom alkaloids and body and head hydrocarbons—were analyzed by gas chromatography and mass spectrometry (GC-MS) by injecting 1 μL of each extract in a GCMS-QP2010 (SHIMADZU) system equipped with a RTX-5MS silica capillary column (30 m × 0.25 mm × 0.25 μm). Helium was the carrier gas, used at a flow rate of 1.0 mL/min on split mode. The MS were taken at 70 eV, and scanning speed was set to 1228, from *m/z* 50 to 700. The interface temperature was maintained at 280°C. The injector temperature was 250°C. Oven temperature was programmed to increase at 12°C/min from 50°C to 330°C, with a final hold time of 1 min.

2.5. Retention Indexes. Resulting alkaloids and cuticular hydrocarbons were identified by matching their retention indices and acquired mass spectra with entries in the internal mass spectra library (Wiley 275) and published literature [21, 23, 26, 30], while the *n*-alkane external standards were used to bracket the retention indexes as whole numbers: 1600–3100 [26].

Table 1: Venom alkaloids identified from hexane extracts of *Solenopsis saevissima* from different regions within Brazil.

Compounds	Short name	Diagnostic ions, *m/z* (relative abundance)	RT (min)	RI (calc.)
2-Me-6-undecenyl piperidine	C11:1	251 (M^+), 236, 180, 124, 111, 98	17.40	1819
cis-2-Me-6-undecyl piperidine	*cis*-C11	253 (M^+), 252, 238, 98	17.61	1841
trans-2-Me-6-undecyl piperidine	*trans*-C11	253 (M^+), 252, 238, 98	17.94	1884
cis-2-Me-6-tridecenyl piperidine	*cis*-C13:1	279 (M^+), 278, 124, 111, 98	19.11	2021
cis-2-Me-6-tridecyl piperidine	*cis*-C13	280 (M^+), 266, 98	19.30	2052
trans-2-Me-6-tridecenyl piperidine	*trans*-C13:1	279 (M^+), 264, 180, 124, 111, 98	19.41	2066
trans-2-Me-6-tridecyl piperidine	*trans*-C13	280 (M^+), 266, 98	19.60	2091

Notes: RT (min): retention times in minutes; RI (calc.): calculated retention indexes based on [21] using external standards as in Methods. Compounds are identified by comparing with mass spectra from [22, 23].

Table 2: Cuticular hydrocarbons from hexane extracts of fire ants *Solenopsis saevissima* obtained from different regions of Brazil.

Compounds	Short name	Diagnostic ions, *m/z*	RT (min)	RI (calc.)	RI (lit.)
docosane	C22	310 (M^+), 113, 99, 85, 71, 57	20.43	2200	2200
tricosane	C23	324 (M^+), 113, 99, 85, 71, 57	21.14	2300	2300
11-Methyl-tricosane	11-Me-C23	338 (M^+), 323, 196, 168, 140	21.40	2336	2336
3-Methyl-tricosane	3-Me-C23	338 (M^+), 323, 309, 281	21.65	2372	2375
tetracosane	C24	338 (M^+), 113, 99, 85, 71, 57	21.84	2400	2400
12-Methyl-tetracosane + 11-methyl-tetracosane	12-Me-C24 + 11-Me-C24	352 (M^+), 337, 323, 210, 196, 182, 168	22.14	2434	2435
12-Pentacosene	12-C25:1	350 (M^+), 111, 97, 83, 69, 55	22.34	2477	—
10-Pentacosene	10-C25:1	350 (M^+), 111, 97, 83, 69, 55	22.36	2483	—
Pentacosane	C25	352 (M^+), 113, 99, 85, 71, 57	22.49	2500	2500
11-Methyl-pentacosane	11-Me-C25	366 (M^+), 351, 224, 196, 168	22.70	2532	2534
3-Methyl-pentacosane	3-Me-C25	366 (M^+), 351, 337, 309	22.95	2570	2574
Hexacosane	C26	366 (M^+), 113, 99, 85, 71, 57	23.15	2600	2600
13-Methyl-hexacosane	13-Me-C26	365 (M^+), 351, 210, 196, 182, 168	23.34	2630	2633
12-Heptacosene	12-C27:1	378 (M^+), 111, 97, 83, 69, 55	23.64	2670	—
13-Heptacosene	13-C27:1[c]	378 (M^+), 111, 97, 83, 69, 55	23.67	2671	—
Heptacosane	C27	380 (M^+), 113, 99, 85, 71, 57	23.80	2700	2700
13-Methyl-heptacosane	13-Me-C27	394 (M^+), 379, 224, 196, 168	24.08	2736	2733
n-Octacosane	C28:1	392 (M^+), 111, 97, 83, 69, 55	24.18	2747	—
13,15-Dimethyl-heptacosane	13,15-DiMe-C27	408 (M^+), 239, 197	24.25	2756	2756
3-Methyl-heptacosane	3-Me-C27	394 (M^+), 379, 365, 337	24.38	2772	2774
Octacosane	C28	394 (M^+), 295, 267, 239, 196, 168	24.60	2800	2800
3,7,11-Trimethyl-heptacosane	3,7,11-TriMe-C27	422 (M^+), 393, 323, 253, 197, 127	24.82	2830	2833
tridecane	C30	422 (M^+), 113, 99, 85, 71, 57	26.01	3000	3000

Notes: Compounds identified by comparison with mass spectra from [12]/[24, 25]. RT: retention times; RI (calc.): calculated retention indexes (based on Van den Dool and e Kratz, 1963 [21]); RI (lit.): retention indexes as recorded in [26–28]. For further information on the collection sites, refer to narrative.

2.6. Derivatization by Dimethyl Disulfide/Iodine. Alkenes were derivatized according with the methodology described in [31].

3. Results

3.1. Samples from Different Geographical Localities. The samples with workers of mixed sizes revealed the existence of two radically different chemical profiles (see Figure 2) within *S. saevissima*, which shall be referred heretofore as *variety A* and *variety B*. The venom alkaloids of both varieties were composed by isomers of 2-methyl-6-undecylpiperidine (C11) and 2-methyl-6-tridecenylpiperidines (C13), but isomers of C13 prevailed in the venom profile of variety A (with only trace amounts of 2-methyl-6-pentadecylpiperidines), while isomers of C11 prevailed in variety B. Cuticular

hydrocarbons were also different to the same extent (see Figure 2). A complete list of identified venom alkaloids is given in Table 1, and a complete list of the cuticular hydrocarbons observed is given in Table 2 (shorter names for each compound are given in these tables). Relative proportions of the main cuticular hydrocarbons identified in field samples of *S. saevissima* varieties A and B are given in Table 3. Variety A was found in Brasilia and Paraná, while variety B was retrieved from Rio de Janeiro. Molecular evidence (not shown) confirmed that the obtained varieties correspond to two consistent and distinct mtDNA haplotypes within the *S. saevissima* clade—that is, they are cryptic species. These molecular results will be dealt with in a separate publication.

Preliminary tests (not shown) with 100% ethanol demonstrated that this solvent is also useful for recovering hydrocarbons and alkaloids from fire ants, as long as the mixture is injected in the GC-MS no later than within 2 weeks of collection.

3.2. Whole-Nest Samples. Further field inspections (not shown) revealed that *S. saevissima* varieties A and B were abundant at the mountains of Rio de Janeiro; thus, a strategic collection point at the city of Pedro do Rio was elected for obtaining whole nests. *Solenopsis saevissima* was the only fire ant species found around that area, and nests of the two cryptic varieties were present. For sorting in the field between the varieties, we devised the following method: (i) a 15 cm wide carton circle (the "arena") was placed in a plastic or glass bowl; (ii) a sample of one nest was collected with a spoon and placed on one side of the paper arena; (iii) another spoonful sample, from the other nest, was placed on the opposite side of the paper arena; (iv) the degree of aggressiveness of the ants was observed for a few moments. Fighters invariably proved being of different varieties when later analysed by GC-MS. An example of the test can be seen online at http://archive.org/details/AggressivenessTestsFireAnts.

3.3. Intraspecific Variation Range

3.3.1. Venom Alkaloids. Minor workers yielded ~7 μg per venom sac (N = 2 groups of 1,000 ants), while media workers yielded ~12 μg per venom sac (N = 1 group of 1,000 ants), and major workers yielded ~25 μg per venom sac (N = 2 groups of 400 ants). Gynes yielded ~90 μg of venom alkaloids per venom sac (N = 15). Table 4 illustrates the relative amounts of each venom alkaloid among workers of different sizes and gynes. Relative proportions of alkaloid isomers of workers varied with size: minor workers always had higher proportions of *trans* isomers (Table 4), while greater size classes had increased amounts of *cis* isomers; *cis* isomers were always predominant in the venom of gynes. Venom alkaloids were not detected in neither body wash nor head extracts of males. Larvae of both varieties contained detectable amounts of the same venom alkaloids of workers (not shown).

3.4. Cuticular Hydrocarbons from Head and Body. Workers always yielded 12–14 μg of head hydrocarbons and ~32 μg of body cuticular hydrocarbons (N = 10). Males yielded ~83 μg (head) and ~135 μg (body) (N = 3) of cuticular hydrocarbons, and queens yielded ~84 μg (head) and ~ 238 μg (body) (N = 3).

Table 3: Relative abundance (%) of cuticle hydrocarbons from random worker samples of *Solenopsis saevissima* varieties A (n = 5) and B (n = 13) obtained from different nests from three different states in Brazil (see Figure 1). Values given in bold were tested for each species variety using Students' t-test, and differing means were attributed to different letters.

Cuticular hydrocarbon	*S. saevissima* variety A	*S. saevissima* variety B
C23	—	12.16 ± 2.48
11-Me-C23	—	2.10 ± 0.69
3-Me-C23	—	21.42 ± 1.94
C24	—	1.75 ± 0.51
12-Me-C24 + 11-Me-C24	—	0.77 ± 2.21
C25:1	**8.52 ± 3.32 a**	**50.74 ± 6.69 b**
C25	**4.06 ± 2.47 a**	**4.38 ± 0.95 a**
11-Me-C25	**9.73 ± 2.19 a**	**2.198 ± 0.89 b**
3-Me-C25	**12.91 ± 1.60 a**	**5.66 ± 0.84 b**
C26	3.24 ± 1.70 a	tr
C27:1	25.49 ± 8.47	tr
C27	4.04 ± 2.30	tr
13-Me-C27	24.19 ± 7.98	tr
11,15-DiMe-C27	2.00 ± 1.27	—
3-Me-C27	3.23 ± 0.62	—
13,15-DiMe-C27	4.48 ± 1.65	—
C30	2.01 ± 0.24	—
Total	100.00	100.00

Notes: (—): not found; (tr): trace amounts. For information on the compounds, collection sites, and species varieties, refer to narrative.

Based on relative abundance within chromatograms, workers had 1-2 μg of total hydrocarbons, while males had about 5 μg, and queens had about 6 μg. Relative abundance between total alkaloids and total hydrocarbons proved quite variable between different individuals. Hydrocarbons from head yielded alkaloid-free clear chromatograms.

Hydrocarbons obtained from the hexane extracts of head and body extracts were always the same (not shown), thus these results were qualitatively equivalent. A comparison of the head extracts of major workers of *S. saevissima* varieties A and B is shown in Figure 3. Main cuticular hydrocarbons of *S. saevissima* variety A were 13-heptacosene, 13-methyl-heptacosane, and 3-methyl-pentacosane, while the main cuticular hydrocarbons of *S. saevissima* variety B were tricosane, 3-methyl-tricosane, 10-pentacosene, pentacosane, and 3-methyl-pentacosane (see Figures 2 and 3).

The relative proportions of cuticular hydrocarbon obtained from body extracts of workers of different sizes and castes of *S. saevissima* varieties A and B are shown in Table 5. The relative amounts of cuticular hydrocarbons varied sensibly among workers of different sizes and castes. A full chromatogram illustrating the differences between cuticular

Table 4: Relative abundance (%) of piperidinic alkaloids obtained from hexane extracts of small workers (SWs), medium workers (MWs), and major workers (LWs) and gynes (Gs) from different nests of *Solenopsis saevissima* varieties A ($n = 3$) and B ($n = 4$) from Pedro do Rio, Rio de Janeiro state, Brazil.

Piperidine	*S. saevissima* variety A				*S. saevissima* variety B			
	SW	MW	LW	G	SW	MW	LW	G
C11:1	—	—	—	—	—	—	—	0.35 ± 1.15
cis-C11	tr	—	—	33.22	4.16 ± 2.16	7.51 ± 4.27	12.21 ± 5.70	61.79 ± 12.99
trans-C11	tr	tr	tr	35.15	95.84 ± 2.16	98.48 ± 4.28	87.79 ± 5.70	tr
cis-C13:1	tr	31.52–35.76	49.69–55.00	17.68	tr	tr	tr	33.15 ± 15.92
cis-C13	tr	tr	tr	4.04	—	—	tr	1.37 ± 0.94
trans-C13:1	97.80–98.00	60.02–64.24	45.53–50.31	7.76	tr	tr	tr	tr
trans-C13	—	—	—	2.15	—	—	—	tr
Unknown	—	—	—	—	—	—	—	tr
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Notes: Compounds identified by comparing with mass spectra from Brand et al. 1972 [29] and Leclercq et al. 1994 [23]. (—) = not found; (tr) = trace amounts. No venom alkaloids were found on males. For further information on the extraction and species varieties, refer to narrative.

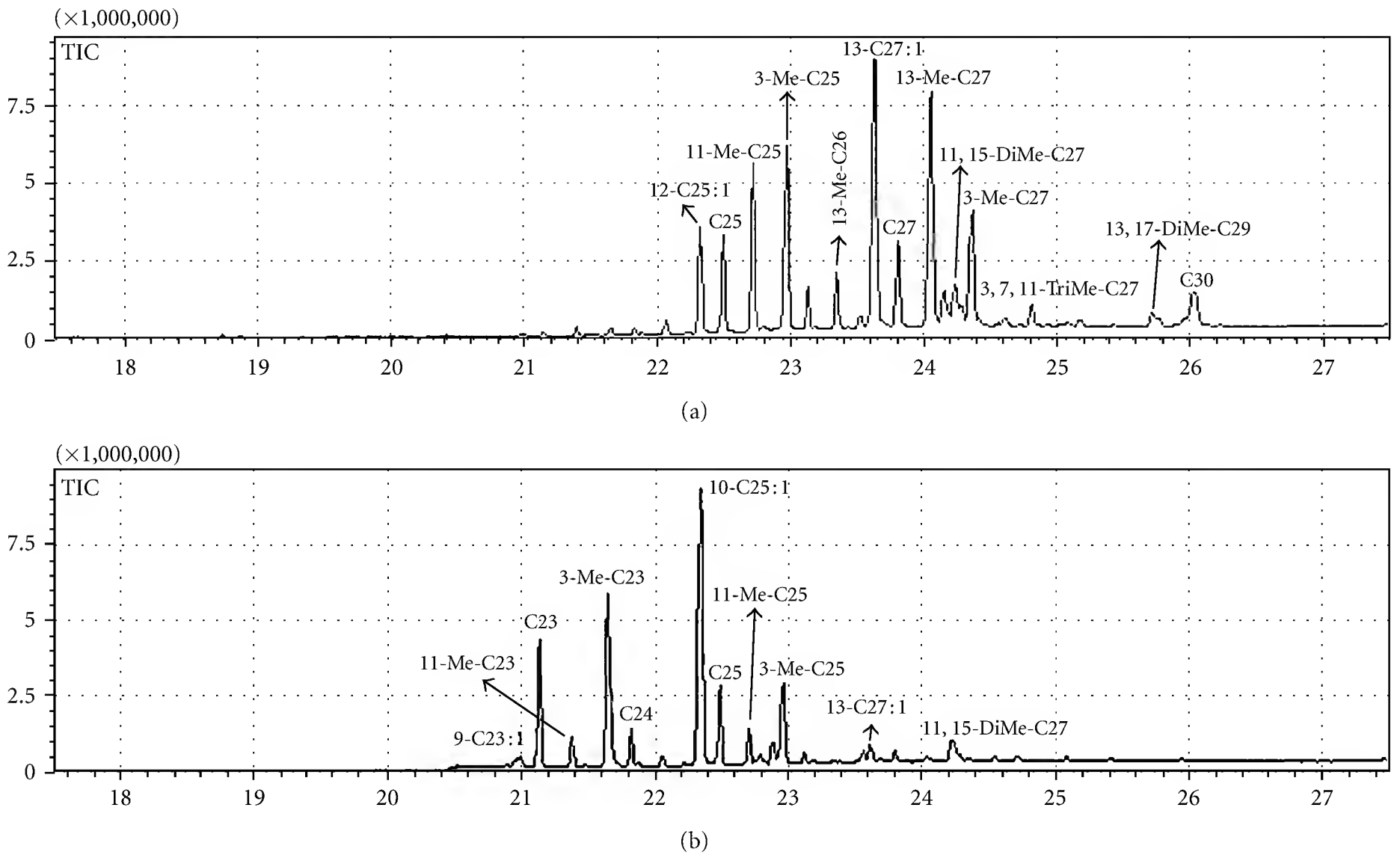

Figure 3: Total ion current chromatograms of hydrocarbons from head extracts of major workers of *Solenopsis saevissima* varieties A (top) and B (below) from Rio de Janeiro, Brazil.

hydrocarbons and venom alkaloids of major and minor workers of *S. saevissima* variety B is given in Figure 4.

4. Discussion

4.1. Samples of Different Geographical Localities. Random samples of mixed worker sizes suggested that the described cryptic varieties are widespread and common over Brazil, in agreement with the scenario predicted by [7] using other tools. It remains to be investigated if the cryptic species appointed by [7] would correspond to similar chemical profiles. A complete scenario can be easily obtained by further sampling from other localities. Other varieties within nominal *S. saevissima* are probably also present. The practice of testing workers for aggressiveness was very helpful in locating a nest of the less prevalent *S. saevissima* variety A within the study area, as it dispensed the need for returning several samples back to the laboratory for GC/MS analyses.

Table 5: Relative abundance (%) of cuticle hydrocarbons from small workers (SWs), medium workers (MWs), and major workers (LW), gynes (Gs) and males (Ms) of *Solenopsis saevissima* varieties A and B, collected in Pedro do Rio, Rio de Janeiro, Brazil.

Cuticular hydrocarbon	*Solenopsis saevissima* variety A (minimum–maximum interval)					*Solenopsis saevissima* variety B (n = 5; mean ± SD)				
	SW (n = 4)	MW (n = 4)	LW (n = 4)	G (n = 2)	M (n = 3)	SW	MW	LW	G	M
C22	—	—	—	—	—	—	—	—	tr	—
C23	1.52–1.87	—	—	tr	—	12.45 ± 4.6	12.02 ± 7.13	9.77 ± 3.06	13.92 ± 1.93	21.81 ± 1.86
11-Me-C23	—	tr	—	tr	—	—	—	—	—	—
3-Me-C23	1.87–2.22	—	—	tr	—	17.37 ± 1.22	16.87 ± 1.18	18.77 ± 2.56	19.27 ± 2.56	18.33 ± 3.11
C24	—	—	—	tr	—	2.62 ± 1.10	2.34 ± 1.75	2.00 ± 1.00	3.40 ± 0.23	4.70 ± 0.60
12-Me-C24 + 11-Me-C24	—	tr	tr	tr	—	—	—	—	—	—
12-C25:1	6.31–7.48	3.91–4.20	4.99–5.21	3.75–45.38	17.06–31.77	—	—	—	—	—
10-C25:1	—	—	—	—	—	43.23 ± 10.86	42.15 ± 13.12	43.77 ± 6.14	34.92 ± 0.92	28.63 ± 5.89
C25	8.17–9.00	4.47–5.31	6.20–9.29	1.18–16.77	3.51–15.33	9.97 ± 5.16	9.20 ± 6.70	8.02 ± 4.34	10.45 ± 0.72	19.57 ± 5.62
13-Me-C25	—	—	—	—	—	1.37 ± 0.69	1.68 ± 0.84	1.88 ± 0.94	1.75 ± 0.88	—
11-Me-C25	6.05–9.23	11.58–13.18	10.8–13.01	7.53–9.82	2.24–7.76	—	—	—	—	—
3-Me-C25	12.22–13.37	10.04–11.08	9.02–9.58	13.27–16.83	10.10–20.73	7.40 ± 2.50	7.34 ± 2.18	6.85 ± 0.62	7.46 ± 0.47	6.48 ± 0.68
C26	—	—	—	1.08–3.02	—	—	—	—	—	—
13-Me-C26	1.45–2.10	1.93–3.28	1.72–2.57	0.00–1.30	10.29–10.63	—	—	—	—	—
12-C27:1	—	—	—	—	—	1.00 ± 1.00	1.10 ± 0.55	1.17 ± 0.59	1.00 ± 1.00	—
13-C27:1	20.53–22.30	26.88–28.83	19.27–22.20	16.28–30.44	—	—	—	—	—	—
C27	15.06–16.08	2.33–3.1	4.60–5.00	0.00–6.56	—	tr	tr	tr	tr	tr
13-Me-C27	15.12–17.8	25.93–28.69	25.30–28.05	0.00–14.36	—	—	—	—	—	—
n-C28:1	—	1.00–1.39	1.08–2.43	tr	—	1.57 ± 0.79	1.97 ± 0.99	2.27 ± 1.14	1.75 ± 0.87	—
13-15-DiMe-C27	3.17–4.00	3.98–4.08	4.26–5.06	0.00–6.85	2.96–12.11	tr	tr	tr	tr	—
3-Me-C27	—	—	—	—	—	tr	tr	tr	tr	tr
C28	—	tr	1.84–2.08	tr	—	—	—	—	—	—
C29	—	—	—	tr	—	—	—	—	—	—
3,7,11-TriMe-C27	—	tr	tr	tr	—	—	—	—	—	—
C30	—	1.32–2.21	tr	tr	—	—	—	—	—	—
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Notes: (—): not found; tr: trace amounts; n: number of nests evaluated.

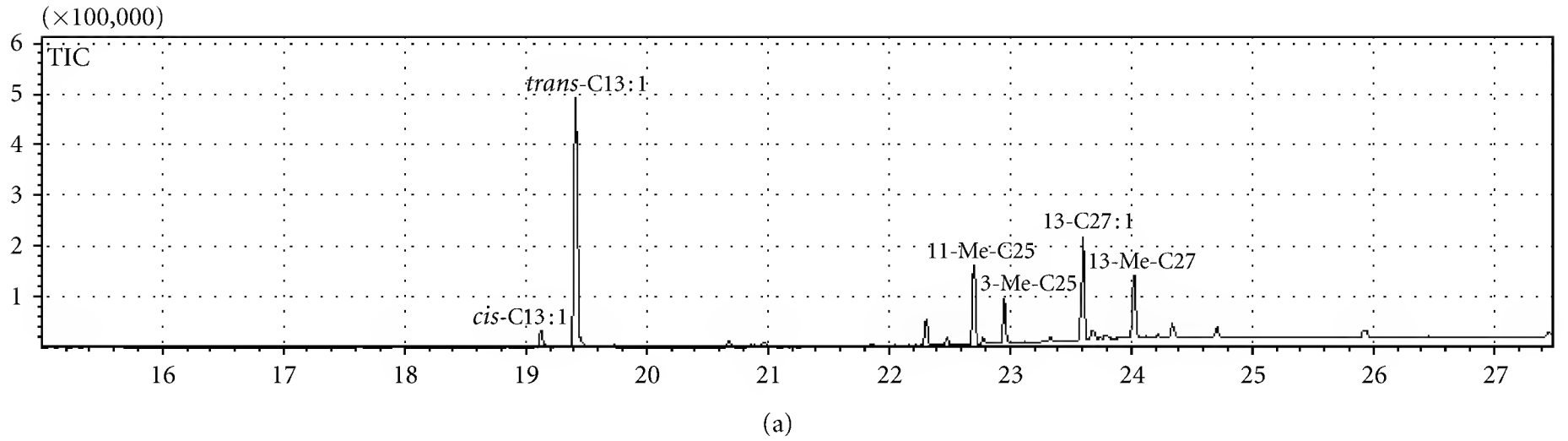

FIGURE 4: Total ion current chromatograms of body washes of workers of *Solenopsis saevissima* variety A from Rio de Janeiro, Brazil: (top) Minor workers; (below) Major workers.

The fact that ethanol provided good chemical profiles of samples collected in the field is interesting, as it is a more common, cheaper, and less volatile solvent. This opens the possibility of checking the alkaloids present in long-standing fire ant samples deposited in wet collections. It should be noted, however, that care should be taken in such cases as polar solvents can be harmful to some analytical systems, for they can interact with the column matrix.

4.2. Venom Alkaloids and Cuticular Hydrocarbons. The reported amount of cuticular hydrocarbons extracted from fire ants is dramatically superior—sometimes >200 times—to other much larger ants such as *Ectatomma ruidum* Roger [32] and *Ectatomma brunneum* Smith F. and *Atta laevigata* Smith F. (unpublished results of E.G.P.F.). This disparate abundance of waxes on the body of fire ants must have some biological implication, which probably explains why they can conveniently be separated by nest debris by flotation [20]. In comparison with previous studies with fire ants, in the present report we found they were in agreement with cuticular hydrocarbons reported by [13] from similar extractions with *S. invicta* and *S. richteri*, yet another study [33] found as little as 300 ng per ant. We think that such difference could be explained by either populational or specific differences, but the fact that the reported values proved also quite variable between different studies with the same species indicates that the matter would merit further comparative investigation. We ought however to emphasize that our chromatograms with different samples within the same nest suggest that there is considerable intraspecific variation in the alkaloids/hydrocarbons ratio.

The abundance of venom alkaloids in the body wash extracts is also elevated, and it should be noted that these ants apparently discharge the contents of their venom glands upon immersion in organic solvent. However, we herein recovered about one-third of the amount reported by [22] with *S. invicta*. Again, it is possible that such difference be due to specific variations, but it should be minded that these authors employed different methods for their venom estimates. The fact that venom alkaloids were also recovered from bodies with excised abdomens indicates that they accumulate for some time on the exoskeleton of the insects. This alkaloidal covering might serve as armor against entomopathogenic fungi and predators, or even have some important role in nestmate recognition, given their abundance.

4.3. Venom Alkaloids and Cuticular Hydrocarbons as Taxonomic Tools. The sampled regions of the present study, from being distant from each other, present marked differences in climate, soil, and vegetation. The different patterns of venom alkaloids and cuticular hydrocarbons of the collected samples indicate they belong to two different cryptic species (compare Tables 1, 2, and 4). These patterns were yet markedly different from reports for *S. saevissima* by [14] based on samples from São Paulo, thus indicating the existence of other cryptic species in Brazil. The additional profile published by [19] further indicates the existence of another cryptic species in French Guyana.

The existence of cryptic species in this clade was solidly demonstrated in the broad study of [7], using other methods. Our finds with chemical characters thus add evidence to the conclusions of Ross et al. (2009) that different evolutionary

entities were grouped in the nominative species *S. saevissima* because of morphological similarity. The actual number of cryptic species and the range of their variation could only be established by investigations employing further different approaches (e.g., isoenzymes, larval characters) based on extensive sampling from other regions of South America. This is an issue of major interest as it pertains the biological boundaries of individual species, how many actually exist, and how they are set in nature and thus warrants immediate attention.

Moreover, the present report also imposes exceptions to the belief (e.g., [10, 34]) that venom alkaloidal composition in fire ant venoms is species specific. Based solely on the pattern of venom alkaloids and following the results in these studies, *S. saevissima* variety B would have been assumed to be *S. geminata* or some close relative, like *Solenopsis xyloni* McCook; variety A would have been likewise mistaken for *S. richteri*.

It seems unlikely that venom alkaloid composition of the different species could be interpreted as remissive of the phylogenetic relationships among fire ants, as first proposed by [29] and discussed in [18]. These authors suggested that the chemical structure of venom alkaloids from different species might reflect evolutionary relationships within fire ants; species with most diverse venom alkaloids would stand a step higher in the taxonomic history of the group. As remarked by [18], this assumption was based on studies of the few North American species. From regarding the herein described patterns of *S. saevissima* in face of preterit reports, it follows that there are at least three completely different alkaloidal patterns recorded within this nominal taxon and that such patterns are strikingly similar to the venom patterns of other species considered distant from each other as, for example, *S. geminata* versus *S. invicta*, which belong to different groups of fire ants species.

4.4. Caste Variations. As mentioned, workers of fire ants range in size (polymorphism) over a broad continuum [35], and thus we decided to adopt an arbitrary division into minor, medium, and major workers. Moreover, worker size also depends on the physiological status and age of the nest [35, 36]. This implies that the arbitrary size class of media workers includes specimens of minor and major workers. Considering this aspect, intermediary workers were predominant in the collected samples. The obtained results confirm previous observations that the worker venom alkaloid *trans/cis* isomeric ratio is dependent on the ant size, with minor workers having higher *trans/cis* isomeric ratios (Tables 1 and 2), and are suggestive that the trans/cis ratio gradually changes at some point within the intermediary size range. A clear trend of ever-increasing relative amounts of venom *cis*-piperidinic alkaloids is perceived towards larger females, queens. Similar trend was observed with other species of fire ants, including *S. invicta* and *S. geminata* [4, 22, 29, 37]. Venom alkaloids were not detected in the male body nor head extracts.

The pattern of cuticular hydrocarbons of *S. saevissima* variety A was quite different from the one obtained for *S. saevissima* variety B. Moreover, intercaste differences in the patterns of cuticular hydrocarbons of *S. saevissima* were detected in both varieties, illustrated by small variations in the relative amounts of the main compounds. For instance, there was a clear tendency for reduction in the relative amounts of C_{23} from minor workers towards major workers (Table 2). Gynes presented a wider range of different cuticular hydrocarbons, whist males presented marked proportion alterations on the same compounds (Table 2).

The present study generally depicts the knowledge gap about fire ant populations in their native South American range. Most of what is currently assumed about the group was extrapolated from laboratory experimentation with North American *S. invicta* and based on a few scattered observations with other species in South America. The validity of currently accepted fire ant species must be revisited, as should their diagnostic features. Chemical characters are indeed valuable parameters that aid species identification, but given the similarity between the obtained chromatograms and those of other distinct species (*S. saevissima* versus *S. geminata* or *S. xyloni*) and the considerable dissimilarity among nominal *S. saevissima* populations, at present chemical characters have to be employed with great caution and also in association with other characters and techniques (e.g., mtDNA). We also hope to have contributed with practical methods and guidance to expand the survey of these characters with other distant fire ant populations.

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Review Article

Chemical Ecology of Egg Parasitoids Associated with True Bugs

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Parasitoids representing some 15 families of Hymenoptera develop in insect eggs; three of these families, Platygastridae (= Scelionidae), Mymaridae, and Encyrtidae, are associated with Heteroptera. Several species of heteropteran egg parasitoids are or may be important for biological pest control. Successful parasitism of insect herbivores by insect parasitoids arises through several phases of host searching, which lead female wasps to the vicinity of, or in contact with, their hosts. During the host location process, females encounter and explore a variety of stimuli, among which chemical cues (i.e., semiochemicals or infochemicals) play a pivotal role. Female parasitoids are under selection pressure to efficiently invest their limited time on the location and exploitation of host-derived stimuli. In general, the levels of reliability and detectability of a particular stimulus are inversely correlated. Female parasitic wasps adopt differing strategies to solve this dilemma. In this paper we focus on the various host selection strategies employed by heteropteran egg parasitoids and possible means whereby the chemically mediated behavior of these wasps may be exploited to enhance biological pest control.

1. Introduction

Egg parasitoids are the largest group of entomophagous insects associated with Heteroptera. Thus, considering that they attack the host before it develops and inflict feeding damage, egg parasitoids show remarkable potential as biological control agents of Heteroptera, as well as other pests [1]. These parasitoids, however, also attack predaceous bugs which, of course, is counterproductive for the efficacy of these important natural enemies [2, 3].

Of the approximately 15 hymenopteran families that include egg parasitoids, those most commonly associated with Heteroptera are Platygastridae, Mymaridae, and Encyrtidae [1]. Most studies of their chemical ecology concern species associated with herbivorous bugs and, to a much lesser extent, predacious Heteroptera. The majority of semiochemical research on egg parasitoids associated with true bugs has been limited to species belonging to the genera attacking economically important pentatomid, scutellerid, mirid, alydid, and coreid plant pests (Table 1): *Trissolcus*, *Telenomus*, *Gryon* (Platygastridae = Scelionidae; see Sharkey [4] and Murphy et al., 2007 [5]), *Anaphes* (Mymaridae), and *Ooencyrtus* (Encyrtidae).

Generally, in order to reproduce, a female parasitoid must find its host at a stage suitable for parasitization. The host selection process involves a sequence of phases mediated by physical and chemical stimuli from the host, the substrate, and/or associated organisms, eventually leading to successful parasitism [6–9]. Because parasitoid foraging time is limited and the potential cues available are numerous, the parasitoid faces the need to optimize exploitation of available cues and discriminate those most reliable in indicating the presence of a suitable host [8, 10]. However, the location and recognition of a suitable host is a complex process, especially for egg parasitoids, because of major constraints due to the small sizes of both the host and the parasitoid itself. Eggs are usually unapparent, especially when they are small, dispersed in the habitat, and concealed in plant tissue. As such, cues that are directly related to the presence of eggs may have low detectability, but high reliability [8, 11, 12]. Additionally, suitable host eggs are generally available for only a short time due to their rapid development [7]. Therefore, egg parasitoids have developed specialized strategies to overcome the reliability-detectability dilemma in order to efficiently parasitize host eggs. Successful parasitism is accomplished

Table 1: Host or host-associated semiochemicals exploited by egg parasitoids of Heteroptera for host location or recognition (modified and updated from Colazza et al. [14]).

Parasitoid	Host	Plant	Function	Origin	Chemistry	Parasitoid response	References
Mymaridae							
Anaphes iole	*Lygus hesperus*	*Gossypium hirsutum* + other	Volatile induced synomone	Plants with feeding damage	Several HIPV, mostly α-farnesene, (Z)-3-hexenyl acetate, methyl salicylate	Attraction in olfactometer (effect of experience); response to EAG	[43, 44, 67]
			Short range synomones	Wounds in different substrates		Ovipositor probing	[52, 53]
			Short range kairomones	Host egg and adult		Ovipositor probing	[52–54]
Encyrtidae							
Ooencyrtus nezarae	*Riptortus clavatus*		Volatile kairomone	Host male attractant pheromone	(*E*)-2-Hexenyl (*Z*)-3-Hexenoate	Attraction in field, increased parasitism	[34–36]
Ooencyrtus telenomicida	*Nezara viridula*		Volatile kairomone	Host male pheromone + ovipositing female		Attraction in Y-tube olfactometer	[38]
Platygastridae							
Gryon boselli	*Gonocerus acuteangulatus*		Contact kairomone	Host footprints		Arrestment and increased searching in open arena	Colazza et al., unpubl.
Gryon pennsylvanicum	*Leptoglossus australis*		Volatile kairomone	Host pheromone		Attraction in field	[56]
Telenomus podisi	*Euschistus heros*	*Glycine max, Cajanus cajan*	Volatile induced synomone	Plants with feeding damage; *cis*-jasmone treatments on resistant plants	Quantitative differences especially (*E,E*)-α-farnesene, methyl salicylate, (*Z*)-3- hexenyl acetate, and (*E*)-2-octen-1-ol	Attraction in Y-tube olfactometer	[41, 42, 66]
			Volatile kairomone	Compounds from host defensive secretion	(*E*)-2-hexenal, 4-oxo-(*E*)-2-hexenal	Attraction and increased searching	[33]
			Kairomone	Host male sex pheromone	Methyl 2,6,10-trimethyltridecanoate	Choice in closed arena	[85]
			Contact kairomone	Host footprints		Arrestment and increased searching in open arena	[46]
			Contact kairomone	Acetone extract of host eggs	2,6,10-Trimethyltridecanoate		[51]
	Euschistus conspersus		Volatile kairomone	Host male attractant pheromone	Methyl(E,Z)-2,4-decadienoate	No parasitism increase in field	[86]
Telenomus calvus	*Podisus neglectus*		Volatile kairomone	Host pheromone			[39]
	Podisus maculiventris		Volatile kairomones	Host male attractant pheromone; host female		Attraction in field allowing egg parasitism; phoresy	[2, 3, 87]
Trissolcus basalis	*Nezara viridula*	*Vicia faba, Phaseolus vulgaris*	Volatile induced synomone	Plants with oviposition + feeding	Increase of (*E*)-β-caryophyllene	Attraction in Y-tube olfactometer	[20, 21]

Table 1: Continued.

Parasitoid	Host	Plant	Function	Origin	Chemistry	Parasitoid response	References
			Volatile kairomone	Host defensive allomone	(*E*)-2-Decenal, 4-oxo-(*E*)-2-hexenal	Attraction in Y-tube olfactometer, increased searching	[30, 33]
			Volatile kairomone	Host attractant pheromone		Attraction in Y-tube olfactometer	[31]
			Contact kairomone (highly specific)	Host footprints (especially females)	Cuticular hydrocarbons	Arrestment and increased searching in open arena	[31, 71, 72]
			Contact kairomone	Follicular secretion and egg extracts	Mucopolysaccharide-protein complex	Host recognition	[50, 88, 89]
Trissolcus brochymenae	*Murgantia histrionica*	*Brassica oleracea*	Short-range induced synomone	Plants with feeding damage		Increased searching behavior	[22, 62, 63],
			Short-range induced synomone	Plants with feeding + oviposition (systemic induction)		Increased searching behavior	[22, 62, 63],
			Volatile kairomone	Pheromone from different host instars		Attraction in Y-tube olfactometer	[28]
			Contact kairomone (highly specific)	Host footprints (especially mated females)	Cuticular hydrocarbons	Arrestment and increased searching in open arena	[28, 32, 47]
			Volatile kairomone	Host egg masses		Attraction in Y-tube olfactometer	[28]
			Contact kairomone	Follicular secretion and egg extracts		Host recognition	[28]: Conti et al., unpubl.
Trissolcus euschisti	*Euschistus conspersus*		Volatile kairomone	Male attractant pheromone	Methyl(*E*,*Z*)-2,4-decadienoate	No parasitism increase in field	[86]
Trissolcus grandis	*Eurygaster integriceps*		Volatile kairomones	Host male attractant and female sex pheromones		Attraction in the field	[90]
	Eurygaster spp.		Contact kairomone	Host egg			[91]
Trissolcus simoni	*Eurydema ventrale*		Volatile kairomone	Host attractant pheromone		Attraction in Y-tube olfactometer	[92]
			Contact kairomone	Host footprints		Arrestment and increased searching in open arena	[32]
Trissolcus utahensis	*Euschistus conspersus*		Volatile kairomone	Host sex pheromone	Methyl(*E*,*Z*)-2,4-decadienoate	No parasitism increase in field	[86]

through the combined exploitation of cues that are directly and indirectly related to host eggs [7, 8, 13, 14]. First, parasitoids may detect volatiles from nontarget instars of the host, that is, adults or juveniles, to reach the vicinity of the host eggs (infochemical detour *sensu* Vet & Dicke [8]), eventually enabling them to pin-point eggs using additional long- and/or short-range cues. A particular and interesting example of such detour behavior of egg parasitoids is phoresy on adult host females; via this strategy, not only are relevant cues more detectable, but the adult itself is also exploited by the parasitoid as a vehicle to arrive at host eggs [15–17]. Second, parasitoids may exploit plant volatiles induced as a consequence of herbivory, which are emitted in large quantities and are, therefore, easily detectable by foraging parasitoids but not necessarily highly reliable [13]. For example, recent investigations have shown that some egg parasitoids are capable of exploiting plant chemicals emitted as a result of egg deposition, thus rendering such highly detectable cues also highly reliable [18–24]. Third, egg parasitoids have been observed to associate, through learning, highly detectable but less reliable cues with the presence of suitable hosts, thus increasing reliability of such cues in experienced wasp females [25, 26].

The complex of stimuli that are used by parasitoids for host seeking and acceptance, originating from the host, the associated substrate and/or organisms, and their possible interactions, has been called the "host-egg unit" [27–29]. The host-egg unit is often quite complex and is related to the parasitoid strategies described above. Thus, the same host might represent different host units for different parasitoids, depending on whether they are specialist versus generalist parasitoids, or whether they have evolved capabilities to exploit adult kairomones or induced plant synomones, or whether they have developed phoretic strategies. In this paper we will focus on the behavioral steps of host selection strategies and the chemical cues exploited by egg parasitoids of emerging pest species of Heteroptera.

2. Exploitation of Indirect versus Direct Host-Related Chemical Cues

The most common indirect host-finding tactic known thus far for egg parasitoids is the infochemical detour strategy based on the parasitoid ability to detect chemical cues associated with stages other than the egg [7, 8]. Exploitation of pheromones and/or allomones from host adults has been demonstrated for both Platygastridae [28, 30–33] and Encyrtidae [34–38] (Table 1). These stimuli provide indirect information on the presence of the host community, leading the wasp female to the vicinity of host eggs. In spite of their low reliability, pheromones and allomones are produced in large amounts, and, therefore, these cues are relatively easy to detect by the female wasps from long to medium distances [39].

Phoresy is a different, but highly specialized bridge-in-time (and bridge-in-space) strategy exploited by egg parasitoids to reduce the spatial and temporal discontinuity between where host adults mate and where host females oviposit [7, 40]. One well-documented case of phoresy by a heteropteran egg parasitoid is that of *Telenomus calvus* Johnson (Platygastridae) females that parasitize eggs of the predacious spined soldier bug, *Podisus maculiventris* (Say) (Pentatomidae). Female *T. calvus* wasps go to the male-produced *P. maculiventris* attractant pheromone, wait nearby for a conspecific soldier bug female to arrive and mate, and then become phoretic on the mated host female until she eventually oviposits [2, 3].

Another indirect means to locate host eggs is exhibited by *Trissolcus basalis* (Woll.) and *Telenomus podisi* Ash. (Platygastridae), which exploit plant synomones induced by oviposition and/or feeding of their hosts, *Nezara viridula* L. [20] and *Euschistus heros* (Fabr.) [41, 42] (Pentatomidae) (Table 1). In a hierarchical context, whether volatiles from host adults or from the host plants of host adults are exploited from the furthest distance has yet to be elucidated. In the case of *Tr. basalis*, because host oviposition is necessary for volatile induction in bean plants, such synomones appear more reliable compared to kairomones from nontarget instars or to feeding-induced synomones [20, 21]. Therefore, it can be hypothesized that kairomones from adults and feeding-induced plant synomones act as a long-distance, indirect cue used to localize the host community (or host habitat), whereas oviposition-induced plant synomones are shorter-range cues used to find plants that actually have host eggs.

Induced plant volatiles are also exploited by mymarids [43, 44] (Table 1). Feeding on cotton, and several other plants, by either sex of *Lygus hesperus* Knight (Miridae) results in the induction of volatiles that are behaviorally and physiologically active towards *Anaphes iole* Girault (Mymaridae). Oviposition appears unnecessary to induce active volatiles [44]; therefore, these synomones should also be considered as indirect cues, exploited to localize the host community.

Once close to a potential host, female parasitic wasps in flight preferably should alight on a plant that probably has host eggs and then commence searching on the substrate for suitable eggs. Different strategies, and cues, are used during this phase (Table 1). For example, *Trissolcus brochymenae* (Ashm.) exploits short-range chemicals that are induced in cabbage plants by *Murgantia histrionica* (Hahn) (Pentatomidae) during oviposition [22]. Such a directly host-related synomone that is systemically emitted by the plant provides reliable information on the presence of suitable host eggs, but not necessarily precise information on where the eggs have been laid [22].

While searching for the host eggs on the plant, different Platygastridae were shown to use chemical traces left behind from the adults and/or juveniles (Table 1). The typical response to such cues, which are perceived through gustatory sensilla [45], is arrestment behavior followed by increased searching intensity [28, 31, 32, 46]. These cues are not directly related to the host eggs, but because of finely tuned adaptations of the parasitoid, these cues may become quite reliable. In fact, although responding to the "footprints" of both males and females of the host species, *Tr. basalis* and *Tr. brochymenae* are able to discriminate host adult sex and

the physiological conditions of host adults (e.g., virgin versus mated females) [28, 31, 47, 48]. Interestingly, in addition to chemical footprints, *Te. podisi* also uses vibratory signals mediating sexual behavior of the host species, *E. heros* [49].

Short-range host location is the next and final step, and female wasps may use visual cues at this point or short-distance kairomones directly related to the host to finally reach the target egg. The presence of volatiles from *M. histrionica* eggs is detectable by *Tr. brochymenae* whereas, in this species, visual cues do not appear to play an important role [28] (Table 1). When a host egg mass is encountered, host recognition by *Trissolcus* species is elicited by contact kairomones present on the egg surface, although physical factors such as shape and size may also affect wasp behavior [28, 50, 51] (Table 1). Short-range physical and chemical stimuli from the plant and host egg are also exploited by *A. iole* to locate and recognize *L. hesperus* eggs embedded in plant tissue [52–54].

3. Long-Range Kairomones from Nontarget Instars of the Host

As mentioned above, a well-known solution for the egg parasitoids to overcome the low detectability of host egg cues is to eavesdrop on the pheromonal communication (sex and other attractant pheromones), or the allomonal defenses, of nontarget stages of their hosts. This strategy was initially studied for egg parasitoids of Lepidoptera (reviewed by Fatouros et al. [13]; Colazza et al. [14]), but several cases are also known under laboratory and field conditions for species associated with Heteroptera (Table 1).

In early laboratory experiments using Y-tube olfactometers, *Tr. basalis* was found to be attracted by volatiles from adults of *N. viridula* [55]. A subsequent study revealed that (*E*)-2-decenal, a component of the defensive secretion from the metathoracic scent gland of *N. viridula*, is responsible for this dose-dependent attraction [30]. More recently, both *Tr. basalis* and *Te. podisi* were found to be attracted to and increase their searching behavior in the presence of defensive compounds from metathoracic scent gland secretions of their hosts, *N. viridula* and *E. heros* [33]. *Trissolcus basalis* showed a significant preference for (*E*)-2-decenal and 4-oxo-(*E*)-2-hexenal, while *Te. podisi* responded positively to (*E*)-2-hexenal and 4-oxo-(*E*)-2-hexenal [33]. In addition to volatiles from metathoracic glands, those from the dorsal abdominal glands of nymphs also appear to be exploited as kairomones by *Tr. basalis*, but nymphal secretions may be attractive at intermediate range rather than long range [39].

Detailed investigations of *Tr. basalis* responses to volatiles from both sexes of *N. viridula* showed that the female egg parasitoid is attracted to the males and to preovipositional females, whereas it is not attracted to virgin females [31]. When males and preovipositional females were assayed in a two-choice test, the parasitoid preferred females [31]. Similar results were obtained in a Y-tube olfactometer with *Tr. brochymenae*, as this parasitoid was attracted by cues from differing stages (eggs, nymphs, and adults) and sexes but showed significant preference for gravid females when compared to males [28]. Therefore, volatile allomones and sex pheromones from host males can direct female wasps toward host aggregates, whereas volatiles from gravid females act hierarchically on a subsequent step, representing a more reliable indicator of the potential presence of host eggs [28, 31].

The generalist egg parasitoid, *Ooencyrtus telenomicida* (Vassiliev) (Encyrtidae), is attracted in Y-tube olfactometer to odors of virgin male and, less intensely, of mated *N. viridula* females in preovipositional state, suggesting that the parasitoid exploits the host male-produced attractant pheromone [38]. When exposed to tomato plants treated with *N. viridula* or untreated control plants, *O. telenomicida* females did not respond to healthy or damaged plants, but only to plants with adult bugs, indicating that active volatiles originate from the host rather than the plant [38]. Field experiments confirm parasitoid attraction towards host adults. Traps baited with the synthetic attractant pheromone of male *Riptortus clavatus* (Thunberg) (Alydidae) captured females of the encyrtid egg parasitoid *Ooencyrtus nezarae* Ishii [34]. Males of *R. clavatus* emit an aggregation pheromone, composed of a blend of three compounds that attract adults of both sexes and nymphs. One compound, (*E*)-2-hexenyl (*Z*)-3-hexenoate, attracts females of *O. nezarae* and resulted in higher parasitism in treated fields compared with untreated fields [35, 36]. This tiny parasitoid has the remarkable ability to fly just above the plant canopy in nonhost habitat, while exploiting the above cues to reach the host habitat [37], although the exact flight mechanisms are unknown. In a different system, the use of traps baited with live adult *Leptoglossus australis* F. (Coreidae) resulted in increased parasitism efficacy by *Gryon pennsylvanicum* (Ashmead) (Platygastridae) [56].

Field confirmation of egg parasitoid response to the attractant pheromone of *P. maculiventris* [2] was also achieved [3]. The phoretic females of *Te. calvus* Johnson parasitized significantly more host eggs in pheromone-baited versus nonbaited traps, whereas the generalist *Te. podisi* did not show any significant differences. As described earlier, *Te. calvus* females exploit the male attractant pheromone of *P. maculiventris* to locate females during mating and then become phoretic on mated female bugs [2, 3].

4. Plant Synomones Induced by Feeding or Oviposition

Host-induced plant synomones are reliable and readily available cues for foraging parasitoids that attack feeding stages of hosts [8, 57, 58]. Egg parasitoids may respond to plant synomones induced by feeding [22, 41, 43]. However, not only feeding but also oviposition by herbivores induces emission of plant compounds acting as synomones between the primary and tertiary trophic levels towards their respective egg parasitoids [18–22, 59–65] (Table 1).

Each of the above systems has unique characteristics *vis-à-vis* induced plant defenses. Important differences are the cause of induction (i.e., oviposition, feeding, or a combination of the two, i.e., direct versus indirect cues),

the type of oviposition (i.e., exposed or embedded), and the relationship between egg and plant (i.e., magnitude of synomone emission, local or systemic emission, and activity range of synomone). Other important differences involve timing of synomone release (often reliably related to host suitability), the elicitor source, and, if known, the chemistry of the induced synomone.

4.1. Egg Parasitoid Exploitation of Feeding-Induced Synomones. Feeding likely induces higher synomone levels than does egg deposition. Regardless, exploitation of such indirect host-related cues seems to have evolved because it allows parasitoids to minimize the searching area, thus maximizing efficiency.

Tritrophic plant/bug/egg parasitoid systems involving Heteroptera have been described for *Glycine max* and *Cajanus cajan* (Leguminosae)/*E. heros*/*Te. Podisi*/[41, 42, 66]; *Brassica oleracea* (Cruciferae)/*M. histrionica*/*Tr. brochymenae* (including oviposition-induced synomones) [22, 62, 63]; *Gossypium hirsutum* (Malvaceae) and other plants/*L. hesperus*/*A. iole* [43].

In olfactometer tests, *Te. podisi* responded to volatiles from soybean and pigeon pea fed upon by adults and nymphs of *E. heros* [41]. Application of *cis*-jasmone elicited a similar volatile profile from soybean plants after 96 hours with quantitative, rather than qualitative, chemical differences and resulted in the concomitant attraction of egg parasitoids [66]. Remarkably, when resistant versus susceptible soybean cultivars were compared, parasitoids were only attracted to resistant cultivars; volatile profiles in damaged plants differed between cultivars. In addition, volatiles from oviposition-damaged plants did not attract *Te. podisi* females [42].

Trissolcus brochymenae females' response to plant volatiles induced by host feeding is different than that of *Te. podisi* females. What is known for the former parasitoid indicates that females mainly exploit plant compounds systemically induced by a combination of oviposition, feeding punctures, and footprints of its host, *M. histrionica*, to elicit host searching. Nevertheless, failure to actually oviposit by the host still induces emission of leaf-surface volatiles but, in such cases, parasitoid response was observed only on the damaged leaves [22]. These types of cues appear less reliable compared with oviposition-induced cues, and would act hierarchically at a lower level than other semiochemical cues.

Olfactometer research with *Anaphes iole* Girault (Mymaridae) demonstrated that this species of egg parasitoid also employs volatiles from cotton and other herbaceous plants infested by adults of its host, *L. hesperus* [43]. Eggs of *Lygus* spp. are embedded through an incision made with the ovipositor in plant tissue, with only the operculum exposed, and compounds from these wounds elicit parasitoid behavioral responses even if no eggs have been laid [52, 53]. Both oviposition and feeding damage cause volatiles to be emitted from cotton plants although oviposition appears to induce release of constitutive terpenes from specific glands in cotton leaves adjacent to the oviposition incision, whereas feeding resulted in systemic induction of different volatiles [67]. The volatile blend induced by *L. hesperus* salivary gland extracts is similar to that induced by volicitin, an elicitor isolated from the regurgitant of moth larvae [68], although chemical analyses of the salivary glands from *Lygus* spp. have shown no evidence of a volicitin-type of fatty acid-amino acid conjugate [67]. In electroantennogram (EAG) assays, *A. iole* responded to the majority of herbivore-induced plant volatiles tested, but most intensely to (*Z*)-3-hexenyl acetate, and methyl salicylate; females responded more than males [44]. Olfactometer and wind tunnel bioassays showed that the female wasps were positively stimulated by (*Z*)-3-hexenyl acetate, methyl salicylate and α-farnesene, although response to (*Z*)-3-hexenyl acetate was exhibited only after preconditioning females to blends of host-plant odors. In field trials, host eggs baited with α-farnesene and (*Z*)-3-hexenyl acetate were more heavily parasitized than were untreated eggs [44].

4.2. Egg Parasitoid Exploitation of Oviposition-Induced Synomones. Oviposition-induced synomones, which are directly related to the target stage, are highly reliable and detectable for egg parasitoids. Herbivore oviposition induces emission of plant compounds that act as synomones towards a variety of egg parasitoids [18–22, 59–65]. This "early herbivore alert" [69] by the plant denotes a particular type of indirect induced defense, which, among parasitoids of Heteroptera, has been observed in two tritrophic systems: *Vicia faba* and *Phaseolus vulgaris* (Leguminosae)/*N. viridula*/*Tr. basalis* [20, 21] and, *Brassica oleracea* (Cruciferae)/*M. histrionica*/*Tr. brochymenae* [22, 62, 63] (Table 1).

In the Heteroptera, the first case of plant volatile induction by a bug gluing eggs on leaves without causing mechanical damage is that of *Nezara viridula* ovipositing on legumes [20]. *Trissolcus basalis* was attracted by oviposition-induced volatiles in an olfactometer, and the volatile emission was systemic (i.e., from damaged and adjacent undamaged leaves) [20]. By maximizing the release surface, the plant may have evolved to increase synomone volatilization, thereby increasing herbivores apparency to egg parasitoids. Over time, synomone activity seems to be finely tuned to parasitoid behavior and biology since the attraction fades when host eggs are near to eclosion. Oviposition by *N. viridula* females on *Vicia faba* L. and *Phaseolus vulgaris* L., combined with feeding punctures, induced release of (*E*)-β-caryophyllene, as well as two other sesquiterpenes. Only the fraction containing (*E*)-β-caryophyllene attracted *Tr. basalis* [21]. Whether the elicitor originates from the eggs, follicular tissue, or elsewhere is unknown; however, the combined presence of feeding punctures is necessary for synomone induction [21].

The other known case of an oviposition-induced synomone for an egg parasitoid of true bugs is quite different from the one just described because the induced compounds act at a very short distance. *Tr. brochymenae* perceives the induced synomone only when it alights on a damaged plant [22, 62]. Compared to healthy plants, females of this parasitoid intensely antennated and searched on leaves having a host egg mass, plus nearby feeding punctures and

chemical footprints (treated surface). Parasitoid response was tested on the upper (adaxial) leaf surface, opposite to the treated (abaxial) surface. Female wasps also responded in a static olfactometer at near contact range to volatiles perceived through olfaction, but host-damaged plants in a Y-tube olfactometer were not attractive to wasps. As with *N. viridula* on leguminous plants, the induction is both local and systemic, but the origin of the elicitor and the mechanisms involved remain unknown. However, in the case of *M. histrionica* on cabbage, egg mass deposition is sufficient, as are feeding punctures, to elicit parasitoid response, although the combination of oviposition, feeding, and footprints increases parasitoid response [22]. Parasitoid reaction to compounds emitted as a consequence of host feeding appears to be a response to damaged host plants [22]; leaves with feeding punctures exhibit alteration of tissues and photosynthesis [70].

5. Short-Range Kairomones from Nontarget Host Stages

Indirect host-related cues originate from adults or juveniles of the host and, in general, elicit arrestment and searching behavior in the parasitoid. Trichogrammatidae and Platygastridae species responses to lepidopteran moth scales were studied earlier (reviewed by Colazza et al. [14]). However, a comparable strategy was also discovered for the heteropteran egg parasitoids, *Trissolcus* spp. [28, 31, 32, 71] *Te. podisi* [46], and *Gryon boselli* Mineo & Szabo (Colazza, Lo Bue, and Cusimano, personal observation), which respond to chemical footprints of pentatomid bugs (Table 1). Both *Tr. basalis* and *Tr. brochymenae* females are able to discriminate chemical footprints left by host females, to which they respond more strongly than to chemical traces left by walking males or nymphs [28, 31, 47, 72]. In addition, *Tr. brochymenae* is able to detect cues from mated females in the preovipositional state, which are preferred to virgin females and parous females (i.e., those that have already produced offspring), thus finely tuning their searching to the host stage most likely to lead to host eggs [47]. This preference was strictly related to the transfer of sperm and associated substances from the conspecific male bug to the female during copulation. The compounds mediating arrestment of *Tr. brochymenae* females are from host cuticle, and those that play a role as gender-specific cues are most abundant on the legs of the host adult [47].

Associative learning plays an important role in host footprint recognition behavior. Oviposition experience increased the arrestment response of *Tr. basalis* females to footprints of *N. viridula* females, whereas prior experience not followed by oviposition led to the gradual fading of the learned behavior [25]. In contrast, previous experience with the footprints of host males did not result in a change of parasitoid response, indicating that residues from males only provide general information for the parasitoid, which is not directly associated with host eggs [25]. There is significant variation in the learning ability of *Tr. basalis* females as a function of environment and spatial distribution, but learning always helps make foraging more successful [26].

Footprint chemistry was investigated in the *Tr. Basalis-N. viridula* relationship. Analysis of extracts of cuticular lipids from *N. viridula* revealed the presence of normal alkanes, with quantitative and qualitative differences between the sexes. One compound, *n*-nonadecane, was recovered only from the cuticle and footprints of males. When added to cuticular extracts of *N. viridula* females, *n*-nonadecane caused *Tr. basalis* females to significantly reduce their residence time in the arena, similar to the behavior of female wasps in the presence of hexane extracts of male hosts [72]. Parasitoid response to host footprints is mediated by adsorption of the contact kairomone in the epicuticular wax layer of plants walked upon by host bugs [48, 73].

6. Short-Range and Contact Kairomones from Host Eggs

Semiochemicals from or on host eggs are likely present in faint amounts and, thus, are probably exploited by egg parasitoids only at close range or upon contact. Short-range and contact kairomones are best known for egg parasitoids of Lepidoptera [14]. Among the Heteroptera, this semiochemical level has been investigated for the egg parasitoid of the harlequin bug (*M. histrionica*), *T. brochymenae*, in both a Y-tube olfactometer and an open arena. In the olfactometer the *T. brochymenae* females were attracted to volatiles from host egg masses, whereas, in the open arena, the female parasitoids oriented towards egg clusters or dummies treated with chemical extract of host eggs. When the egg extract was applied without dummies, it elicited the same response, whereas dummies without extract did not influence parasitoid behavior, indicating that visual factors are unnecessary for this last step in host location [28] (Table 1).

Parasitoid host recognition to egg contact kairomones is much more obvious than are responses to egg volatiles (Table 1). When in contact with the heteropteran host egg mass, *Trissolcus* spp. use both physical and chemical cues; egg size and the shape are important cues, but chemicals on the egg surface are fundamental for host acceptance [50]. The recognition kairomones are contained in the adhesive secretion from the follicular cells of heteropteran hosts [28, 50, 51], composed of mucopolysaccharide-protein conjugants [50], but their chemical nature has not been defined yet. Surprisingly, (*E*)-2-decenal, a component of the defensive secretion from the metathoracic scent gland of *N. viridula* that was attractive to *Tr. basalis* in an olfactometer, also elicited parasitoid antennation and ovipositor probing of egg-sized glass beads [30].

When host eggs are embedded in plant tissue, semiochemicals may originate from the egg, the damaged plant, or from their interaction. Females of the mymarid, *Anaphes iole*, respond to *Lygus* eggs inserted into plant tissue with arrestment, increased antennation, and ovipositor probing even if oviposition wounds do not contain eggs, although probing is much more intense if the incision contains a host

egg. Artificial wounds are also probed by the parasitoid, and so are eggs removed from the substrate and placed on a surface [52, 53]. Although chemical cues appear to play a major role, physical cues are also important in this phase; *A. iole* females adopt a probing posture when chemicals from host or plants are combined with appropriate shapes [52–54]. Electroantennogram assays showed that the parasitoid antennae sense several plant volatiles, including green leaf volatiles, confirming the importance of plant chemicals during host searching in this species [44]. Ovipositor probing behavior is the final step of host searching by *A. iole*, rather than merely host recognition; perhaps this explains why these mymarid females insert their ovipositor, although less frequently, even in artificial wounds made in a parafilm substrate containing neither host eggs nor contaminated by host material [52]. Moreover, learning host and plant cues increases *A. iole* oviposition efficiency [53]; indeed, a preference for (*Z*)-3-hexenyl acetate occurs only after preconditioning with host-induced plant volatiles [44].

7. Conclusions

The importance of egg parasitoids as biological control agents for herbivorous insect pests is widely recognized. Nevertheless, their success in classical biological control programs is slightly lower than that for other kinds of introduced parasitoid species [74]. Several Mymaridae and Platygastridae species have been used for augmentative biological control of Heteroptera [74], the most successful of which involved releases of *Tr. basalis* against *N. viridula* on soybean in Brazil [75, 76].

While several egg parasitoids of Heteroptera are potentially effective biological control agents, there are still constraints preventing the realization of this goal. Incomplete behavioral and ecological knowledge for most parasitoid species remains problematic and, perhaps most importantly, mass production of egg parasitoids remains inefficient [77–79]. Understanding the host selection strategies of egg parasitoids and the chemical stimuli involved could lead to improved biological control efficacy through behavioral manipulation of egg parasitoids in the field and to development of better *in vivo* and *in vitro* rearing methods. An estimated 16 million ha of cropland worldwide currently receives inundative releases of egg parasitoids, primarily involving species in the genus *Trichogramma* [77]. Efficient mass rearing of *Trichogramma* spp., as well as intensive, focused research preceded this achievement, including elucidation of the chemicals necessary for acceptance of artificial host media and the parasitoid development [79]. The success of *Trichogramma* biocontrol programs is a model of what may be achieved with other parasitoids given the required research investment.

Successful implementation of egg parasitoids against true bugs will depend on judicious applications of synthetic semiochemicals, particularly synomones and kairomones, and appropriate strategies to overcome existing constraints, including treatments facilitating parasitoid rearing, conditioning, and manipulation [61, 74, 80, 81]. Other interesting possibilities include the development of plants with elevated expression of indirect induced resistance factors (i.e., the induction of plant synomones exploited by the herbivores' parasitoids) or spraying fields with resistance elicitors [61]. Accurate knowledge of parasitoid chemical ecology will be important in all phases of biocontrol and integrated pest management [82]; important elements toward implementation include procedures for selection of egg parasitoids, evaluation of their specificity [27, 32, 71], risk assessment of new introductions [83], release methods [80], and quality control [80, 84]. Implementation of knowledge gleaned from laboratory studies must finally be scaled up and transferred to growers.

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