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SAMPLING TERRESTRIAL GASTROPODS USING CARDBOARD SHEETS

J. W. Hawkins, M. W. Lankester & R. R. A. Nelson

Department of Biology, Lakehead University, 955 Oliver Road, Thunder Bay, Ontario, P7B 5E1, Canada

ABSTRACT

Cardboard sheets are an efficient way of collecting large numbers of terrestrial gastropods and are useful for estimating relative densities and determining species composition of snails and slugs active on the surface of the forest floor, provided sampling is conducted under optimal weather conditions. Sheets may be less reliable, however, for quantitatively assessing the subterranean component of gastropod communities. Cardboard sheets placed on the forest floor sampled approximately 1/50 the number of gastropods estimated, using soil cores, to be in the upper 10 cm of soil beneath the sheets. The numbers collected by the two methods were not correlated ($p = 0.96$), but cardboard sheets produced up to 30 times as many specimens per unit of sampling time. Gastropod numbers beneath cardboard sheets peaked at near-ground temperatures around 15°C and were augmented by animals moving horizontally over the surface of the litter from the surrounding area and vertically from the underlying soil, particularly when conditions were wet. Although mean ($\pm$ S.E.) gastropod densities determined using sheets allowed to weather for a year (27.3 ± 4.1 m^{-2}) and new sheets (16.2 ± 2.6 m^{-2}) were not significantly different ($p = 0.09$), larger samples might confirm a tendency to prefer weathered sheets. In addition, 4 of 20 species were collected in greater numbers on weathered sheets, suggesting differential use of cardboard sheets by those species.

Key words: terrestrial gastropods, sampling, cardboard sheets, horizontal movement, temperature.

INTRODUCTION

Corrugated cardboard sheets have frequently been used in parasitological studies to collect terrestrial gastropods that serve as intermediate hosts of metastrongyloid nematodes (Lankester & Anderson, 1968; Gleich & Gilbert, 1976; Kearney & Gilbert, 1978; Upshall et al., 1986; Beach, 1992; Lankester & Peterson, 1996). For this purpose, cardboard sheets have provided a convenient, time-efficient method of collecting large numbers of snails and slugs, but whether sample data can be used to accurately estimate gastropod population densities and species composition remains unknown.

Boag (1982) compared the use of cardboard sheets and hard masonite boards for sampling terrestrial gastropods and concluded that cardboard sheets were effective for repeatedly sampling the same area over time but that the technique was subject to potential limitations. He suspected that cardboard sheets may not sample all gastropod species and life stages equally and that horizontal movement to the sheets may bias density estimates. In addition, Boag (1990) suspected that sheets may become progressively more attractive to gastropods the longer they are exposed to the weather.

Our purpose was to evaluate some concerns raised about using cardboard sheets to estimate population densities and determine the species composition of terrestrial gastropod communities. We tested the hypothesis that the number of gastropods collected using cardboard sheets is correlated with that deeper within the underlying litter and soil. In addition, we investigated whether gastropods migrate horizontally across the surface and accumulate beneath cardboard sheets, thereby inflating population estimates. The relationship between temperature and the number of gastropods collected was explored, and collections from new and weathered cardboard sheets were compared.

MATERIALS AND METHODS

Two study sites, in recently restocked spruce (*Picea* spp.) plantations, were located in Fraleigh Township (48°08′N, 89°49′W), about 60 km southwest of Thunder Bay,

Ontario, within the Great Lakes-St. Lawrence Forest Region (Rowe, 1972). Site 1 was on a south facing slope, had shallow, sandy loam soils and deciduous regrowth consisting primarily of trembling aspen (*Populus tremuloides*) and mountain maple (*Acer spicatum*). Site 2 was also on a south facing slope but had deep, silty soils and regrowth consisting of trembling aspen, willow (*Salix* spp.), alder (*Alnus* spp.), fireweed (*Epilobium angustifolium*), raspberry (*Rubus* spp.), and white birch (*Betula papyrifera*). Additional information on the study sites can be found in Bell et al. (1996).

Terrestrial gastropods were collected using unwaxed, corrugated cardboard sheets (80 × 107 cm) placed on the surface of the forest floor and weighed down with rocks. Gastropods located beneath and on top of each cardboard sheet were identified, with the aid of Pilsbry (1939–1948), Oughton (1948), and Burch (1962), counted and removed. Species too small to be identified in the field were preserved in glycerin alcohol and identified later under a dissecting microscope at 16×. Voucher specimens were sent to the Royal Ontario Museum, Toronto, where identifications were confirmed.

To determine whether the number of gastropods collected using cardboard sheets was correlated with that in the soil beneath them, 10 cardboard sheets, in place for five days on site 1, were examined for gastropods on September 14, 1993. Immediately thereafter, three soil cores (8 × 7 × 10 cm) were taken at random locations from beneath each sheet. The top and bottom halves (5 cm each) of each core were placed in separate bags, labelled, and stored frozen at –5°C for 2–20 days. Before freezing, an attempt was made to collect any slugs present in the soil samples by placing fresh lettuce leaves in the bags and leaving them overnight in a controlled environment chamber at 20°C. The inner surface of the bag and the lettuce were then inspected for slugs and snails that moved out of the soil sample.

Thawed soil samples were placed in an enamel dissection tray (25 × 45 × 6 cm) half-filled with water and visually examined for gastropods. Following this preliminary visual search, samples were washed through a series of sieves (smallest = mesh No. 120, 0.125 mm opening) and the remaining material examined for the presence of gastropods under a dissecting microscope (6×). Gastropods without a visible tissue mass within the shell were considered dead at the time soil cores were collected and were not counted in density estimates. In the case of smaller specimens, this was determined with the aid of a dissecting microscope.

An experiment was designed to determine whether cardboard sheets sample only those gastropods residing in the litter and soil directly beneath them, or if collections also include animals moving laterally from the surrounding area. Ten pairs of cardboard sheets were placed randomly at approximately 10 m intervals on the ground vegetation at site 2. Each pair consisted of one sheet enclosed tightly by a strip of sheet metal (22 gauge, 12 cm high) penetrating 7 cm into the soil and protruding 5 cm above the soil surface, plus a control sheet (unenclosed), placed 4 m away.

The above-ground surface of the sheet metal barrier was coated with automotive bearing grease and covered with a mixture of coarse grain, black pepper and cayenne pepper (2:1) to discourage gastropods from crawling over it. All overhanging ground vegetation was removed. Pairs of cardboard sheets were sampled simultaneously for gastropods, between 0800 hrs. and 1000 hrs., every 2–3 days from July 22 to August 18, 1994, for a total of 11 sampling days. Gastropods were removed and each sheet was returned to its original position.

The relationship between temperature and numbers of gastropods collected beneath cardboard sheets was examined. At each of 30 cardboard sheets on site 2, three type-T copper-constanten thermocouple leads were positioned, one 2 cm above the sheet to measure ambient air temperature, one directly beneath, and one 2 cm deep in the litter beneath the sheet. Thermocouple temperatures were measured, using a Cole-Parmer digital thermometer (Model 08500-41), five times throughout the summer of 1994 (0700–1100 hrs.), immediately before collecting all gastropods on the sheet.

An experiment designed to determine if weathered cardboard sheets sample more gastropods than new sheets was initiated in early June 1993. Ten cardboard sheets were randomly placed on the ground vegetation at site 2 and sampled five times over the summer of 1993 as part of a larger gastropod population study (Hawkins et al., 1996). All were left exposed to the weather over winter. On May 2, 1994, a new cardboard sheet was positioned approximately 4 m from each weathered sheet. At the same time, the weathered

TABLE 1. Gastropods collected on cardboard sheets and from soil cores beneath sheets

Species	Cardboard sheets*		Soil cores†	
	Total	Density (/m^2)‡	Total	Density (/m^2)‡
Zonitoides arboreus	71	8.3 ± 92.1	103	613.1 ± 138.6
Discus cronkhitei	16	1.9 ± 0.8	57	339.3 ± 81.4
Striatura milium	16	1.9 ± 0.7	49	291.7 ± 70.1
Deroceras laeve	48	5.6 ± 1.0	1	6.0 ± 6.0
Vitrina limpida	29	3.4 ± 1.0	19	113.1 ± 39.1
Strobilops labyrinthica	14	1.6 ± 0.8	9	53.6 ± 24.2
Vertigo gouldi	13	1.5 ± 0.8	5	29.8 ± 23.9
Euconulus fulvus	13	1.5 ± 0.5	3	17.9 ± 12.7
Cochlicopa lubrica	0	0	14	83.3 ± 33.4
Columella edentula	13	1.5 ± 0.5	0	0
Zoogenetes harpa	0	0	7	41.7 ± 19.9
Striatura exigua	2	0.2 ± 0.2	1	6.0 ± 6.0
Vertigo modesta	0	0	1	6.0 ± 6.0
Carychium exile canadense	0	0	1	6.0 ± 6.0
Total	235	27.5 ± 5.2	270	1607.1 ± 272.3

*10 sheets of unwaxed corrugated cardboard (80 × 107 cm)
†30 soil cores (8 × 7 × 10 cm)
‡mean ± S.E. (/m^2 of surface area)

sheets were moved approximately 1 m from their over-winter position and any gastropods adhering to the sheets were removed. Each of the 10 new sheets was sampled, simultaneously with its corresponding weathered sheet, four times during the 1994 field season (May 20, June 29, July 12, and July 25, 1994).

Simple linear regression (Neter et al., 1989) was performed to determine whether any relationship existed between density estimates obtained using cardboard sheets and those obtained using soil cores. Density estimates from soil cores were used as the independent variable. The null hypothesis of no difference between enclosed cardboard sheets and control sheets was tested using the Wilcoxon Rank Sum test (Bradley, 1968). A Kruskal-Wallis analysis of variance (Bradley, 1968) was performed to test for differences in gastropod densities between the 11 sample periods. A repeated measures analysis of variance (Gumpertz & Brownie, 1993) was performed to detect differences in near-ground temperature between the three different thermocouple positions. The dependent variable in this analysis was normalized using a square root transformation. The Wilcoxon Rank Sum test was also used to test the null hypothesis of no difference in the use of new and weathered cardboard sheets by gastropods. All statistical differences were considered significant at $p < 0.05$. Statistical procedures were performed on SPSS PC-6.1 (Norusis, 1992a, 1992b).

RESULTS

Cardboard Sheets vs. Soil Cores

The mean (± S.E.) density of gastropods active on the surface of the forest floor and estimated using the cardboard sheet method was 27.5 ± 5.2 m^{-2} whereas a mean (± S.E.) of 1607.1 ± 272.3 m^{-2} was estimated using the soil core method to be in a 1 m × 1 m × 10 cm volume of soil (Table 1). Fourteen species, including 13 snails and one slug (*Deroceras laeve*), were collected. Eighty-eight percent of gastropods collected from the soil cores were found in the upper 5 cm of the soil and the remainder in the bottom 5 cm. Linear regression analysis revealed no correlation between the densities of gastropods estimated using cardboard sheets and soil cores ($F = 0.003$; $p = 0.958$). Checking a cardboard sheet required 5–15 minutes with yields ranging from 7–49 gastropods per sheet; 3–4 hours were required to examine each soil core with a range of 0–27 gastropods being recovered.

Barrier-Enclosed Sheets

Total mean (± S.E.) gastropod density was lower on sheets enclosed with a sheet metal

TABLE 2. Mean (± S.E.) densities (/m^2) of terrestrial gastropods collected from cardboard sheets enclosed with a metal barrier and control sheets (unenclosed)

	Barrier enclosed sheets*	Control sheets*	p-value†
Deroceras laeve	1.18 ± 0.152	1.90 ± 0.280	p = 0.097
Euconulus fulvus	0.23 ± 0.060	0.20 ± 0.058	p = 0.472
Columella edentula	0.12 ± 0.034	0.27 ± 0.076	p = 0.328
Zonitoides arboreus	0.14 ± 0.047	0.23 ± 0.056	p = 0.077
Striatura milium	0.12 ± 0.062	0.04 ± 0.026	p = 0.462
Succinea ovalis	0.06 ± 0.025	0.09 ± 0.036	p = 0.974
Vitrina limpida	0.03 ± 0.018	0.10 ± 0.034	p = 0.121
Vertigo gouldi	0.02 ± 0.015	0.09 ± 0.033	p = 0.088
Strobilops labyrinthica	0.04 ± 0.021	0.02 ± 0.015	p = 0.409
Cochlicopa lubrica	0.02 ± 0.015	0.04 ± 0.021	p = 0.409
Discus cronkhitei	0.03 ± 0.018	0.02 ± 0.015	p = 0.652
Pallifera dorsalis	0	0.04 ± 0.034	p = 0.156
Zoogenetes harpa	0.04 ± 0.026	0	p = 0.082
Vertigo ovata	0.02 ± 0.015	0	p = 0.156
Anguispira alternata	0.01 ± 0.011	0	p = 0.317
Gastrocopta tappaniana	0	0.01 ± 0.011	p = 0.317
Total	2.07 ± 0.228	3.05 ± 0.349	p = 0.038

*sampled with removal 11 times between July 22 and August 18, 1994
†p-values based on a Wilcoxon Rank Sum Test

barrier (2.1 ± 0.2 m^{-2}) than on control sheets (3.1 ± 0.4 m^{-2}) (p = 0.038), although no difference was detected when individual gastropod species were considered (Table 2). Cumulative totals of 195 and 287 gastropods were collected from enclosed and control cardboard sheets, respectively, over the course of the experiment. *Pallifera dorsalis* and *Gastrocopta tappaniana* were not collected from the sheets enclosed with a barrier; *Zoogenetes harpa, Vertigo ovata,* and *Anguispira alternata* were not collected from the control sheets. The mean density of snails and slugs, from both enclosed and control cardboard sheets, varied over the 11 sample periods (p < 0.0001) (Fig. 1). Over the first five collection days, mean (± S.E.) gastropod density was lower under enclosed (2.0 ± 0.21 m^{-2}) than control sheets (3.9 ± 0.26 m^{-2}) (p = 0.0029). However, densities increased sharply under both, and particularly under control sheets, following heavy rains on August 3rd and 7th totalling 35.2 mm and on August 16 following 3.8 mm of rain (Fig. 1).

Temperature Beneath Sheets

Gastropod collections peaked when the temperature beneath cardboard sheets was approximately 15°C and decreased at lower and higher temperatures. Temperature was more variable on clear days (4°C to 30°C, $\bar{x}$ = 13.5 ± 0.56) than on overcast days (11°C to 22°C, $\bar{x}$ = 15.8 ± 0.33).

Mean (± S.E.) temperature beneath the cardboard sheets (14.4 ± 0.4°C) was slightly cooler than that 2 cm above the forest floor (15.4 ± 0.6°C) throughout July, while both were similar in August. However, interaction in temperature among the three thermocouple positions was observed over the summer (F = 4.99; p < 0.05). Throughout July, the temperatures 2 cm above the forest floor and directly beneath (0 cm) the cardboard sheets were higher than those measured 2 cm deep in the humus layer but, for the first half of August, the reverse was true.

Weathered Sheets

Total mean (± S.E.) gastropod densities on new (16.2 ± 2.6 m^{-2}) and weathered (27.3 ± 4.1 m^{-2}) cardboard sheets were not significantly different (p = 0.09). However, of the twenty species of terrestrial gastropod collected (Table 3), *Euconulus fulvus, Vertigo gouldi, Carychium exile canadense,* and *Striatura milium* were present in greater densities on the weathered sheets than on the new sheets.

DISCUSSION

Results reported here demonstrate that the number of terrestrial gastropods present within the litter and underlying 10 cm of soil is more than 50 times as great as the number of

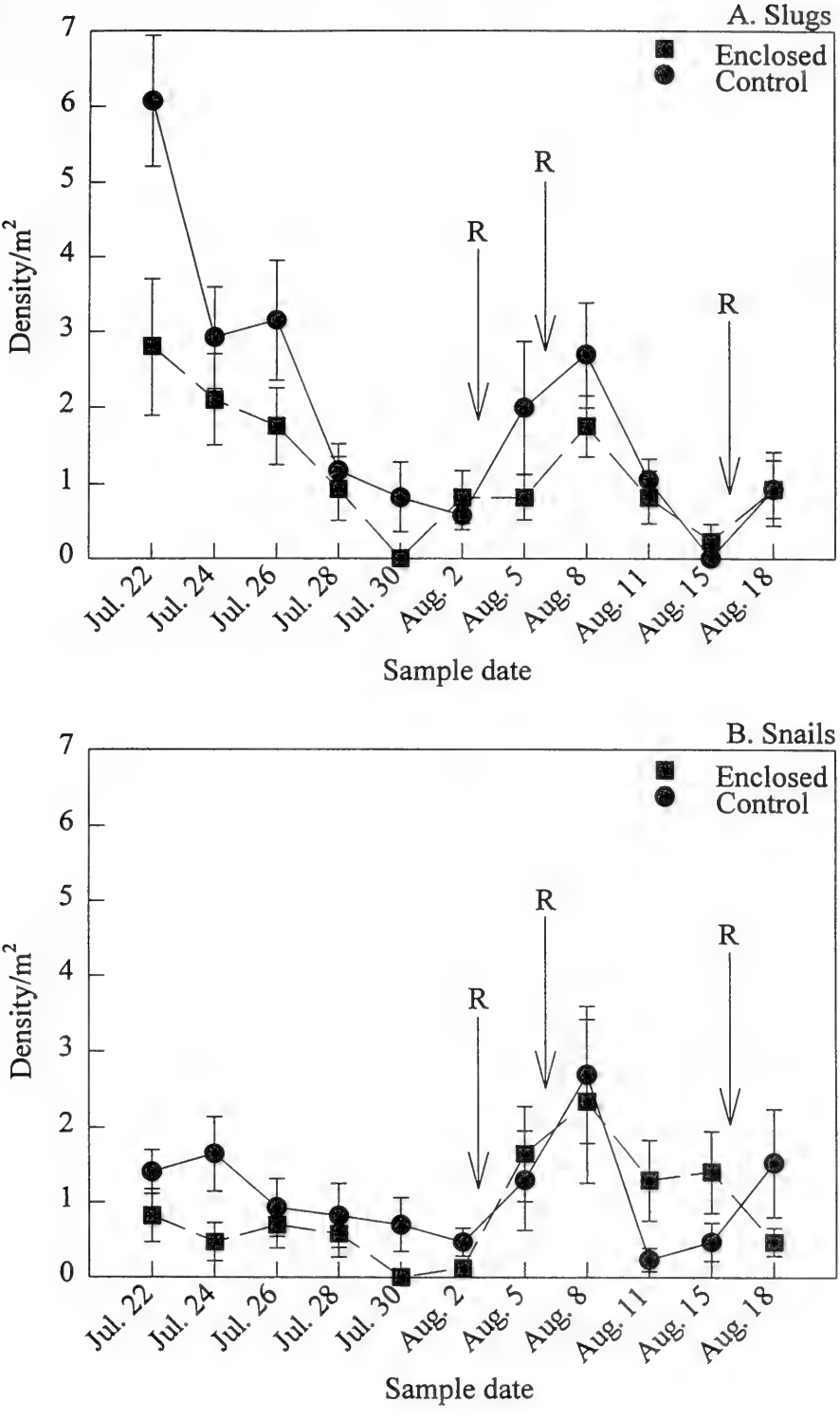

FIG. 1. Mean (± S.E.) densities of gastropods removed from cardboard sheets enclosed with a metal barrier and control sheets (unenclosed), on 11 sample days, July 22 to August 18, 1994. [R = rain greater than 3 mm]. (A) Slugs. (B) Snails.

TABLE 3. Mean (± S.E.) densities (/m^2) of terrestrial gastropods collected from beneath new and weathered cardboard sheets

Species	New sheets*	Weathered sheets*	p-value†
Zonitoides arboreus	5.12 ± 1.03	6.53 ± 1.04	p = 0.187
Strobilops labyrinthica	1.85 ± 0.46	4.51 ± 1.08	p = 0.072
Euconulus fulvus	1.71 ± 0.42	4.51 ± 0.85	p = 0.001
Deroceras laeve	2.72 ± 0.49	2.23 ± 0.35	p = 0.712
Vitrina limpida	1.30 ± 0.49	1.10 ± 0.45	p = 0.889
Discus cronkhitei	0.40 ± 0.14	1.30 ± 0.38	p = 0.110
Vertigo gouldi	0.35 ± 0.18	1.10 ± 0.36	p = 0.018
Carychium exile canadense	0.09 ± 0.09	1.24 ± 0.38	p < 0.001
Striatura milium	0.20 ± 0.15	1.07 ± 0.35	p = 0.008
Succinea ovalis	0.75 ± 0.28	0.52 ± 0.12	p = 0.665
Columella edentula	0.46 ± 0.19	0.78 ± 0.32	p = 0.703
Anguispira alternata	0.29 ± 0.12	0.69 ± 0.29	p = 0.631
Striatura exigua	0.23 ± 0.12	0.64 ± 0.24	p = 0.278
Cochlicopa lubrica	0.23 ± 0.08	0.52 ± 0.30	p = 0.863
Gastrocopta tappaniana	0.06 ± 0.04	0.20 ± 0.07	p = 0.079
Vertigo ovata	0.14 ± 0.08	0.12 ± 0.09	p = 0.655
Vertigo modesta	0.14 ± 0.10	0.06 ± 0.04	p = 0.960
Zoogenetes harpa	0.06 ± 0.04	0.06 ± 0.04	p = 1.000
Punctum minutissimum	0.06 ± 0.06	0.06 ± 0.06	p = 1.000
Pallifera dorsalis	0.03 ± 0.03	0.03 ± 0.03	p = 1.000
Total	16.19 ± 2.61	27.25 ± 4.13	p = 0.086

*sampled with removal four times throughout the summer of 1994 (May 20, June 29, July 12, and July 25)
†p-values based on a Wilcoxon Rank Sum Test

snails and slugs active on the surface of the forest floor and detectable using the cardboard sheet sampling technique. Although the cardboard sheet and soil core sampling methods were not correlated, each provides a relative density estimate of a particular component of the gastropod community, one active on the surface of the forest floor and one present within the litter and underlying soil. Kralka (1986), working in the boreal forest of Alberta, examined 5 cm deep soil cores and estimated a total mean gastropod density of 80 m^{-2}, with maximum densities of *Discus cronkhitei* and *V. gouldi* reaching 340 m^{-2} and 460 m^{-2}, respectively. Estimates of gastropod population densities in boreal forests using cardboard sheets have ranged from 2–38 m^{-2} (Kearney & Gilbert, 1978; Hawkins, 1995; Lankester & Peterson, 1996).

Cardboard sheets provided a more time-efficient method of collecting terrestrial gastropods, yielding up to 30 times as many specimens as found in soil cores in similar time periods. In addition, the majority of gastropods found adhering to sheets can be identified in the field, with the exception of smaller species, such as *Vertigo* spp. and *Columella* spp., which must be identified beneath a microscope. Soil cores, however, not only involve a greater effort in the field but also require considerably more time to extract specimens from the samples in the laboratory.

When using cardboard sheets to sample terrestrial gastropods, horizontal movement across the surface of the litter could affect the number of snails and slugs collected. With repeated sampling and removal, the mean number of gastropods collected beneath both barrier enclosed and control sheets would be expected to decline if no horizontal movement of gastropods towards the sheets were occurring. However, if individuals were continually immigrating from the surrounding area, the mean density of individuals found beneath the enclosed sheets should fall to a lower level than that under control sheets. In fact, this was observed over the first five collection days. The mean density recovered beneath enclosed sheets (2.0 ± 0.21 m^{-2}) was half that from unenclosed sheets (3.9 ± 0.26 m^{-2}) suggesting that horizontal movement does occur. Numbers increased, however, under both enclosed and control sheets following separate rainfalls totalling 35 mm and 4 mm. Although the increase on both occasions was greatest beneath the control sheets, indicating that some increase in horizontal movement had probably occurred, an increase beneath the enclosed sheets suggests that individuals also moved vertically in response to the wet-

ter conditions (Locasciulli & Boag, 1987). A more direct demonstration of horizontal movement was provided by Boag (1990), who marked and released snails beneath masonite boards and observed that 5% of *D. cronkhitei* and 12% of *E. fulvus* moved from beneath the boards onto the surrounding litter.

The extent to which gastropods might actually be attracted to cardboard sheets has not been determined, yet there is some empirical evidence that they do accumulate beneath more permanent sampling structures (Boag, 1990). Snails and slugs would accumulate beneath cardboard sheets if more individuals move under a sheet than leave, implying that conditions beneath cardboard sheets are generally more favourable than those encountered on the surrounding forest floor. These animals can be observed moving openly across the surface of the litter at night and on overcast and rainy days until moisture, temperature, and/or light conditions become unfavourable (Boag, 1985; personal observation). Under natural daily conditions of increasing light intensity and decreasing humidity, their most direct route to refuge would be downward into the litter and soil. Conditions that discourage gastropod movement on the surface may be delayed in onset and be less severe beneath a cardboard sheet. However, sheets seldom remain suitable refuge for long. They dry readily in the sun, and the vegetation beneath them dies back if left covered for more than 2–3 weeks. In some circumstances, the litter beneath a cardboard sheet may be drier than its surroundings, for example if a sheet has been put in place before a light rain or heavy dew occurs. This expected variability can best be minimized by placing already dampened sheets in a new location during, or immediately after, a rainy period, and by checking the sheets within a few days and only in the early hours of the morning.

The temperature beneath cardboard sheets influences the number of gastropods that can be collected using this method. The mean temperature immediately beneath the cardboard sheets was slightly lower than the air temperature 2 cm above them throughout July, suggesting that the shading effect of the sheets keeps temperatures cooler than those on the surrounding forest floor. Collections were greatest when the temperature beneath the sheets was approximately 15°C. Boag (1990) reported the greatest number of gastropods beneath masonite boards at ambient air temperatures between 7.5°C and 17.5°C and direct observation of snails in terraria indicated greater activity on the surface of the litter at temperatures ranging from 6°C to 15°C (Boag, 1985). Snails and slugs most likely take refuge deeper in the litter and underlying soil when temperatures reach lower or higher extremes. The time of day and amount of cloud cover will clearly play a role in influencing the temperature beneath the sheets. On sunny, hot days, favourable temperatures beneath the sheets will only prevail for a limited period of time early in the morning. On overcast days, however, daytime temperatures will remain cooler and larger numbers of gastropods are likely to be found beneath cardboard sheets later into the day. The apparent relationship between temperature beneath the sheets and the number of gastropods collected should be considered when using this technique in field studies of terrestrial gastropods.

Overall, mean densities of gastropods collected from weathered and new cardboard sheets were not significantly different. However, the low power of the test ($n = 10$) and a reasonably large difference between the mean ($\pm$ S.E.) densities (weathered sheets = $27.3 \pm 4.1\ m^{-2}$; new sheets = $16.2 \pm 2.6\ m^{-2}$), suggest that snails and slugs may in fact favour weathered sheets. Four species (*E. fulvus, V. gouldi, C. exile canadense,* and *S. milium*) may preferentially use weathered sheets. Weathered sheets appeared to absorb more moisture and retain it longer following a rain. A similar increase in use of weathered masonite boards was seen by Boag & Wishart (1982) and Boag (1990). It was suggested that boards exposed for long periods to the elements may become more attractive as a result of having dissipated any possible repellant chemicals, or because they acquire fungal hyphae and slime trails on their lower surface. Boag (1990) also suggested that various gastropod species may use sampling boards differentially, which may explain, in part, the greater numbers of four species collected from weathered sheets and underscores a potential limitation of using cardboard sheets to estimate relative numbers and densities of terrestrial gastropods.

We conclude that the cardboard sheet sampling technique is a time-efficient method of collecting large numbers of terrestrial gastropods that otherwise become difficult to find in daylight hours. If sampled under optimal weather conditions, cardboard sheets provide an acceptable method of quantifying the relative abundance and species composition of gastropods active on the surface litter. This

method allows comparison between gastropod communities in different locations and habitats provided collections are made under similar weather conditions and in the same season to account for different reproductive life histories (Comfort, 1957; Berry, 1966; Umiński & Focht, 1979; Livshits, 1983). Cardboard sheets are less reliable, however, for quantitatively assessing the subterranean component of gastropod communities.

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REPRODUCTION AND EMBRYONIC DEVELOPMENT TIME OF *BATHYPOLYPUS ARCTICUS,* A DEEP-SEA OCTOPOD (CEPHALOPODA: OCTOPODA).

J. B. Wood[1], E. Kenchington[1,2] & R. K. O'Dor[1]

. . . *Worse, in terms of outright scariness, Are the suckers multifarious* . . . Bill Watterson, Calvin and Hobbes

ABSTRACT

Mating, brooding, and embryonic development rate of *Bathypolypus arcticus,* a deep-sea octopod, are described. Live specimens of *B. arcticus* were collected in the Bay of Fundy, Canada, and kept in a flow-through system in the lab. Two of the octopods laid and brooded viable eggs. Brooding and embryological development took over a year at average temperatures of 7.3°C and 7.8°C. Brooding females ate occasionally and left their eggs shortly before dying. Hatchlings weighed 208 ± 17 (SD) mg from the first batch and 283 ± 20 (SD) mg from the second batch. There was no evidence of multiple spawning.

Mating of *B. arcticus* was also observed. The usually smaller male sits upon the female, enveloping much of the female's mantle in his web, and he inserts his large ligula into her mantle. One or two large spermatophores are transferred by a combination of mantle pumping and arm groove peristalsis. A filmed mating sequence lasted 140 seconds.

Key words: cephalopod, Octopoda, Octopodidae, *Bathypolypus arcticus,* deep-sea, mating, embryonic development, brooding.

INTRODUCTION

Bathypolypus arcticus (Prosch, 1849) is a small octopodid that rarely exceeds 200 g (O'Dor & Macalaster, 1983). This species has been found to depths of 1,543 m (Voss, 1988a,b) and is classified as a deep-sea octopus by Voss (1988b). *Bathypolypus arcticus,* most common at depths of 200–600 m, is widely distributed in the Atlantic Ocean (O'Dor & Macalaster, 1983). Assuming the three-year life span estimated by O'Dor & Macalaster, females brood eggs for a larger percentage of their life than any octopus studied thus far, with the possible exception of the iteroparous *Octopus chierchiae* (Jata, 1889) (Rodaniche, 1984). Like all deep-sea octopods, female *B. arcticus* lay large eggs, from which well-developed young hatch.

Mature male *B. arcticus* have the largest ligula relative to body size of any octopodid. The ligula is part of the hectocotylus, the modified third right arm that males use to transfer their equally large spermatophores while mating. Mating has not been previously described for *B. arcticus*—what males do with their hectocotylus was unknown. Two mating positions have been observed in the family Octopodidae: a distant position in which the male and female are separated except for the hectocotylus, and one in which the male mounts the female (Mangold, 1987). Mangold notes that mating may last from a few minutes to several hours.

As much as there is to be learned from cephalopods we can easily obtain, perhaps even more is yet to be learned from species that dwell in the deep-sea (Forsythe & Van Heukelem, 1987). Little laboratory work has been done with deep-sea cephalopods due to difficulties in collecting undamaged live specimens and continually providing cold water. The only previous laboratory information on brooding and embryonic development of deep-sea octopods is from O'Dor & Macalaster (1983). They reported that a single female *B. arcticus* laid eggs in August 1978 and brooded them until they hatched in July 1979. They state that the temperature varied between 3°C and 10°C, but temperatures were not recorded. The female was essentially ignored until the spring of 1979 when the eggs

[1]Biology Department, Dalhousie University, Halifax, Nova Scotia B3H 4J1, Canada
[2]Science Branch, Department of Fisheries and Oceans, P.O. Box 550, Halifax, Nova Scotia, Canada

were discovered to be developing. She only had 4 of 40 eggs left in July 1979 and was not offered food while brooding. This female *B. arcticus* died shortly after her eggs hatched.

Very little is known about the life history of deep-sea octopods. This report describes the first detailed observations of mating, brooding, and embryonic development time of *Bathypolypus arcticus.*

METHODS

Eighteen *B. arcticus* were collected from the Bay of Fundy off Digby (*circa* 44.70°N and 65.90°W) on June 8–21 and below Brier Island (circa 43.80°N and 66.30°W) on August 22–September 1, 1994. The FRV *J. L. Hart,* a 20 m trawler belonging to the Department of Fisheries and Oceans (DFO), was used. Animals were collected in scallop trawls as incidental catch during DFO scallop stock surveys. The majority of animals came from tows at depths of 75–100+ m. While on the boat, specimens were housed in a portable cooler for as long as a week and kept at temperatures below 10°C. Additional specimens were collected in the same manner in the summer of 1995.

The *B. arcticus* collected in 1994 were kept together in a flow-through system at DFO's Halifax laboratory. The animals were housed in a 91 by 91 cm fibreglass tank (internal dimensions). The walls of the tank were 4 cm thick and contained chilling coils. Water depth was 31 cm. Water temperature was controlled by adjusting the amount of ambient and heated water that entered the system. Temperature varied with the incoming water and user demand.

Initially, temperature was recorded with a mercury thermometer (Fig. 1). In late December 1994, a min./max. thermometer was added to the system to record temperature fluctuations.

The octopoda collected in 1995 were kept in two flow-through fiberglass tanks at Dalhousie. Most of the females collected laid fertile eggs. Temperature was recorded but is not presented here. The number of eggs laid in these fertile broods was assessed in May 1996 by removing the females and photographing their eggs with an underwater camera. Eggs were much easier to count in static photographs. (We knocked eight eggs off when removing the females from their broods, and these are included in the totals for the appropriate females.)

During the first months after capture, the octopodids were hand-fed live crustaceans and mollusks removed from their shell, and they were offered amphipods *ad libitum.* Hand feeding consisted of bumping the food into the octopuses arms. Later, sand shrimp (*Crangon*) and brittle stars (ophiuroids) were added in *ad libitum* quantities to the tank, and polychaetes, mussels, crabs, amphipods, and other small invertebrates were added as they became available.

Octopodids that laid eggs were observed and occasionally offered food by hand. However, brooding octopodids were disturbed as little as possible. Once the eggs started hatching, brooding octopodids were filmed with a 24-hour time-lapse VCR under red light.

To measure mantle length (ML), mantle width (MW), and interocular width (IOW) of day-old individuals, they were filmed and measurements were made with an Optimas Video Analysis System. This system was used to reduce stress on the animals. Summers (1985) used a somewhat similar method, and he briefly discussed the reliability of using photographic size determination.

Wet weights ± 1 mg of 15 hatchlings from each brood were obtained with a Mettler P163 scale. Members of the first batch of hatchlings were dried with a tissue to absorb excess water before being weighed. Many animals weighed with this method died; several of the dead hatchlings had tears in their skin. Because this method damaged the hatchlings and caused mortality, it was abandoned. Individuals from the second batch were weighed in a weighing tray with a micron screen bottom. This tray was placed on a tissue before being weighed to absorb excess water. Preserved and recently dead juvenile *B. arcticus* were weighed with both methods to quantify the difference between the two methods. The second method produced results that were 10.1% ($n = 26$) higher. Weights of the first batch were converted so that they could be compared to those of the second batch.

Mating and hatching was filmed by Dave Gaudet (Halifax Cable) with a housed Sony CCD 3-chip DX3 camera using high-8 format. The primary author induced several eggs to hatch by handling them and/or adding sugar to the water.

RESULTS

Adult mortality was highest within the first few weeks of capture. By September 1994, 12 of the 18 octopodids collected during the pre-

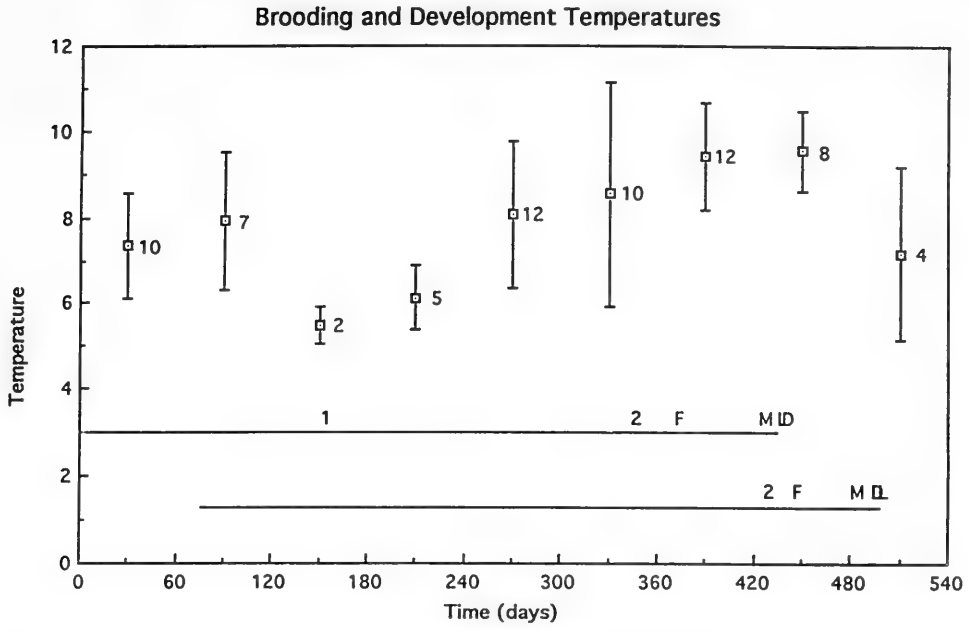


FIG. 1. Average and standard deviation of temperatures at which brooding octopuses were kept. The number next to the average temperature is the sample size for that 60-day period. The horizontal lines show the brooding period for the two females (1 = first inversion, 2 = second inversion, F = first hatchling, M = median hatchling, L = last hatchling, D = death).

vious summer were still alive. Of those, eight were still alive in September 1995. Of the 18 *B. arcticus* collected in 1994, only one was male. However, in 1995 the sex ratio was 50/50 (n = 32).

Average temperature during the brooding period was 7.3°C for the first female and 7.8°C for the second female (Fig. 1). During the experiment, the min./max. thermometer recorded a minimum temperature of –1.8°C on September 28, 1995, caused by a pump failure. A similar problem that dropped the temperature to 0.0°C occurred in fall 1994. A maximum temperature of 16.7°C was recorded by the min./max. thermometer during July 17–24, 1995. These extreme temperatures probably lasted for only a short time.

On August 16, 1994, a female *B. arcticus* was discovered brooding at least three eggs that were laid the previous night. The female refused food. There were about ten eggs on August 17. The female laid more eggs by August 22 and ate a few amphipods. Due to our effort to disturb the female as little as possible while she was laying eggs, eggs were not counted until several months later. Although precise observations could not be made, no noticeable additions of eggs took place after two weeks. To count the eggs, it was necessary to remove the female for a brief period. Fifty-five eggs were counted, but only 54 were accounted for at hatching. This discrepancy is likely due to difficulties in counting the eggs while keeping the female away from them. Eggs were glued individually to the side of the tank by the female. The glue was initially clear but it turned green after a few days.

While brooding eggs, the female occasionally ate food offered to her. She took fish pellets, amphipods, *Crangon,* and crushed mussels. Food was offered by hand-feeding as described above.

Brooding females would sit on their eggs and cover most of them with their web. They often directed their funnel down, which provided a water current around the eggs. The video tapes of brooding females revealed that, although they occasionally moved slightly away from the eggs, they would always keep a few arms on the eggs while exploring the perimeter around the brood with the other arms. When a probe was used to try to get a

better look at the eggs, the female would actively defend them, sometimes by blasting jets of water at the probe and/or grabbing it.

An egg was taken on January 20, 1995, 158 days after the first eggs were laid, to determine if the batch was fertilized. Incirrate embryos, except Argonautidae, flip position in the egg twice during development (Boletzky, 1987). The egg was viable and the embryo was in the first inversion. By July 31, 1995 (day 350), most of the embryos had flipped the second time. At least one egg had not flipped by August 8, 1995. On August 27, 1995 (day 377), the first octopus hatched. By October 23, 1995 (day 434), all the octopodids in the first batch had hatched, although six of the last seven were induced to hatch so they could be filmed (Figs. 2–5).

Eggs were 11 mm long and 6 mm wide before hatching. Hatchlings (n = 15) weighed 208 ± 17 (SD) mg, had a mantle length of 7.71 ± 0.49 (SD) mm, a mantle width of 7.14 ± 0.39 (SD) mm, and an interocular width of 2.30 ± 0.20 (SD) mm. The median day of hatching was October 14, 1995 (day 425), and assuming the median day of egg laying was one week after the first egg was laid, the average octopus in the first batch took 419 days at an average temperature of 7.3°C. This female died three days after the last egg in her brood hatched.

On October 30, 1994, a slightly larger female started laying viable eggs in the lower left corner of the same tank. Over 50% of these eggs flipped the second time by October 23, 1995 (day 359 from first laying of eggs). The first egg in the second batch hatched on or a few days before November 8, 1995 (day 375), and the last on December 30, 1995 (day 427) (Fig. 1). The median day of hatching was December 17, 1995 (day 414). The average octopus in the second batch took 407 days at 7.8°C to develop. This batch, counted as they emerged, yielded 36 hatchlings with an average weight of 283 ± 20 (SD) mg (n = 15), mantle length of 8.91 ± 0.43 (SD) mm, mantle width of 8.60 ± 0.84 (SD) mm, and an interocular width of 2.84 ± 0.23 (SD) mm. The second female died two days before her last egg hatched. Brooding behaviour was as noted for the first female, except a few days before the second female died she left her eggs, at one point for several hours, and then returned to them. She was in very bad condition by this time and appeared to be having trouble breathing and orienting herself. Both females moved away from the eggs shortly before dying. Females were preserved in formalin after they died. The preserved specimens weighed 20.82 g and 17.55 g respectively. The two females weighed an estimated 30–40 g prior to laying eggs.

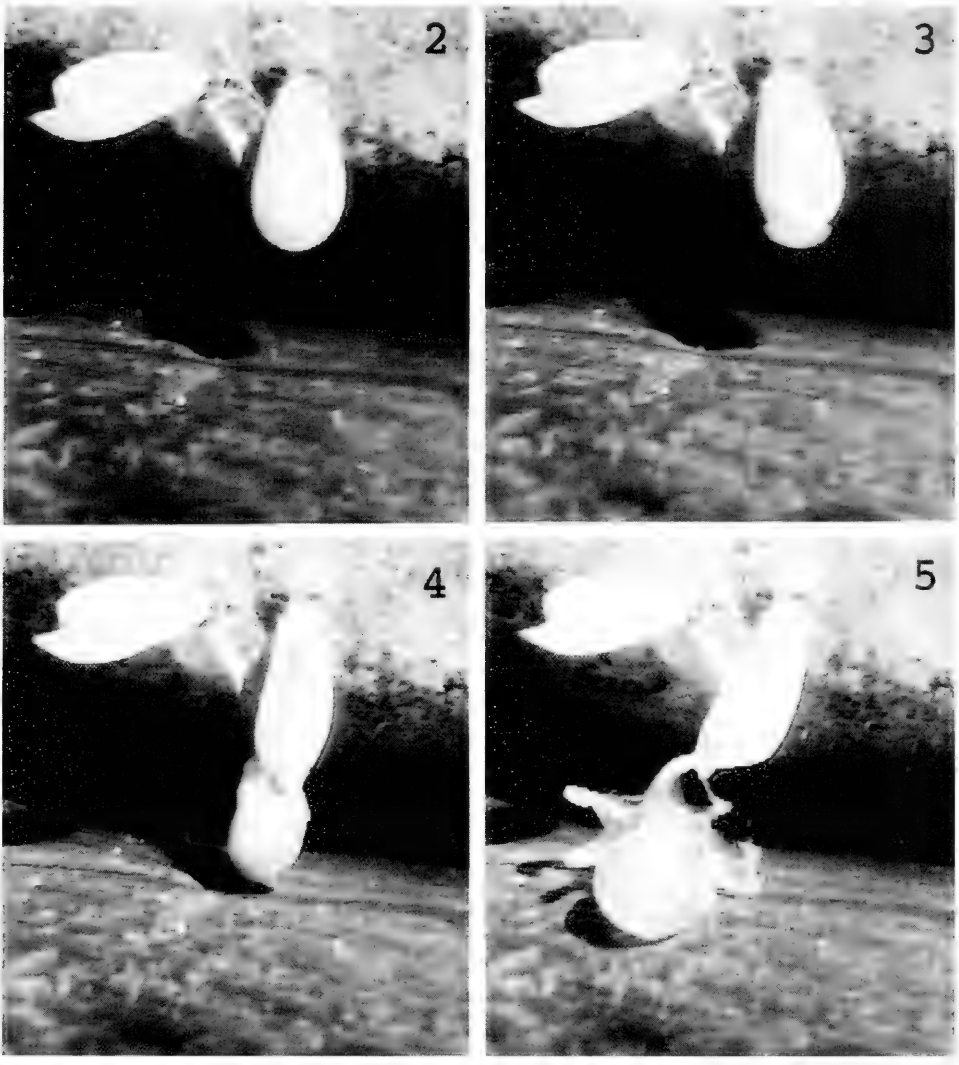
None of the hatchlings from either batch had any of the outer yolk sac remaining upon hatching. Video tapes revealed that hatchlings are able to hang upside down from the water surface (see Marliave, 1981; Van Heukelem, 1976). A variety of foods were offered to the first batch of hatchlings. They were initially hand-fed fresh mussel meat, and bits of gammaridean amphipods, mysid shrimp, and *Crangon septemspinosus*. Plankton and live gammaridean amphipods were offered to some of the hatchlings. Later, they were offered the small burrowing gammaridean amphipod *Corophium volutator [ad libitum]*. Hatchlings from the second batch were rarely hand-fed and were offered *C. volutator ad libitum* within a few days of hatching.

Several other females that were collected in 1994 laid eggs on the side of the tank or in a clay pipe. These eggs disappeared after a few months. An egg was removed from one of these females. When this egg was examined several months later, it was found to be unfertilized. Presumably these batches were not viable and the females ate them. A single male was kept in the tank with the females.

Nine of the females that survived collection in 1995 were brooding eggs in May 1996. All of these broods were fertile, and seven were able to be counted (the eighth and ninth octopus laid eggs in a plastic pipe). Therefore, the nine *B. arcticus* in this study laid 12, 13, 18, 19, 36, 48, 54, 89, and 105 fertile eggs. However, we estimate that none of these cephalopods weighed 70 or more grams when they laid their eggs. Macalaster (1976) reports that 70 g is the average size of mature females.

The following observations were made on the 1995 females that laid eggs. Females brooding their own eggs can be moved away from their eggs and then returned to them. Also, females that are brooding eggs can be moved to care for another octopuses brood of fertile eggs. While females don't differentiate between their own fertile eggs and other broods of fertile eggs, they seem to be able to recognize infertile eggs, which we presume they eat. One female was briefly observed to be slightly out of arm reach of her eggs but was rapidly moving back toward them. This occurred shortly after amphipods were added to the tank.

Male *B. arcticus* uses the mounting position to mate. The smaller male initiated mating by

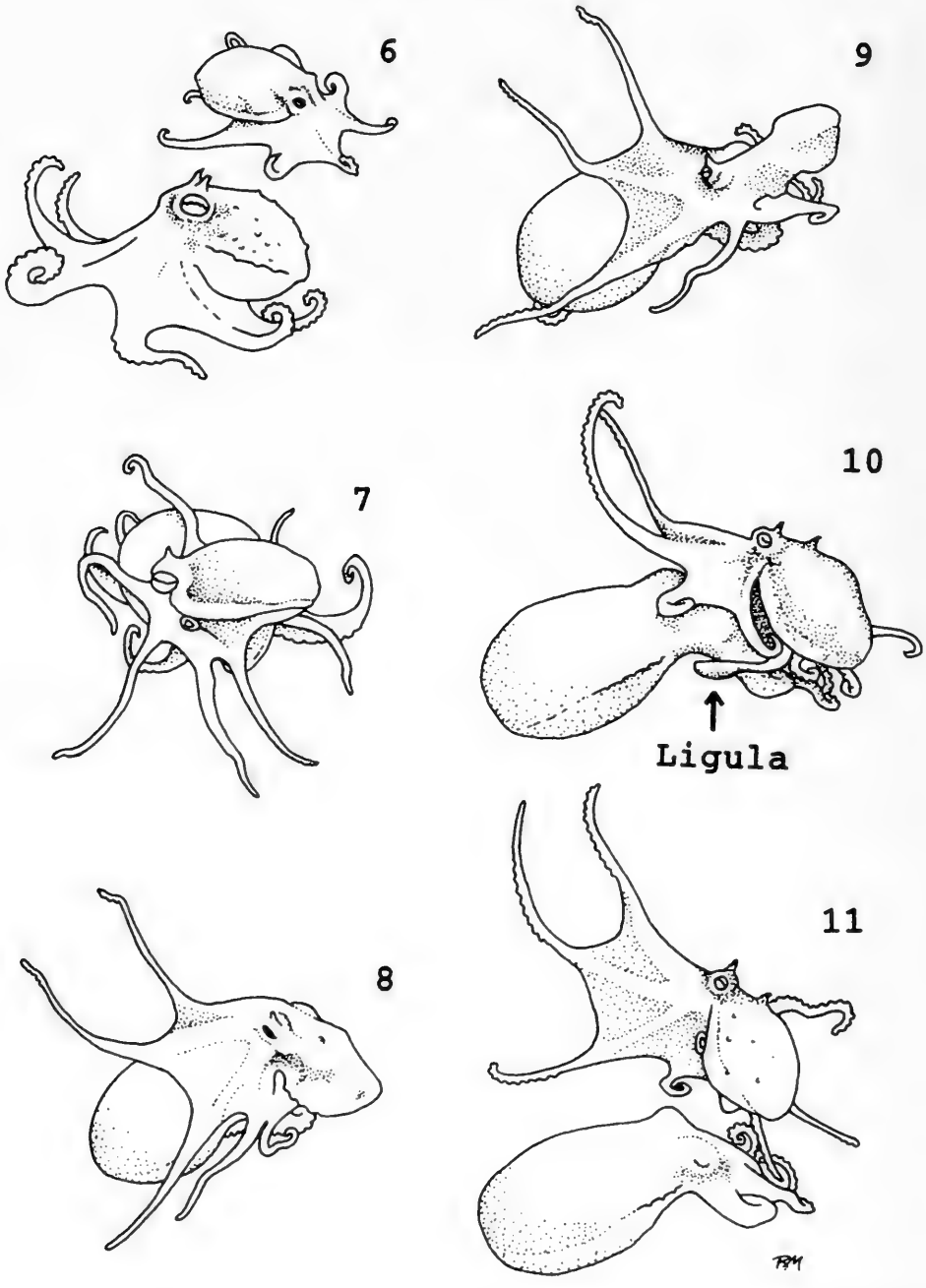


FIGS. 2–5. Hatching. FIG 2. Swollen egg just prior to hatching. FIG. 3. During hatching the pressure in the swollen egg pushes out the end of the mantle. FIG. 4. The octopus frees himself from the egg. FIG. 5. The fully functional hatchling emerges from the egg. Photographs from high-8 video by D. Gaudet.

mounting the female and enveloping her mantle in his web (Figs. 7–9). He then inserted his hectocotylus into the mantle of the female. Its folded shape suggests that it was actually inserted into the oviduct to open it. During mating, the male contracts his mantle in a dramatic pumping arch motion (Fig. 9); this may help the large spermatophore pass to the funnel. We presume that the spermatophore is then passed from the funnel to the large grove on the hectocotylus and that through peristalsis it is transferred to the ligula which is in or on the oviduct. The female remained motionless during mating, which lasted 140 seconds. *Bathypolypus arcticus* mates readily—specimens collected the following year mated in the cooler that they were kept in while still at sea. These matings followed the pattern outlined above.

DISCUSSION

This study presents the first detailed data on the brooding and development time for a deep-sea octopodid. *Bathypolypus arcticus* brood eggs for over 400 days, and if they live



FIGS. 6–11. Mating *Bathypolypus arcticus*. FIG. 6. The male octopus (foreground) sees the female octopus and pounces on her (FIG. 7). FIG 8. He mounts her and inserts his ligula into her mantle cavity. FIG. 9. The male stretches his mantle dramatically (this happened twice) presumably to help pump the spermatophore to his ligula. FIG 10. The male removes his ligula and departs (FIG. 11). Illustrations by Rebekah McClean.

for three years, as estimated by O'Dor & Macalaster (1983), these octopodids spend over a third of their life brooding. Additionally, this study describes for the first time the mating behavior of *B. arcticus*. Mating in *B. arcticus* is of interest because these little cephalopods have the largest ligula relative to body size of any octopod.

In this experiment, brooding behaviour and developmental time were very consistent between the two females despite differences in egg size, female size, and time of laying. This suggests that the length of the brooding period is relatively fixed at a given temperature.

Brooding *B. arcticus* in this experiment were kept at average temperatures of 7.3°C and 7.8°C, which is significantly higher than the 4 ± 2°C SD that O'Dor & Macalaster (1983) reported for wild specimens. Therefore, one might expect *B. arcticus* to brood eggs for even longer in nature, because length of embryonic development has been shown to depend on temperature in cephalopods (Boletzky, 1987, 1994). Ken Drinkwater (personal communication) reports that average monthly bottom temperatures in the area where the octopodids were collected were as warm as 11–12°C in August and September 1994 and that the average annual temperature was 8.0°C in 1994—these temperatures are approximately 1.1°C higher than usual. O'Dor & Macalaster stated that laboratory specimens experienced mortality with only brief periods of temperatures from 10°C to 12°C, whereas our specimens survived periods of weekly average temperatures in the 11°C range. Although O'Dor & Macalaster (1983) found no evidence of migration, we point out that migration to warmer water to spawn has not been ruled out. Villanueva (1992) found evidence of up-slope ontogenic migration in *Bathypolypus sponsalis*.

Brooding *Bathypolypus arcticus* occasionally take food to offset the extremely high energetic cost of brooding eggs for over a year. The octopus reported on by O'Dor & Macalaster (1983) may have eaten her own eggs to have sufficient energy to brood the remaining four through to hatching. If this is true, it shows that *B. arcticus* can brood for a year by eating its own eggs. The cost in such a protracted brooding period is a sharp decrease in fecundity. An alternative explanation is that this female only had a few viable sperm from her mating, which occurred at least five months earlier. Perhaps she ate the other 36 eggs because they were not viable.

The long brooding period in *B. arcticus* must have substantial costs of time, energy, and risk of predation. This period may limit *B. arcticus* to a semelparous strategy, because the costs of brooding are not worth the effort unless the number of eggs and their chance of survival is sufficiently large. Historicity, or phylogenetic legacy (Williams, 1992), may also limit many octopodids to a semelparous strategy.

Parental care is necessary for all incirrate octopod eggs because they lack a protective egg case. Without the mother caring for and defending her eggs, they would be rapidly suffocated by fouling organisms (Boletzky, 1994). However, the length of this brooding period varies. *Bathypolypus arcticus* benefit in several ways from their large young and long brooding period. Sibling and non-sibling competition favour smaller broods of larger eggs (Stearns, 1992). Longer brooding periods may be selected for in *B. arcticus* because the egg stage is likely to have a high survival rate compared to hatchling. Stearns (1992) noted that "selection should increase the proportion of time spent in the safest developmental stages." Yampolsky & Scheiner (1996) discussed demographic reasons that favour large offspring in cold environments for exothermic animals.

Because *Bathypolypus arcticus* are not found in high densities (O'Dor & Macalaster, 1983) and because they are not very mobile, chances to mate may be rare. The female's ability to store viable sperm for at least five months (often incorrectly cited as ten months; O'Dor & Macalaster, 1983) and the male's large spermatophores and ligula are likely adaptations to maximize fitness under such conditions. It is possible that the large ligula and spermatophores are selected for in sperm competition. Cigliano (1995) noted that octopodids meet the criteria for sperm precedence and that the spoon-shaped ligula could be used to scoop out competitors sperm. Another (non-exclusive) possibility is that the large ligula expands once in the oviduct to anchor the hectocotylus during mating. Further investigation will be needed to confirm or reject these theories.

The successful manipulation of females between broods suggests that experiments manipulating brood and egg size may be possible. An explanation of the experimental significance of manipulation of offspring size can be found in Stearns (1992). Such experiments could answer such questions as whether hatchling behaviour (e.g., planktonic versus benthic) is a function of hatchling size, species, or hatchling age. Also, the effect

of egg size on development time could be tested within a brood, which would minimize or eliminate many confounding factors. Workers should test to see if other octopus species can be manipulated in this manner, because the long brooding period and cold temperatures required for keeping *B. arcticus* make it a poor choice for such experiments.

The fact that a soft-bodied cephalopod can survive collection in a scallop trawl, days at sea, and acclimation to laboratory conditions, indicates the hardiness of *B. arcticus*. Boletzky & Hanlon (1983) provided a general review of low trauma cephalopod collection techniques and general culture information. Less traumatic collection techniques that specifically target deep-sea cephalopods are allowing investigators to study more species of deep-sea octopods in the lab. Researchers at the Monterey Bay Aquarium and Monterey Bay Aquarium Research Institute used an ROV specifically to capture and film cirrate octopuses, and they have been able to keep these octopuses alive for several months (Stein-Hunt and Hochberg, personal communication).

We are just beginning to understand the life history of deep-sea octopods. Additional studies involving such modern sampling techniques as video monitoring from submersibles and remote operated vehicles (Vecchione & Roper, 1991) will continue to complete the picture of how deep-sea cephalopods behave in nature. However, because deep-sea cephalopods cannot always be found, workers must hope for chance encounters. Telemetry has provided more continuous records of the behaviour of *Loligo* and *Nautilus* (Carlson et al., 1984; O'Dor et al., 1993, 1994) and could also be employed to study other deep-sea cephalopods. Cost and logistical problems limit the use of these techniques.

It is widely acknowledged that laboratory conditions can bias results. However, studies of cephalopods in their natural environment are difficult due to their mobility, excellent vision, and nocturnal habits (Boletzky & Hanlon, 1983). Laboratory studies are currently the best, most direct way to look at development time, life span, reproductive strategy, growth rates, and other processes that occur in individuals over time. Also, the effect of such variables as temperature, sex, and diet can be analyzed and separated. Laboratory conditions allow the experimenter to observe and manipulate such behaviour as mating or hatching. Further laboratory studies of hardy deep-sea species are needed if we want to understand how these organisms live in their cold, dark world.

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THE INHERITANCE OF AN EMBRYONIC LETHAL MUTATION IN A SELF-REPRODUCING TERRESTRIAL SLUG, *DEROCERAS LAEVE*

Richard M. Lebovitz

c/o L.C. Evogen, 3515 Washington Boulevard, #317, Arlington, Virginia 22201, U.S.A.

ABSTRACT

A new lethal developmental mutation (EZL-1, embryonic zygotic lethal-1) in *Deroceras laeve* was used as a genetic marker to determine whether self-reproduction is by parthenogenesis or self-fertilization. An average of 25.7% of the progeny of individuals (N = 38) apparently heterozygous for the mutation exhibited the EZL-1 lethal phenotype, a result that can only be explained if reproduction is meiotic. Histological sections of a fertile slug revealed the presence of both sperm and eggs in the gonad, consistent with self-fertilization, rather than parthenogenesis. In addition to EZL-1, other developmental defects were observed in progeny.

Key words: *Deroceras,* development, mutation, self-fertilization, slug.

INTRODUCTION

Self-reproduction is widespread, particularly among molluscs (Heller, 1993). *Deroceras laeve,* the grey garden slug, a pulmonate, reproduces predominantly, if not exclusively, by self-reproduction (Foltz et al., 1982). It is of particular interest to malacologists because it is unsettled whether they self-reproduce by self-fertilization or by parthenogenesis (Heller, 1993; Hoffmann, 1983; Foltz et al., 1982; Nicklas & Hoffmann, 1981). To identify the mechanism of reproduction in this species, the inheritance of a recessive lethal mutation, embryonic zygotic lethal-1 (EZL-1), was studied. This is the first report of an embryonic mutation in a terrestrial slug.

EZL-1 mutant embryos complete about 21% of the embryonic stage, arresting after the acquisition of most of the rudimentary adult structures, including the tentacles, mantle, and foot. An average of 25.7% of the progeny of apparently heterozygous individuals (N = 38) for the mutation exhibited the EZL-1 lethal phenotype, a result that can only be explained if reproduction is meiotic (Asher, 1970). Histological sections of a fertile individual revealed the presence of both sperm and eggs in the gonad, consistent with self-fertilization, rather than parthenogenesis (Hoffmann, 1983; Asher, 1970). In addition to EZL-1, other developmental defects were observed in progeny.

MATERIALS AND METHODS

The founder slugs were isolated from the wild and maintained on a diet of fresh romaine lettuce. Subsequent progeny of the founders were isolated after hatching and cultured individually in tupperware dishes containing a piece of moistened towel. There were no opportunities for cross-fertilization. Eggs from mature, isolated, animals were collected every one to two days and transferred to a moistened towel in a petri dish, where they were stored until hatching. The phenotype of unhatched eggs was observed under a dissecting microscope and scored.

Slugs were fed fresh salad leaves every one to two days. Environmental conditions were not controlled, although the slugs were generally maintained at room or seasonal temperatures, between about 16°C to about 28°C.

Karotyping was performed on three- to four-day-old embryos. After removal from the egg case, embryos were incubated for 30 min in 0.125% colchicine dissolved in 0.45% KCl, washed in 0.045% KCl for about 30 min, and then fixed in a cold mixture of 3 parts methanol to 1 part acetic acid. Fixed embryos were placed in a drop of 60% acetic acid, squashed under a siliconized coverslip, and dried on a hot plate between about 37°C and 47°C. When the slides were completely dried, the coverslip was removed, and the slides

were stained with aceto-orcein for microscopic examination.

For histology, a fertile slug was fixed in 4% formaldehyde, dehydrated, and embedded in paraplast. Six μm sections were prepared and stained with Harris hematoxylin-phloxine-eosin for light microscopic examination.

The values for lifespan, sexual maturity, and egg number were calculated as the mean with a standard deviation (±). Statistical analysis was performed by chi-square, using the test for heterogeneity described in Mather (1951).

RESULTS

Although five different founder lines were maintained for a four-year period by self-reproduction alone, only one line, 1F, is described here. This line was selected because of its possession of a recessive lethal mutation, providing an opportunity to characterize the mutation and use it as a genetic marker to study self-reproduction.

The animals in this study were identified as *Deroceras laeve* by H. Lee Fairbanks of Pennsylvania State University according to their description by Pilsbry (1948). In all three animals dissected, an inspection of the dorsal surface of the internal cavity revealed that the ovotestis was hidden under the digestive gland, a characteristic of *D. laeve* but not *D. reticulatum,* a species it closely resembles. In addition, the rectal caecum and terminal male genitalia were absent.

Development of the Adult

Animals collected from eight generations were included in this study. The founder (IF) was a progeny of an animal captured from the wild. Ninety animals, predominantly from the sixth and seventh generations, were selected for analysis. Only animals that reached maturity and produced viable offspring were included. For convenience, the ages discussed below were calculated from the day of egg deposition, rather than the actual day of hatching.

The average lifespan of all 90 animals was 164 ± 40.7 days (about 5 1/2 months), ranging from 90 to 250 days. Sexual maturity, marked by the first deposition of eggs, was reached at about three months (94.8 ± 18.7 days), with a shortest time of 71 days and a longest of 148. Animals produced eggs for an average of 57.2 ± 35.2 days. Egg production continued until an average of 12 ± 8.8 days before death.

Very few metaphase figures were observed in the embryos. Of these, quality of the chromosomal squashes was low and thus precluded detailed karyotyping. However, of 14 spreads sufficiently clear to count, the chromosome number averaged 50 (data not shown). Patterson & Burch (1978) reported that *Deroceras* are diploid, having a haploid chromosomal number of 30. The number here is roughly twice that value, consistent with diploidy.

Viability and Phenotypes of Deposited Eggs

A mean of 66.5 ± 32.5 eggs (N = 90; total eggs = 5,981), with a range of 6 to 164, were produced per animal. In addition to producing eggs with embryos that developed into normal adults, two other primary egg phenotypes were observed. First, eggs were frequently observed that showed very little, if any, embryonic development. Inside such eggs, a small ball-like structure, varying in size, was typically observed. These eggs are referred to as type A. All animals produced this type of egg. Secondly, a category of eggs that undergo about 20% of normal development and then arrest were observed. These are embryonic/zygotic lethal-1 or EZL-1, the mutation described here. Not all individuals produced EZL-1 eggs. It is assumed that those that did were heterozygous for the gene loci and that those that did not were homozygous normal at the EZL-1 locus.

Normal Egg Development

Development of the viable and normal egg occurred substantially as described in Carrick (1938) for *Agriolimax agrestis.* There is no metamorphosis nor a larval stage. Rather, the embryo gradually assumes the adult form, progressing through at least five distinct and identifiable forms. For the purposes of this study, the discussion is focused on the progression of the embryo from stage IV to V, because this is the stage at which embryonic development is arrested in EZL-1.

Embryonic development takes about 14 days. Stage IV, reached at about four days, is characterized by the appearance of most of the rudimentary structures of the adult organism, including the tentacles, mantle, and foot (Fig. 1A, reproduced from Carrick, 1938). The

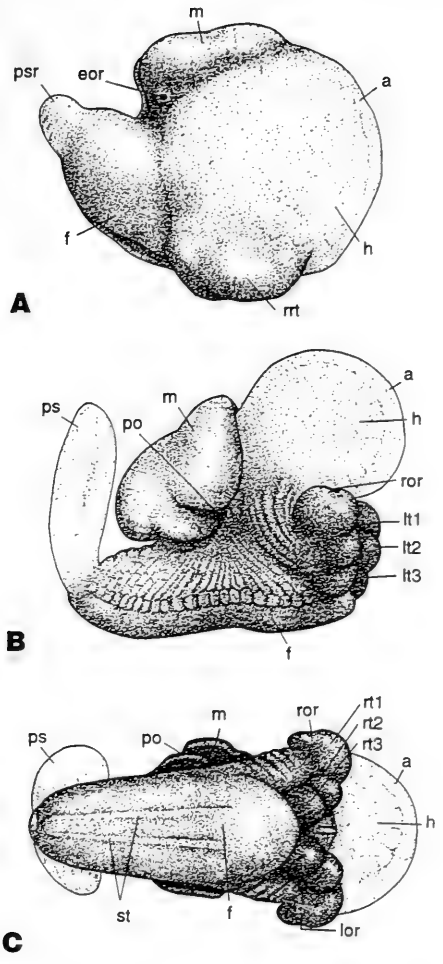

FIG. 1. Development of *Deroceras laeve*. Stage IV. A, Right aspect. psr, rudiment of posterior sac; eor, external opening of right larval nephridia; m, mantle; a, anterior sac; h, hepatic mss; rrt, rudiment of right tentacles; f, foot. Stage V. Maximum development of posterior and anterior sacs. B, Right aspect. C, Ventral aspect. ps, posterior sac; po, pulmonary orifice; ror, optic rudiment of right anterior tentacle; lor, optic rudiment of left anterior tentacle; rt1, right anterior tentacle; rt2, right posterior tentacle; rt3, third tentacle of right side; lt1, left anterior tentacle; lt2, left posterior tentacle; lt3, third tentacle of left side; st, pedal streaks. Reproduced by permission of the Royal Society of Edinburgh and R. Carrick from Carrick (1938–1939).

posterior sac, the respiratory organ of the embryo, and the hepatic mass, another embryonic structure, also become visible at this time. The progression from stage IV to V is gradual, marked by the enlargement of the rudimentary stage IV structures into recognizable and significantly differentiated forms. Stage V is identified by Carrick (1938) when the hepatic mass reaches its maximal size (Fig. 1B).

A primary characteristic of the transition from stage IV to stage V is the differentiation of the posterior sac from the embryonic foot. The foot and posterior sac originate from the same embryonic region. During development from stage IV to stage V, the two structures gradually assume characteristic forms, appearing to comprise different cell types. As the foot morphologically separates from the posterior sac, it thickens, and two streaks (Fig. 1C, st) appear on its ventral surface, running from anterior to posterior. After the appearance of the pedal streaks, the mantle and tentacles begin to enlarge, and the embryo gradually becomes more adult. Concomitantly, the hepatic mass begins to shrink, ending stage V. The entire sequence takes about seven to eight days.

After about seven days, midway through development, pigmented eye spots appear in the first optic tentacles. Subsequently, pigmentation spreads in a wave across the outer embryo epithelium. The remainder of development until hatching is characterized in Carrick (1938) and is not discussed here.

Type A Egg Development

An average of 23 ± 13.8% (N = 90) eggs from founder 1F were type A, showing little, in any, growth past the blastula stage (Stage I according to Carrick, 1938). A type A egg was classified by the appearance in the perivitelline egg fluid of a small, round mass and a long, twisted, wispy membranous thread. The latter was defined by Carrick (1938) as the remains of the sperm body, but this conclusion has not been generally accepted (South, 1992). The type A phenotype cannot be distinguished at deposition from a viable egg, except that the former does not develop any further over the 14-day embryonic period. From all appearances, it is likely that this class represents developmentally inactivated or unfertilized eggs.

A second class of eggs is infrequently identified in 1F progeny. These eggs, type B, undergo a small amount of growth, arresting one to two days after egg deposition. B exhibits the same characteristic ball-like morphology as A, but it is about two to three times larger. It is uncertain at what development type B arrests, but the absence of visible structures suggests

TABLE 1. Percent frequency of egg phenotypes produced by homozygous and heterozygous EZL-1 *Deroceras laeve*.

	TOTAL EGGS	% EZL-1	% Type A (not fertilized or not activated)
Group 1, homozygotes (N = 22)	1141	1.3 ± 2.1 range: 0–7.4	20.8 ± 16.4
Group 2, homozygotes (N = 30)	2181	1.0 ± 1.7 range: 0–7	21.4 ± 13.7
Group 3, heterozygotes (N = 38)	2659	25.7 ± 9.6 range: 8.6–61.5	25.8 ± 11.8

that it has not, or has just, entered stage III (Carrick, 1938) when visible morphological differentiation begins. The tissue mass inside the perivitelline sac of type B eggs often is "fuzzy," rather than a well-defined structure as in type A, possibly indicating tissue necrosis. It is possible that B defines an early embryonic (or maternal effect) mutation, but this has yet to be confirmed. B represents less than 3% of the total eggs and was included in the type A class for all calculations, because it is only qualitatively different and therefore difficult to score separately.

EZL-1 Egg Development

In the line from founder 1F, an embryonic/zygotic lethal mutation (EZL-1) was first observed in the fourth generation, when the phenotypes of unhatched embryos were first inspected for defects. It was observed in all subsequent generations.

The lethal mutation was first distinguished on day 4 of the 14-day development period, about 21% of the way through its development. Developmental arrest occurs during stage V when the embryonic foot and posterior sac start to become distinguishable structures in the rudimentary foot of the zygote (Fig. 1C). In the majority of these embryonic lethals, although the foot and posterior sac became distinguishable from one another, the foot is enlarged but arrests before or when the pedal streaks would become visible in a normal embryo. The posterior sac, on the other hand, looks perfectly normal in EZL-1. Its characteristic movement throughout the normal 14-day period when egg development was examined suggests that the EZL-1 zygote is alive, but that its development halted.

All development appears to cease in EZL-1. For example, during normal development, the optic tentacles begin to enlarge after the appearance of the pedal streaks, when the hepatic mass reaches its maximum (i.e., stage V). In EZL-1, the zygote acquires the rudiments of the optic tentacles, but they do not appear to expand or acquire any visible structure, such as the pigmented eyespots observed in normal embryos.

Frequency of EZL-1 Phenotype

Because animals carrying the EZL-1 mutation could not be distinguished phenotypically from non-carriers, genotype was deduced from the frequency of the mutation appearing in their progeny. To calculate the percentage of eggs exhibiting the EZL-1 lethal phenotype, the number of type A eggs was subtracted from the total eggs and the result was divided into the number of eggs scored as EZL-1. This adjustment was done to eliminate type A eggs from the analysis, on the assumption that they represent a class of eggs that are either unfertilized or unactivated. The results are summarized in Table 1.

Group 1 comprises three generations descended from (and including) a fifth generation animal that apparently did not possess the EZL-1 gene mutation. An average of 1.3% of the eggs were scored as EZL-1. The values ranged from 0% to 7.4%. It is assumed that the eggs scored as EZL-1 (since all data were collected blindly) died for other reasons, but at a stage similar to the one at which EZL-1 arrests. In some of these, it had been noted that the phenotype was not characteristic of EZL-1, that is, later developmental arrest or different-sized embryo.

Groups 2 and 3 were collected from descendants of three fourth-generation animals. These animals were from the same founder as Group 1, but from a lineage that diverged at the third generation. The animals were classified into two groups, using 7% EZL-1 as a cut-off, because that was the highest value observed in Group 1. Based on this value,

Group 2 possessed an average of 1% EZL-1 eggs, similar to Group 1, the animals that do not have the EZL-1 mutation. The average number of eggs with the EZL-1 phenotype in Group 3, however, was 25.7%, with only four of the 38 animals being lower than 17% (that is, 8.6, 12.7, 14.3, and 15). This result for the pooled data (normal = 1,450 eggs; EZL-1 = 481 eggs) is consistent with the expected number for self-fertilization by heterozygotes ($\chi^2 = 0.027$, df = 1, $0.95 > P > 0.90$). When Group 3 was analyzed as a class, using chi-square to test heterogeneity (Mather, 1951), the χ^2 value was high ($P < 0.05$), suggesting that the class was heterogeneous. Homogeneity was established ($\chi^2 = 48.2$, df = 35, $0.30 > P > 0.20$) by eliminating from Group 3 the two animals with the highest EZL-1 frequency (43% and 62%). As discussed below, other lethal developmental defects have been noticed in slug progeny, albeit at a much lower occurrence than EZL-1. Thus, the appearance of EZl-1 in these two slugs (as well as others) could have been inflated by scoring other developmental defects that resemble but are not EZL-1. Figure 2 is a frequency histogram of the % EZL-1 per animal.

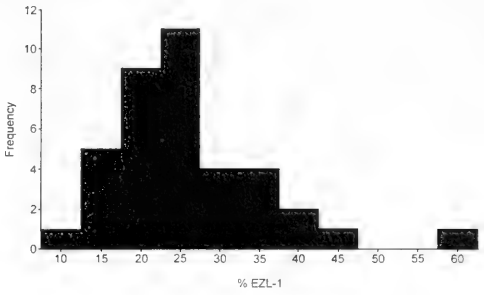

FIG. 2. Frequency histogram displaying percent eggs exhibiting the EZL-1 phenotype from individual slugs. Data collected from 38 slugs characterized as heterozygous for the EZL-1 locus.

Other Developmental Defects

The appearance of an EZL-1 like phenotype was observed an average of 1.1% (± 1.9%, N = 52) in lines apparently homozygous wild-type for the EZL-1 gene locus (Groups 1 and 2). Other lethal developmental defects were noted, as well. These phenotypes included: developmental arrest at earlier stages than EZL-1, either with a distinct morphology or comprising a bulbous, undifferentiated mass; incomplete to fully pigmented embryos; and fully developed embryos that did not emerge from the egg shell. When added to the EZL-1-like phenotype, the mean value of developmental defects for the apparently homozygous wild-type animals was 3.4% (± 3.9%, N = 52).

Histology of the Gonad

The simultaneous presence of both sperm and egg in slug gonads is well documented (South, 1992). In *D. reticulatum,* for example, all individuals are hermaphroditic, and the gonad can contain both eggs and sperm at the same time (Runham & Laryea, 1968). Both sperm and eggs were seen in the gonad of one fertile *D. laeve.* The spermatids are arranged in clusters, suggesting their origin from individual spermatogonia (Fig. 3A). Mature spermatozoa were abundant in the hermaphroditic duct (Fig. 3B). Because aphallic slugs, lacking most of the terminal structures of the male reproductive system, were fertile, it is suggested that sperm production is dissociated from the development of the reproductive tract required for transporting sperm.

DISCUSSION

The EZL-1 mutation in *D. laeve* is clearly a recessive mutation at a single gene locus. First, it is transmitted through successive generations, segregating between carrying and non-carrying slugs. Secondly, an apparently heterozygous individual produces an average of 25.7% eggs exhibiting the EZL-1 phenotype, consistent with either simple Mendelian inheritance by self-fertilization or meiotic parthenogenesis with a high frequency of recombination (Asher, 1970; Hoffmann, 1983). The detection of both sperm and egg in the gonad favors self-fertilization, in agreement with Foltz et al. (1984), and all other pulmonates (Heller, 1993), but contrary to the conclusions of Nicklas & Hoffmann (1981) and Hoffmann (1983).

EZL-1 is an embryonic lethal mutation that arrests during stage V of development (Carrick, 1938). Its progression from the blastula to stage IV is visibly normal, acquiring most, if not all, of the rudimentary adult structures. However, the progression from stage IV to the adult in EZL-1 is arrested. Differentiation, for example, of the foot rudiment into the posterior sac and foot is abnormal in these

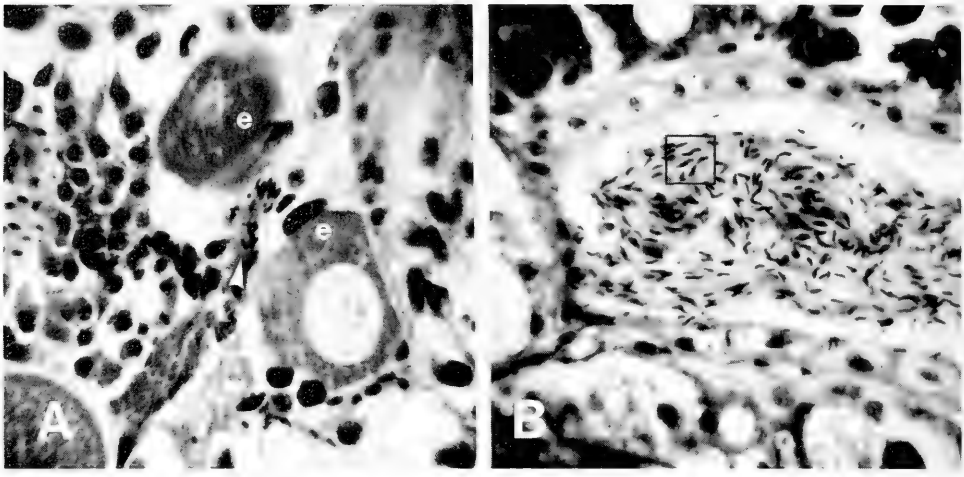

FIG. 3. Light micrograph showing the reproductive tract of *Deroceras laeve*. A, Section through gonad revealing a cluster of spermatids (arrow head) between egg cells (e). B, Hermaphroditic duct filled with mature spermatozoa. Sperm nuclei are darkly stained (box).

mutants, and the optic tentacles do not significantly differentiate from their rudimentary elements.

It is likely that the EZL-1 locus is a zygotically active gene, rather than a maternally active one. Maternally active genes are contributed by the maternal chromosome of the oocyte, responsible for setting up the spatial patterns in the embryo. Once the spatial organization is set out, differential expression of the zygotic genes is triggered, and embryonic development can be completed. In EZL-1 mutations, development is visibly normal until stage IV, when the acquisition of all of the adult rudimentary structures is complete. Further development, however, is aberrant. Thus, the initial spatial patterning of the embryo is normal, suggesting that the performance of the maternal genes is normal. On the other hand, differentiation of the embryo is abnormal, arresting about one-quarter into development, making it likely that the defect is zygotic, a consequence of the malfunction of a zygotically active gene.

The number of eggs in self-fertilizing slugs that failed to hatch was high. For example, in apparently homozygous individuals, about 23% of the eggs did not develop at all, and 3.4% of the developing eggs exhibited a range of lethal phenotypes. The failure of eggs to hatch into viable offspring has been observed with cross-fertilizing snails as well (Doums et al., 1994; Jarne & Delay, 1990; Rollinson et al., 1989). In one of these studies (Jarne & Delay, 1990), a significant difference in egg "hatchability" was observed between self- and cross-fertilizing *Lymnaea peregra* snails. It was stated that the disparity was due to well-formed snails that did not hatch, rather than eggs arresting at an early developmental stage. This result was interpreted as a decrease in fitness produced by self-fertilization, which these authors called self-fertilization depression. The studies presented here suggest the possibility that self-fertilization depression can be caused by a recessive lethal mutation. For example, the number of unhatched eggs produced by EZL-1 heterozygotes (EZL-1 eggs plus type A) is about two-fold greater than for homozygous wild-type slugs (type A eggs only) at the EZL-1 gene locus. Thus, the presence of a lethal mutation decreases egg "hatchability" in self-fertilization, a result that could be avoided by cross-fertilization with non-carrying animals. It is interesting that in the studies reported here with self-fertilizing slugs, the largest class of unhatched eggs exhibit no, or little, embryonic development. Further studies are needed to explain this observation.

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FOREST AND SCRUB SNAIL FAUNAS FROM NORTHERN MADEIRA

R. A. D. Cameron[1] & L. M. Cook[2]

ABSTRACT

The island of Madeira has two major natural vegetation types, a damp forest association at higher altitudes, with below it a dry association of grasses, herbs and low scrub. The open scrub is predominantly on the south coast and the eastern peninsula. The land mollusc fauna of the high forest and the north coast has been surveyed, and compared with that of other regions. Presence or absence of 84 species, 56 of them endemic, has been recorded in 51 samples. The forest samples are very clearly separated from those of the other regions; species in the families Pupillidae and Vitrinidae have radiated there, whereas radiation of Helicidae is characteristic of the other areas. Where non-endemics are present, they increase the species richness and do not displace endemics. There is no evidence of subdivisions within the forest fauna. Previous work has shown that at lower and drier locations an eastern peninsula fauna is differentiated from that of the south coast. The existence of the forest accounts for some of the species richness of the Madeiran archipelago, but much of it is attributable to species proliferation, especially in the Helicidae, between similar scrub habitats on different islands and on different parts of the same island.

Keywords: land snail, Mollusca, Madeira, competition, endemism.

INTRODUCTION

The Madeiran group is in the Atlantic Ocean, 900 km southwest of Portugal and 300 km west of Morocco at 33°N 17°W. The island of Madeira is 58 km at its largest dimension and rises to an altitude of 1860 m. There are two other clusters of islands, Porto Santo with its offshore islets and the three Deserta islands, all considerably lower than Madeira itself. The archipelago is exceptionally rich in land molluscs, with a high frequency of endemic species. In a count by Waldén (1984) there are 261 ± 3 taxa, of which 193, or 73.9%, are endemic. Waldén's (1983) list contains 216 species.

The distribution of the land snail fauna has been examined in detail in the southeast and eastern part of Madeira (Cameron & Cook, 1992; Cook et al., 1990). These areas have a predominantly dry-zone fauna, but with some species found also in the montane forest region. Some montane species occur, along with those characteristic of the modern eastern fauna, in the fossil deposit on the eastern peninsula (Cook et al., 1993; Goodfriend et al., 1994, 1996). The distribution of species in Porto Santo has also been surveyed (Cameron et al., 1996).

The territory from the peninsula to the western extremity of the island in the mountains, mostly on the north side of the main dividing ridge, has now been investigated. This area consists of steeply sloping valleys and mountainsides, covered in many places by indigenous humid laurel forest (Sjögren, 1972). The collections allow comparison with the lower-lying and dryer south coast and the eastern peninsula of Madeira, and also with Porto Santo and the Desertas. In order to understand how the fauna evolved, it is necessary to know the extent to which species are localised within islands and whether there is any evidence of competition, which might limit the diversity at a given locality or lead to selection for divergence between taxa.

MATERIALS AND METHODS

Fifty-one samples were examined, stretching from Porto Moniz in the west to the Boca do Risco to the east of the north coast, and from there along the peninsula and the southeast coast, a linear distance of about 70 km. Site locations are shown in Figure 1. Each site was examined by two people for about half an hour, searches being made on rock faces, litter and living vegetation. About 5 l of soil and litter were collected at each site, tak-

[1]Division of Adult Continuing Education, University of Sheffield, Sheffield S1 4ET, United Kingdom
[2]The Manchester Museum, University of Manchester, Manchester M13 9PL, United Kingdom

ing small amounts from favourable patches within the sample area. Material so collected was air-dried and searched in the laboratory after sieving. Material passing through a 0.5 mm mesh aperture was discarded. Searching strategies of this kind give reasonably robust and reliable indications of presence and absence. Different and more time-consuming techniques would be needed to estimate densities (Cameron, 1973, 1992). For the purpose of this study, presence and absence data are sufficient. A set of specimens of critical endemic species has been deposited with the Manchester Museum. Nomenclature and classification follow Waldén (1983), Cook et al. (1990), Groh & Hemmen (1986) and Holyoak & Seddon (1986). There were 84 species recorded, of which 56 are endemics. The mean number of species in the 51 samples was 14.0, with a standard deviation of 4.7. The richest sample contained 31 species, the poorest 6.

Salient features of the habitat, including dominant vegetation structure, the balance of native and alien plant species, and altitude, were noted and used to classify the sites into the following categories.

A (Sites 1–5): southern coastal scrub, firmly within the *Aeonio-Lyntathion* alliance (Sjögren, 1972), usually with introduced *Opuntia tuna* present. All sites subject to some degree of past agricultural disturbance, including occasional burning.

B (Sites 6–8): sites on the Ponta de São Lourenço, all open with field layer only present and with calcareous sand in the substrate. Floristically, they fall into the *Biserrulae-Scorpiurietum* association within the *Aenio-Lytanthion* alliance (Sjögren, 1972; Hampshire, 1984). These are the richest and least disturbed sites on the Ponta as reported by Cook et al. (1990).

C (Sites 9–15): coastal cliff and slope samples from the north coast. These also fall in the *Aeonio-Lytanthion* alliance of Sjögren (1972), but with more native scrub elements and higher moisture levels than on the south coast.

D (Sites 16–21): coastal cliff and slope samples from the north coast, as for C above but in which there are significant elements of the *Clethro-Laurion* alliance, the vegetation of typical native laurel forest (Sjögren, 1972), which descends to sea level in places along the north coast.

E (Sites 22–33): inland sites (at least 1 km from the sea) below 600 m, clearly within the *Clethro-Laurion* alliance, but generally disturbed and part cleared, with non-native trees often dominating the vegetation.

F (Sites 34–41): native *laurisilva* above 600 m but below 900 m above sea level in which native species typical of the *Clethro-Laurion* predominate.

G (Sites 42–51): native laurisilva above 900 m but below 1,400 m, as for F, but with *Erica* species often more important. At and above 1,300–1,400 m *laurisilva* is frequently replaced by *Erica* scrub or by overgrazed montane grassland (Sjögren, 1972).

The topography and land-use pattern of the island is such that there is some geographical clustering of sites placed in the same category. Categories C, D and E are more heterogeneous than the others, and note should be taken of the following sites.

Site 12 (Group C): extremely arid, very heavily grazed and uniquely on Pleistocene limestone rather than volcanic rock.

Site 14 (Group C): very open, with no surviving laurisilva elements, but with springs and permanently moist soil in places.

Site 20 & 21 (Group D): both with more or less complete native forest cover or shaded, wet rocks with native *Clethro-Laurion* vegetation, although very close to the sea.

Sites 22 & 23 (Group E): both clearly within the *Clethro-Laurion* zone altitudinally, but in an area of long-term forest clearance with much open ground and non-native vegetation typical of grassland and scrub.

RESULTS

General Patterns

Table 1 shows the percentage occurrence of each species in the samples within each sample group as defined above. A full matrix of species and sites is available from the authors, and a copy has been deposited with the collections in the Manchester Museum.

There is evidence of both geographical and ecological patterns in the distribution of species. Table 2 lists the species found only in predominantly *Aeonio-Lytanthion* habitats and their derivatives (groups A, B and C), only in predominantly *Clethro-Laurion* habitats (groups D, E, F and G) and also those found in combinations of C, D and E, which are to some extent intermediate between the extremes of A + B and F + G. Thirty-five species out of 84 show restriction to one or other of the

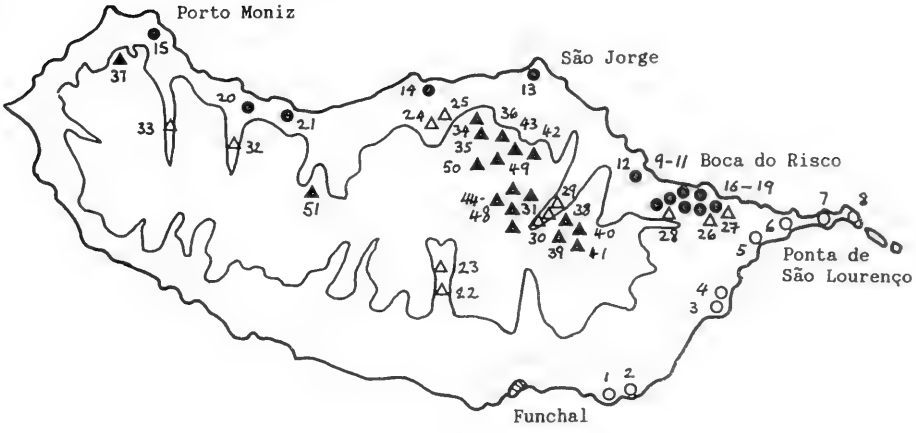

FIG. 1. The island of Madeira showing location of sampling sites. Open circles: sites in groups A and B (south coast and peninsula), closed circles: sites in groups C and D (dry and wetter north coastal samples), open triangles: group E (inland, wooded or disturbed, below 600 m), closed triangles: groups F and G (woodland, 6–1,400 m). Approximate position of 600 m contour is shown. Maximum distance from west to east is 58 km.

major vegetation alliances, and a further 12 are restricted to intermediate habitat types, which may represent a third, extensively disturbed faunal grouping. Helicidae, generally the largest species with the thickest shells, constitute a far higher proportion of restricted species in the open and intermediate habitats than they do in the forests (13 out of 30 in the former, 2 out of 17 in the latter). Relatively few non-endemics are restricted to forest sites.

To investigate the distinctions between categories further, we carried out a cluster analysis on individual samples. The basis for such analyses is a matrix of values of an index of similarity in species composition between all pairs of samples. The Jaccard Index is one such index, calculated by dividing the number of species in common between two sites by the total number of species present in both. When there are different numbers of species in the samples being compared, this index measures both the taxonomic similarity and the difference in number of species, because the maximum possible value is number of species in the sample with the fewer species as a fraction of the total in that with the larger number. Cook et al. (1972) normalized the Jaccard Index by multiplying it by the ratio of the larger species number to the smaller, calling the result maximum similarity. This would be appropriate if all the variation in species number was regarded as sampling error, and we were concerned exclusively with taxonomic similarity. However, a measure that is affected to some extent by difference in species richness as well as species composition would be preferable to either of these indexes, because it gives a better idea of the ecological similarity of the sites. When applied to presence/absence data, the Nei Index (Nei, 1987) is the number of species in common divided by the geometric mean of the number of species in each sample of the pair, providing the right kind of balance. It is therefore an appropriate measure, and has been used to examine pairwise similarity. Figure 2 shows the dendrogram of faunal affinities for all species using the Nei Index and UPGMA clustering. It confirms the habitat distinctions noted above. It is clear that the data fall into two major clusters (Table 3). All samples of groups A, B and C fall into one of these, and all those of groups F and G into the other. Samples in groups D and E are distributed between the two. There are clear separations between groups A and B and between these and groups E, F and G. Groups C, D and E are more scattered; much of the variation being explicable in terms of the specific site characteristics noted above. We therefore conclude that the groups recognise differences in ecology and/or geography that genuinely influence the faunal composition. Intermediate habitats have mixtures of species from the two major faunas.

The Nei index is usually applied to frequency data, and may be used to examine the fraction of sites occupied by each species in each group as shown in Table 1. The result is shown in Figure 3. Again, all species were included; when non-endemics are excluded, the

TABLE 1. Samples grouped into seven geographical and habitat categories, showing fraction of sites occupied in each group (to nearest per cent). Endemic species (1 to 56) are listed before non-endemics (57 to 84), otherwise species are listed in the systematic order given by Waldén (1983). Authorities for the names are in Waldén (1983), Groh & Hemmen (1986), and Holyoak & Seddon (1986).

A	B	C	D	E	F	G	Group
ENDEMICS							
0	66	42	83	91	100	100	1. *Craspedopoma mucronatum*
0	0	14	50	33	50	20	2. *C. neritoides*
20	0	0	0	0	0	0	3. *C. monizianum*
0	0	14	50	16	37	10	4. *C. trochoideum*
0	0	14	33	33	62	80	5. *Columella microspora*
40	33	57	16	0	0	0	6. *Staurodon saxicola*
0	0	0	0	0	37	50	7. *Leiostyla cheilogona*
0	0	0	0	0	0	10	8. *L. filicum*
0	0	0	16	0	0	0	9. *L. vincta*
0	0	14	16	16	0	0	10. *L. irrigua*
0	0	14	33	8	50	30	11. *L. loweana*
0	0	0	0	0	0	20	12. *L. concinna*
0	0	0	0	0	25	10	13. *L. laurinea*
0	0	14	16	8	12	0	14. *L. sphinctostoma*
0	0	14	0	0	0	10	15. *L. arborea*
0	0	14	16	8	0	0	16. *L. fusca*
20	0	57	66	0	0	0	17. *L. recta*
80	33	14	0	0	0	0	18. *L. millegrana*
0	0	0	0	0	25	10	19. *Lauria fanalensis*
0	0	0	0	0	37	50	20. *Hemilauria limneana*
0	0	0	0	0	12	0	21. *Phenacolimax nitidus*
0	0	0	33	75	100	89	22. *P. marcidus*
0	0	14	33	58	62	80	23. *P. ruivensis*
0	0	0	16	16	12	0	24. *P. behnii*
0	0	0	16	8	12	30	25. *P. albopalliatus*
0	0	14	0	0	0	0	26. *Janulus stephanophora*
80	33	42	50	0	0	0	27. *J. bifrons*
0	0	14	33	58	12	20	28. *Amphorella tornatellina*
0	100	0	16	0	0	0	29. *A. cf. minor*
40	0	0	0	0	0	0	30. *A. mitriformis*
0	33	0	0	0	0	0	31. *A. cf. iridescens*
80	33	28	33	0	0	0	32. *Pyrgella leacockiana*
40	0	0	16	0	0	0	33. *Boettgeria deltostoma*
60	66	14	0	0	0	0	34. *B. depauperata*
0	66	71	33	0	0	0	35. *B. exigua*
0	0	0	16	0	62	50	36. *B. crispa*
40	100	28	16	0	0	0	37. *Heterostoma paupercula*
0	0	14	0	0	0	10	38. *Spirorbula latens*
0	0	14	0	0	0	0	39. *S. squalida*
40	100	0	0	0	0	0	40. *Caseolus compactus*
60	0	0	0	0	0	0	41. *C. leptostictus*
20	0	0	0	0	0	0	42. *Disculella maderensis*
0	0	57	16	8	0	0	43. *Actinella lentiginosa*
0	0	14	0	0	0	0	44. *A. actinophora*
100	0	0	0	0	0	0	45. *A. arcta*
0	0	14	16	16	0	10	46. *A. fausta*
0	0	0	16	16	0	0	47. *A. carinofausta*
0	0	0	0	0	0	10	48. *A. obserata*
100	100	100	100	16	0	0	49. *A. nitidiuscula*
0	0	0	0	16	0	0	50. *A. giramica*
20	0	0	0	0	0	0	51. *Discula tabellata*
100	100	85	50	0	0	0	52. *D. polymorpha*
60	0	71	66	66	50	10	53. *Leptaxis erubescens*
0	0	0	16	0	0	20	54. *L. furva*
0	0	14	33	58	87	100	55. *L. membranacea*
100	100	28	0	8	0	0	56. *L. undata*

TABLE 1. (*Continued*)

A	B	C	D	E	F	G	Group
NON-ENDEMICS							
0	0	14	0	0	12	10	57. *Carychium minimum*
0	0	14	33	58	75	20	58. *C. tridentatum*
0	0	57	0	16	0	0	59. *Cochlicopa lubrica*
80	0	57	100	66	75	0	60. *C. lubricella*
20	0	14	33	8	12	0	61. *Columella aspera*
0	0	14	0	0	0	0	62. *Truncatellina callicratis*
60	33	42	16	0	0	0	63. *Vertigo pygmaea*
100	0	100	66	50	0	0	64. *Lauria cylindracea*
80	0	14	0	0	0	0	65. *Vallonia costata*
40	33	14	0	0	0	0	66. *V. pulchella*
0	0	0	0	0	0	10	67. *Acanthinula aculeata*
0	0	0	0	16	87	69	68. *Plagyrona placida*
0	0	0	50	50	37	10	69. *Punctum pygmaeum*
100	33	85	66	75	87	69	70. *P. pusillum*
0	0	0	33	25	12	10	71. *Helicodiscus singleyanus*
20	0	0	0	0	0	0	72. *Hawaiia miniscula*
60	33	85	83	91	87	30	73. *Vitrea contracta*
0	0	14	0	16	12	0	74. *Nesovitrea hammonis*
80	0	100	83	8	0	0	75. *Oxychilus cellarius*
0	0	0	16	16	0	0	76. *Zonitoides arboreus*
0	0	0	0	8	0	0	77. *Oxychilus alliarius*
0	0	14	83	58	87	40	78. *Euconulus fulvus*
60	33	0	0	0	0	0	79. *Cecilioides acicula*
20	0	0	0	0	0	0	80. *Ferrusacia folliculus*
60	33	0	0	0	0	0	81. *Caracollina lenticula*
0	33	0	0	0	0	0	82. *Cochlicella barbara*
0	33	0	0	0	0	0	83. *Theba pisana*
0	0	28	0	8	0	0	84. *Helix aspersa*
5	3	7	6	12	8	10	Total sites in group
19	14	30	31	21	19	23	Endemic species
13	7	16	12	16	11	9	Non-endemic species
32	21	46	43	37	30	32	Total species in group

pattern is almost identical. The impression gained from presence/absence data is confirmed. Groups A and B, representing the faunal composition of the *Aeonio-Lytanthion* alliance on the southern and eastern parts of the island, are similar to each other and form a pair distinct from the rest. At a higher level of similarity, categories F and G, the higher altitude *Clethro-Laurion,* separate off from C, D and E, which contain species characteristic of the laurel forest plus others introduced by disturbance and by natural spreading to those coastal areas resembling the eastern sites. They include sites 22 and 23 (exceptionally dry and open for group E), which resemble the open habitat section, and sites 14, 20 and 21 (in groups C and D), which have above average moisture or *Clethro-Laurion* characteristics. The faunal separation of the south coast and the high laurel forest is due not only to the species restricted to them but also to marked differences in proportion in several of the other, non-restricted species.

There is also a less obvious correlation between similarity and geographical position. Sites within groups A and B are close both geographically and in terms of affinity, and there is a collection of group C and D sites between Boca do Risco and São Jorge, which also show high affinity levels. No geographical pattern is visible in the forest sites.

Ratio of Non-endemics to Endemics

When non-endemics are introduced to an endemic fauna, one possible outcome is a negative relation between numbers of endemic and non-endemic species in samples. This may occur because non-endemics thrive in habitats to which endemics are not adapted, and vice versa, or because there is direct competition between the species. Such a

TABLE 2. Species in the survey which come from (a) *Clethro-Laurion* forest or from lower altitude habitats of similar type (DEFG), (b) intermediate and disturbed habitats (CDE) or (c) from *Aeonio-Lytanthion* scrub or the damper sites of group C (ABC).

Groups DEFG	CDE	ABC
ENDEMICS		
Cyclophoridae		
		Craspedopoma monizianum
Pupillidae		
Leiostyla cheilogona	*L. vincta*	*L. millegrana*
L. filicum	*L. arborea*	
L. concinna	*L. fusca*	
L. laurinea		
Lauria fanalensis		
Hemilauria limneana		
Vitrinidae		
Phenacolimax nitidus		
P. marcidus		
P. behnii		
P. albopalliatus		
Zonitidae		
	Janulus stephanophora	
Ferussaciidae		
		Amphorella mitriformis
		A. cf. iridescens
Clausiliidae		
Boettgeria crispa	*B. depauperata*	
Helicidae		
	Spirorbula squalida	
		Caseolus compactus
		C. leptostictus
		Disculella maderensis
		Actinella arcta
A. obserata	*A. lentiginosa*	
	A. carinofausta	
	A. giramica	
		Discula tabellata
Leptaxis furva		
NON-ENDEMICS		
Cochlicopidae		
	Cochlicopa lubrica	
Valloniidae		
		Vallonia costata
		V. pulchella
Acanthinula aculeata		
Plagyrona placida		
Endodontidae		
Punctum pygmaeum		
Helicodiscus singleyanus		
Zonitidae		
		Hawaiia miniscula
	Oxychilus alliarius	
	Zonitoides arboreus	
Ferussaciidae		
		Cecilioides acicula
		Ferrusacia folliculus
Helicidae		
		Caracollina lenticula
		Cochlicella barbara
		Theba pisana
	Helix aspersa	

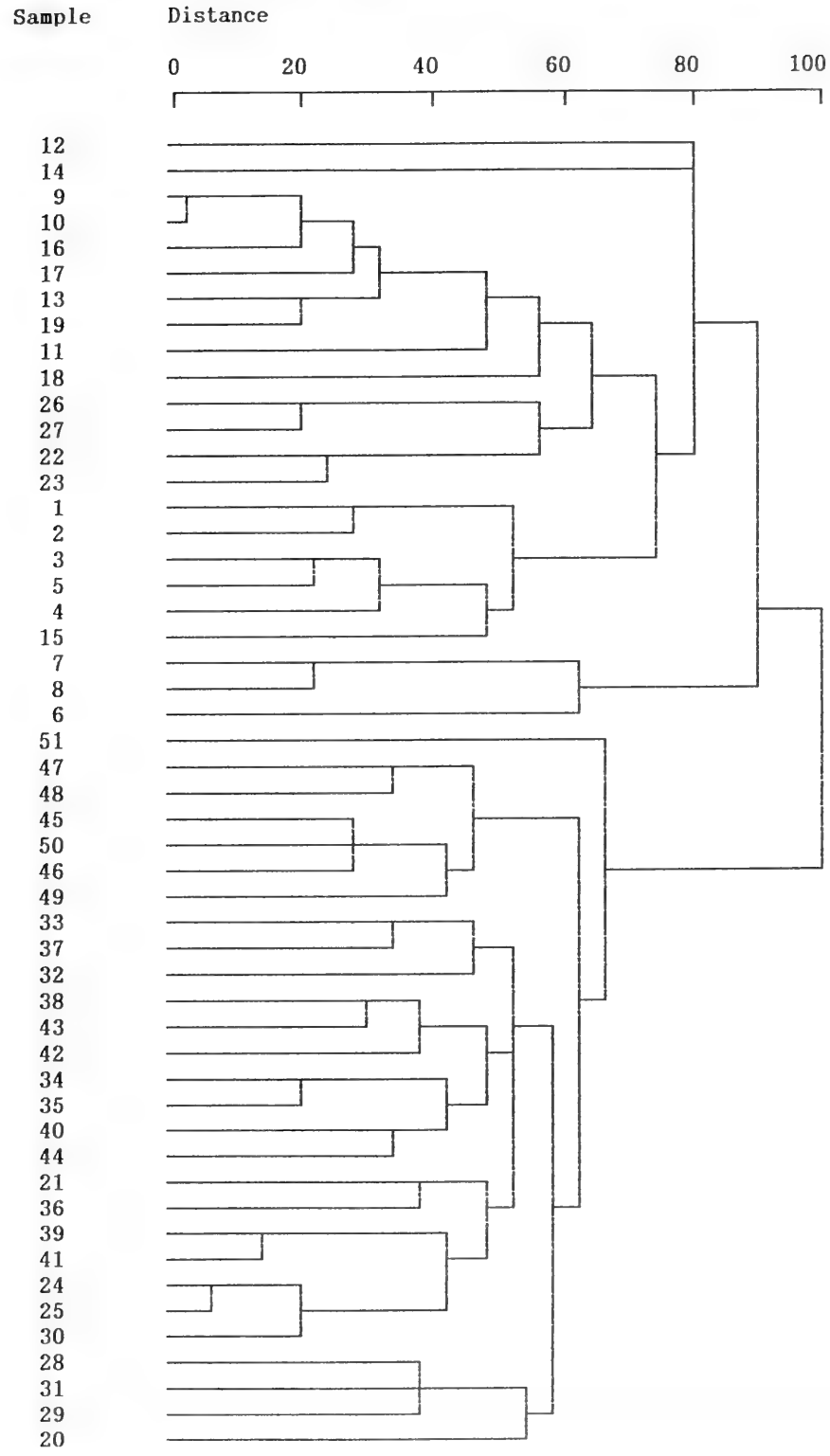

FIG. 2. Similarity of the 51 samples in the survey. Scale shows distance as a fraction of the maximum value for the analysis.

TABLE 3. The seven defined habitat groups divided between the two main clusters of Figure 2. Groups, based on habitat type and reflecting to some extent geographical location, are defined in the text.

	Defined groups							
Derived cluster	A	B	C	D	E	F	G	Total
1	5	3	7	4	4	0	0	23
2	0	0	0	2	8	8	10	28
Total	5	3	7	6	12	8	10	51

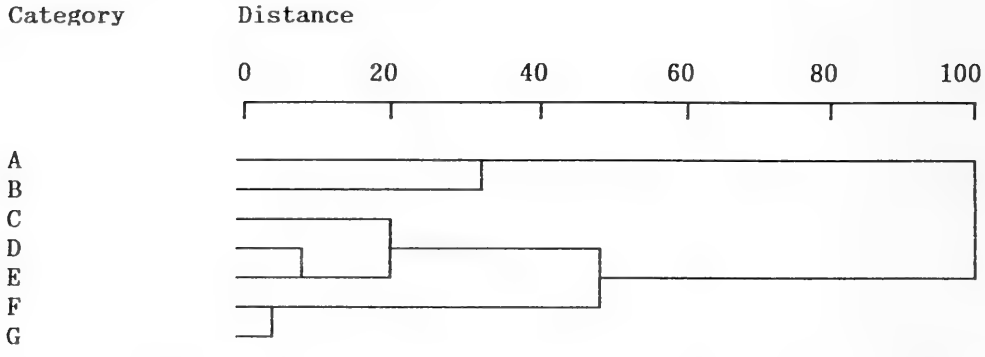

FIG. 3. Similarity of the seven categories of samples derived from the frequency of species in the samples within categories (Table 1). Scale shows distance as a fraction of the maximum value for the analysis.

relation occurs, for example, in our study of the snails of Porto Santo (Cameron et al., 1996), where there is a negative relation of endemics to non-endemics on a rocky/undisturbed to sandy/disturbed axis. To test for a similar effect on Madeira, the association of the number of non-endemic species with the number of endemic species has been examined. The correlation coefficient r is 0.263, for which $t = 1.906$ ($P < 0.06$). Thus, there is no evidence of a negative association; the relation is positive, and nearly significant, the slope of the reduced major axis being 0.757. It could result from the fact that sites vary in their suitability for molluscs, good sites being favourable to non-endemics and endemics alike. The feature most likely to lead to high species number may be microhabitat heterogeneity. At any rate, presence or absence of a species appears to depend on habitat suitability, with no evidence of negative association between the two categories.

DISCUSSION

Cook et al. (1990) demonstrated differences between snail faunas from open habitats in the south and east of Madeira, which suggested a pattern of geographical differentiation independent of present habitats. Similar, indeed more striking differentiation of this kind is found in the neighbouring island of Porto Santo (Cameron et al., 1996). This study extends the survey to the high altitude forest and open and intermediate habitats along the north coast of Madeira.

There is a radical difference between the fauna of the *Clethro-Laurion* forest and of the open habitats and scrubby areas at lower altitudes. In the forest, the climate is cooler, rainfall is higher, and soils are less rich in calcium (Sjögren, 1972). Table 4 illustrates the different balance of families in *Clethro-Laurion* sites from those of open habitats on Madeira and on the other islands of the archipelago, which are even more arid. The faunas have substantial representation of small, thin-shelled species (especially Pupillidae) and semi-slugs (Vitrinidae), and are less dominated by Helicidae species, only six of which were found above 600 m altitude. In the open sites, there is again evidence of geographical differentiation, albeit complicated by disturbance and small-scale mosaics of forest and open habitats. Faunas from mid- to high alti-

TABLE 4. Species distribution between families in samples collected in different parts of the archipelago. Madeira north side (Groups D to G): data from this paper. Madeira fossil: samples 45 thousand years old or older from the sand bed on the eastern peninsula from Cook et al. (1993). Madeira, eastern peninsula and Desertas: contemporary samples from Cook et al. (1990). Porto Santo: contemporary samples from Cameron et al. (1996).

Family	Madeira N side	Madeira fossil	Madeira peninsula	Desertas	Porto Santo
Endemics					
Pupillidae	13	7	4	0	5
Ferussaciidae	3	3	5	1	8
Clausiliidae	3	1	4	1	1
Vitrinidae	5	2	2	0	1
Helicidae	12	18	12	10	32
Others	6	6	5	1	0
Non-endemics (all families)	19	2	21	0	9
Total	61	39	53	13	56

tude forests, free from gross disturbance, do not show such effects, although distances between sites are the same, or greater than those involved in open habitat comparisons.

In drawing the distinction between the relatively open *Aeonio-Lytanthion* and *Clethro-Laurion* alliances, Sjögren (1972) noted that on the drier south side of the island the *Aeonio-Lytanthion* gives way to forest between 300 and 700 m above sea level, whereas on the wetter north it is rare for it to ascend above 300 m; *Clethro-Laurion* or derivatives frequently approach sea-level. While modest changes in altitudinal range of the alliances (for example, in response to Pleistocene climatic changes) could isolate sections of open habitat on the south coast, and extinguish it in the north, the forest area would fluctuate is size but remain as a largely continuous block. Extension of forest faunas in the past is suggested by the subfossil data in Table 4, and confirmed by detailed analysis (Cook et al., 1993).

Although forest faunas are more uniform, there is some altitudinal differentiation, with species characteristic of low or high altitudes. There are some species-rich genera. Of these, *Leiostyla* species are small, rare, cryptic and apparently patchily distributed. Our knowledge of them is as yet too limited to say whether they show geographical differentiation. On the present evidence, however, the extensive proliferation of distinct species of limited distribution but similar habitat, which contributes so much to the species richness of the archipelago, is largely a dry-habitat phenomenon. Forests, especially at higher altitudes, have few non-endemic species, and some of those that do occur may be natives rather than introductions. We have no evidence pointing to adverse effects of non-endemics on endemics or to the presence of habitats which favour non-endemics over endemics. The greater number and higher density of non-endemics in open habitats probably reflects the richer conditions there, created to some extent by human activity, in which both categories can flourish.

ACKNOWLEDGEMENTS

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COMPARATIVE STUDIES ON THE ANATOMY AND HISTOLOGY OF THE ALIMENTARY CANAL OF THE LIMACOIDEA AND MILACIDAE (PULMONATA: STYLOMMATOPHORA)[1]

Ana Maria Leal-Zanchet

Laboratório de Histologia, Centro de Ciências da Saúde, Universidade do Vale do Rio dos Sinos, Caixa Postal 275, 93022-000 São Leopoldo-RS, Brasil

ABSTRACT

The anatomy and histology of the digestive tract of *Deroceras laeve, D. reticulatum, D. rodnae, Lehmannia marginata, Malacolimax tennelus, Boettgerilla pallens* and *Tandonia budapestensis* are described comparatively. The alimentary canal is composed of oesophagus, crop, stomach, intestine and rectum. An intestinal caecum is present in *D. reticulatum, D. rodnae* and *L. marginata.* The intestine is subdivided into four histologically distinct regions. From the oesophagus to the third intestinal region, and in the rectum, the alimentary canal is lined by a simple columnar to cuboidal epithelium. In the fourth intestinal region and in the intestinal caecum, the epithelium is simple squamous to cuboidal. Supporting ciliated and nonciliated cells are found in the epithelium. Both types carry microvilli. Eight gland cell types can be distinguished: mucous cells of types I-V, cystic cells, and intestinal secreting cells of types I and II. The epithelium of the alimentary canal is surrounded by a layer of connective tissue and two muscle layers, an inner longitudinal muscle layer and an outer circular muscle layer.

Key-words: anatomy, histology, alimentary canal, secretory cells, Limacidae, Agriolimacidae, Boettgerillidae, Milacidae.

INTRODUCTION

According to Likharev & Wiktor (1980), the superfamily Limacoidea is formed by the families Limacidae, Agriolimacidae and Boettgerillidae, whereas the family Milacidae belongs to the superfamily Zonitoidea. Little information exists on the anatomy and histology of the digestive system of the limacids and agriolimacids. Simroth (1885). Quick (1960) and Wiktor (1973), in their systematic studies on the group, only described the course of the alimentary canal. Studies on the anatomy and histology of the alimentary canal of *Limax, Arion* and *Helix* have been carried out by Gartenauer (1875) and Baecker (1932). Walker (1972) examined the digestive system of *Deroceras reticulatum,* emphasizing the physiology of the crop, stomach and intestine. More recently, only a few observations have been made on the histology of the alimentary canal of a limacoid species (Babula & Skowronska-Wendland, 1988). No data are found in the literature on the anatomy and histology of the digestive system of the families Boettgerillidae and Milacidae. The purpose of this paper is to report on a comparative anatomical and histological study of the alimentary canal of six limacoid species and a milacid species.

MATERIALS AND METHODS

The following species were studied: *Malacolimax tenellus* (Müller, 1774) and *Lehmannia marginata* (Müller, 1774) (Limacidae), *Deroceras laeve* (Müller, 1774), *D. reticulatum* (Müller, 1774) and *D. rodnae* (Grossu & Lupu, 1965) (Agriolimacidae), *Boettgerilla pallens* (Simroth, 1912) (Boettgerillidae), and *Tandonia budapestensis* (Hazay, 1881) (Milacidae). The animals were collected next to the city of Tübingen, Baden-Württemberg, Germany, and kept in a cool room at 15°C (Leal-Zanchet, 1995). The species are herbivorous, except for *B. pallens* (Leal-Zanchet, in press). In the laboratory, the animals were fed cabbage, lettuce and carrots. For the anatomical studies, the slugs were anaesthetized in a 5%

[1]Part of a thesis submitted to the Lehrstuhl Spezielle Zoologie of the University of Tübingen, Germany, in partial fulfillment of the requirements for the degree of Doctor of Natural Sciences.

solution of menthol for three to four h, fixed in 4% formaldehyde for 24 to 48 h and transferred to 70% ethanol. The slugs were progressively dissected with the aid of a binocular microscope. The digestive tract was uncovered and drawn under a camera lucida. Finally, the alimentary canal was opened longitudinally to observe its internal morphology. For the light microscopy studies, the slugs were anaesthetized in 5% menthol for two and fixed in Susa or 4% paraformaldehyde/glutaraldehyde. Tissues fixed with Susa were dehydrated in ethanol, embedded in Paraplast, and serially sectioned at 6 μm. The sections were stained with haematoxylin/eosin, or with the triple stain methods of Masson-Goldner (MG) and Azan-Heidenhain (AZ) (Romeis, 1989). This method was used to delimit histologically the organs and regions of the digestive system, and also for a first characterization. Small fragments of the various parts of the alimentary canal were fixed in 4% paraformaldehyde/glutaraldehyde, washed in phosphate buffer, dehydrated in ethanol and embedded in historesin. The 2 μm thin sections were stained with methylene blue and basic fuchsin (MF) (Bennett et al., 1976). This second method gives the best results for detailed histological studies.

For measurements of the epithelium and the gland cells we used a micrometric glass. Four animals of each species were measured. All measurements were made on the material fixed in paraformaldehyde/glutaraldehyde and embedded in historesin. They were made on the apices of the folds and between the folds. The mean values were given in the tables.

RESULTS

Anatomical Features

The narrow oesophagus (oe) rises from the buccal mass (Fig. 2, bcm) and widens posteriorly to form the crop (cr). From this emerges the small stomach (s), which is followed by the long intestine (Fig. 2, i). The ducts of the digestive gland open into the stomach (Figs. 3, 4). The intestines of *Lehmannia marginata,* and *Malacolimax tenellus* have two forward-directed loops, whereas the intestines of *Deroceras laeve*, *D. reticulatum*, *D. rodnae, Tandonia budapestensis* and *Boettgerilla pallens* have only one forward directed loop (*sensu* Quick, 1960). From the second intestinal loop of *Lehmannia* and *Malacolimax,* or the single loop of the other species, arises the terminal branch of the intestine, which goes forward and enters into the body wall and is then called rectum (Fig. 1).The rectum goes through the pallial complex for a short way and unites with the ureter into the anus, which is situated in the right anterior part of the body. A blind tube, the intestinal caecum (Fig. 2, c), is associated with the terminal part of the intestine of *L. marginata, D. reticulatum* and *D. rodnae* (Fig. 1).

The oesophagus has longitudinal folds (Fig. 5, lf), and the crop has a smooth wall without folds, containing only narrow elevations (Fig.

KEY TO LETTERING ON FIGURES

as: apocrine secretion
bcm: buccal mass
c: intestinal caecum
ca: clear area
cc: ciliated columnar cells
cct: cells of the connective tissue
ce: cuboidal epithelium
ci: cilia
cia: ciliated area
cm: circular muscle layer
cn: cell neck
cr: crop
ct: connective tissue
cy: cystic cells
dg: digestive gland
dgd: duct of the digestive gland
dgo: opening of the digestive gland
e: elevation
i: intestine
ic: intestinal secreting cells
ic I: intestinal secreting cells of type I
ic II: intestinal secreting cells of type II
lef: leader fold
lf: longitudinal fold
lg: leader groove
lm: longitudinal muscle layer
lu: lumen
mc: mucous cells
mc I: mucous cells of type I
mc II: mucous cells of type II
mc III: mucous cells of type III
mc IV: mucous cells of type IV
mc V: mucous cells of type V
mi: microvilli
n: nucleous
nc: nonciliated columnar cells
nu: nucleolus
oe: oesophagus
s: stomach
sac: strong acidophilic cells
se: squamous epithelium
sg: salivary glands
sgd: duct of the salivary gland
sgr: secretory granules
sl: subepithelial layers
sm: secretion mass
sv: supranuclear vacuoles
t_1, t_2: typhlosoles
tf: transversal fold
va: vacuole containing amorphous material

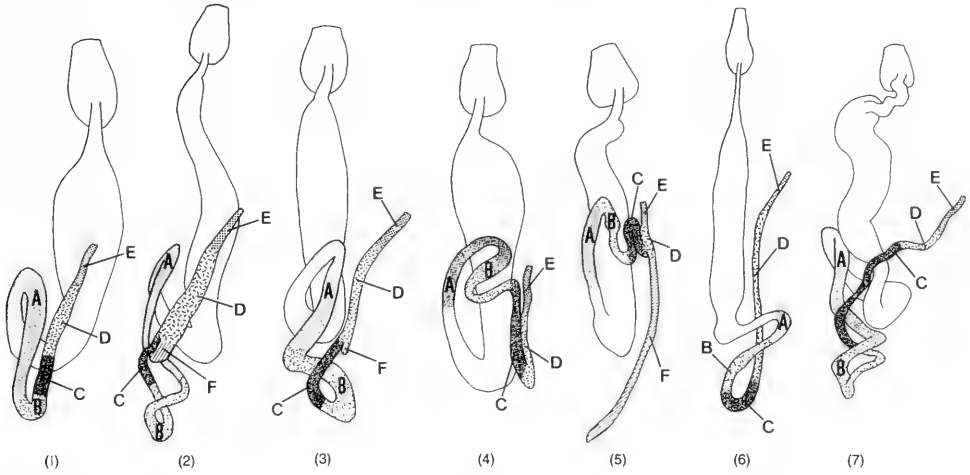


FIG. 1. Schematic diagrams comparing the digestive system of (1) *Deroceras laeve*, (2) *Deroceras reticulatum*, (3) *Deroceras rodnae*, (4) *Malacolimax tenellus*, (5) *Lehmannia marginata*, (6) *Boettgerilla pallens* and (7) *Tandonia budapestensis*. *A*: first intestinal region, *B*: second intestinal region, *C*: third intestinal region, *D*: fourth intestinal region, *E*: rectum, *F*: intestinal caecum.

First intestinal region
Second intestinal region
Third intestinal region
Fourth intestinal region
Rectum
Intestinal caecum.

6, e) due to the irregular height of the epithelium. The crop of *Tandonia budapestensis* is an exception, with the wall having transverse folds (Fig. 28). The stomach contains three conspicuous folds, two longitudinal typhlosoles and a transverse fold (Figs. 4, 7). Each typhlosole originates close to an opening of the duct of the digestive gland and runs posteriorly as far as the posterior limit of the stomach (Fig. 4, t_1, t_2). The transverse fold is closely triangular and extends between the two openings of the ducts of the digestive gland (Fig. 4, f). A leader groove occurs between the typhlosoles, as well as between the transverse fold and the typhlosoles (Fig. 4, lg). The intestine can be subdivided into four regions. Figure 1 shows the arrangement of the intestinal regions in relation to the intestinal loops. The first and second intestinal regions have smooth walls without folds, being differentiated only by their histological features. However, in *L. marginata* and *M. tenellus*, only small longitudinal folds are present in the first intestinal region (Fig. 8, lf). The third intestinal region has numerous longitudinal folds (Fig. 10, lf), and the fourth region has a smooth wall, except for a ciliated area (Fig. 11, cia). This occurs in limacids and agriolimacids, but not in *B. pallens* and *T. budapestensis*. The intestinal caecum has no folds. The rectum has a smooth wall in its proximal third and abundant longitudinal folds in its distal two thirds (Figs. 12–16, lf). Among these, three to five differentiated folds occur in Agriolimacidae, Boettgerillidae and Milacidae (Figs. 13–16, lef). There are five of these folds in *B. pallens* and *T. budapestensis*, where they are most developed (Figs. 13, 14, 43). In *Deroceras*, there are two to three leader folds (Figs. 15, 16, 44).

Histological Features

Epithelium: Oesophagus—The oesophagus is lined by a columnar simple epithelium. In the proximal third of the oesophagus, its surface is cuticularized, but in most of the oesophagus the epithelium carries cilia and microvilli (Fig. 17). The cilia are especially numerous on the crests of folds (Figs. 5, 26).

The epithelium of the oesophagus consists of ciliated columnar cells (cc), nonciliated columnar cells (nc), and mucous cells (Fig. 17, mc l). Both ciliated and nonciliated columnar cells bear microvilli. The columnar cells show an acidophilic cytoplasm, in which supranuclear vacuoles (Fig. 17, sv) with strong acidophilic or cromophobe contents can be seen.

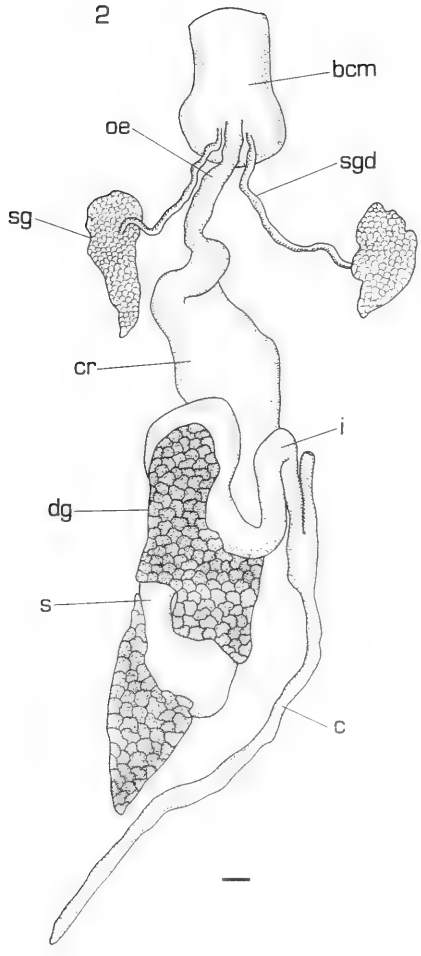


FIG. 2. Dorsal view of the digestive system of *Lehmannia marginata.* Scale bar 1 mm.

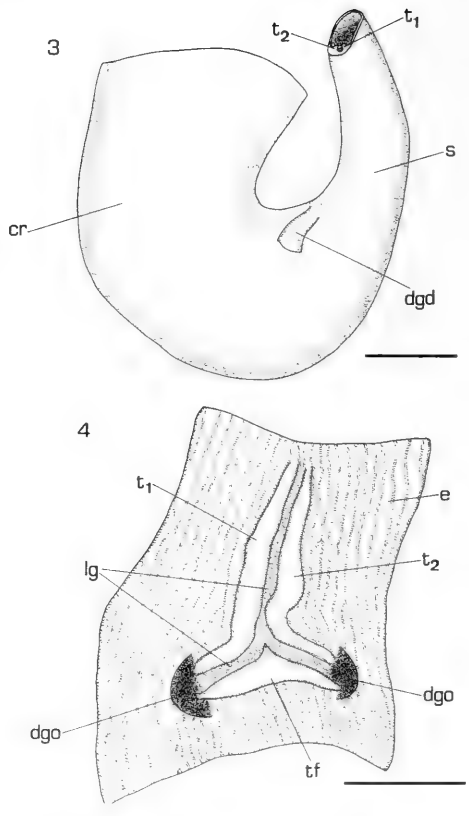


FIG. 3. Ventral view of the crop and the stomach of *Deroceras reticulatum.*

FIG. 4. Internal morphology of the stomach of *Lehmannia marginata.* Scale bar 1 mm.

The most apical part of the cells is a clear area (Fig. 17, ca). The oval nucleus lies in the middle or basal third of the cells (Fig. 17, n).

The columnar cells of the alimentary canal show an apocrine secretion (Figs. 17, 18, 22, as), and eventually a holocrine secretion in which strong acidophilic columnar cells are discharged into the lumen (Figs. 30, 31, sac).

The mucous cells of the oesophagus are termed type I (Fig. 17, mcl). They are intraepithelial and flask shaped, with a broadened base and a long neck, and they contain numerous weakly or strongly basophilic granules (MF). The oval or elongated nucleus is basally located and surrounded by a strong acidophilic cytoplasm. In the paraffin sections, the granules and the remaining cytoplasm of the mucous cells cannot be distinguished; the mucous cells show a foamy content that stains light green with MG and light blue with AZ.

Crop—The columnar epithelium of the crop is higher than that of the oesophagus (Table 1). In the crop, only nonciliated columnar cells (nc) and mucous cells of type I (Figs. 18, 27, mcl) are present. The columnar cells of the crop are clearly distinguishable because of the presence of very abundant large vacuoles containing amorphous material (Figs. 18, 27, va). The contents of the vacuoles can be removed by histological methods. Supranuclear vacuoles (Fig. 18, sv) with strong acidophilic or chromophobe contents, similar to those of the oesophagus, are also present. The oval or

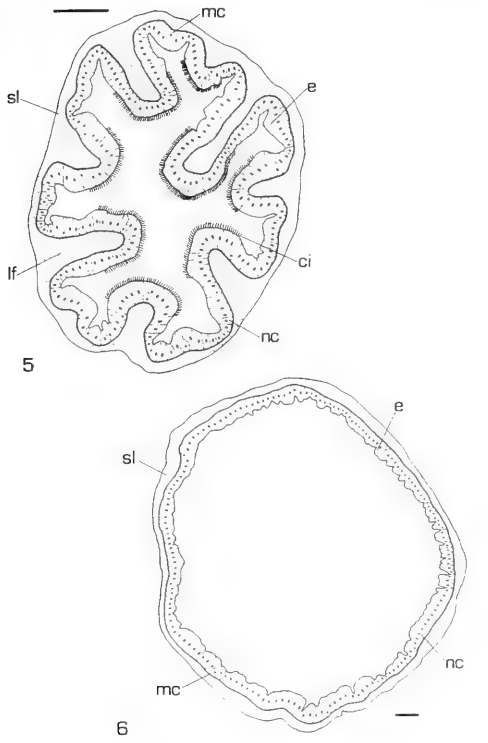


FIG. 5. Cross-section of the oesophagus of *Lehmannia marginata*.

FIG. 6. Cross-section of the crop of *Lehmannia marginata*. Scale bar 0.1 mm.

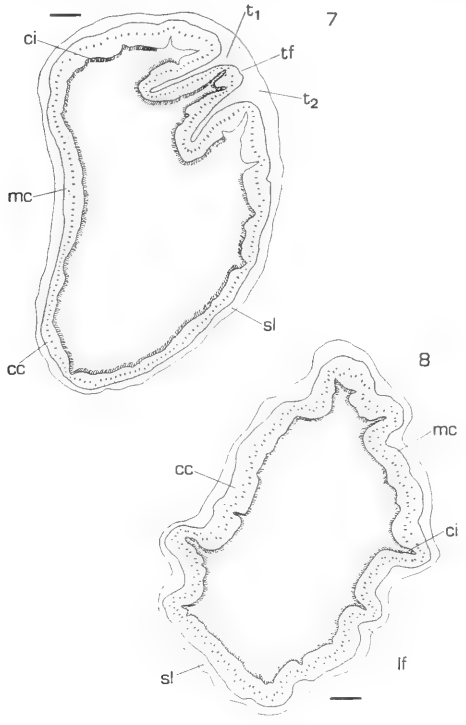


FIG. 7. Cross-section of the stomach of *Lehmannia marginata*.

FIG. 8. Cross-section of the first intestinal region of *Lehmannia marginata*.

elongated nucleus lies in the middle or basal third of the cells (Fig. 18, n). Type I mucous cells are similar to those of the oesophagus (Figs. 18, 27, mcI).

Stomach—The ciliated columnar epithelium of the stomach usually is lower than that of the crop (Table 1). In the stomach, the ciliated columnar cells far outnumber the nonciliated cells. The latter occur only in small areas adjoining the typhlosoles (Fig. 7). The cytoplasm of the columnar cells is similar to that of the corresponding cell types of the crop, but the number of vacuoles containing amorphous material is lower (Fig. 19, va). The columnar cells of the leader groove (Fig. 20), located between the two typhlosoles, differ from the other columnar cells of the stomach by their strong acidophilic cytoplasm containing rare vacuoles. In addition, the epithelium of the leader groove and of the typhlosoles is taller than in the rest of the stomach and has long cilia (Fig. 20).

In the stomach, the mucous cells are more abundant than in the crop (Table 3). Two types of mucous cells can be distinguished. The type I mucous cells are found in most of the stomach and are similar to those of the crop in shape and morphology (Fig. 19, mcI). The type II mucous cells occur only in the leader groove (Figs. 20, 31, mcII). However, in *B. pallens* and *T. budapestensis*, type II mucous cells are found in the entire stomach. They are intraepithelial and have a long base with an acidophilic cytoplasm containing many vacuoles. The nucleus lies distal at the base. The long neck shows numerous small and strong basophilic secretory granules (Table 2, Fig. 20).

First Intestinal Region—This region has a high columnar epithelium (Table 1) with a pseudostratified appearance because the nu-

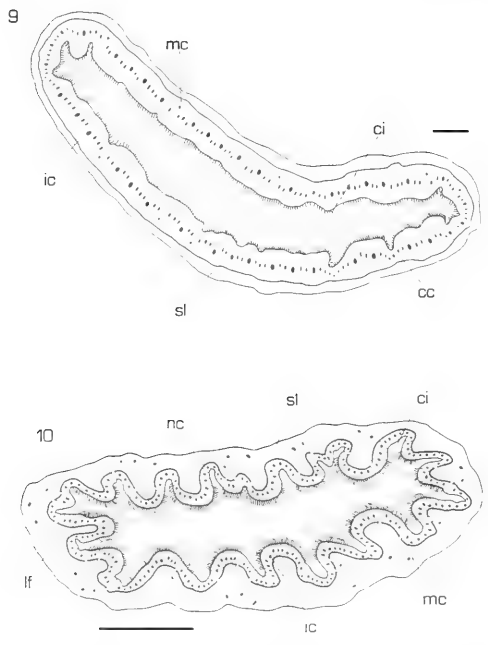


FIG. 9. Cross-section of the second intestinal region of *Lehmannia marginata.*

FIG. 10. Cross-section of the third intestinal region of *Lehmannia marginata.* Scale bar 0.1 mm.

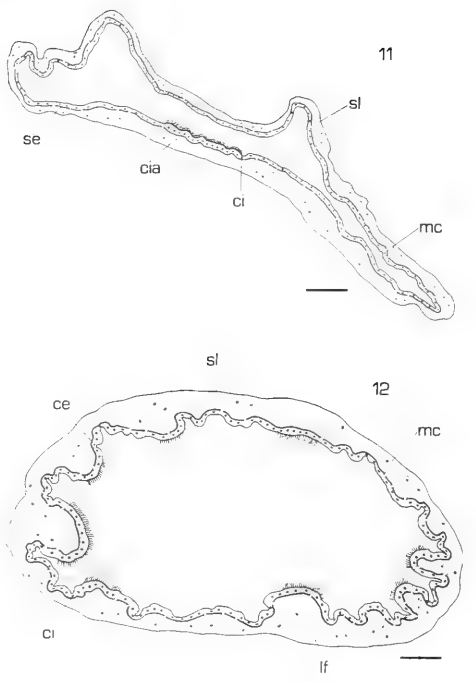


FIG. 11. Cross-section of the fourth intestinal region of *Lehmannia marginata.*

FIG. 12. Cross-section of the rectum of *Lehmannia marginata.* Scale bar 0.1 mm.

clei tend to be arranged in two rows: the round nuclei of the abundant mucous cells form a row at the base of the epithelium, and the elongated nuclei of the columnar cells lie in one or two rows halfway up in the epithelium (Figs. 21, 33, n).

Only ciliated columnar cells occur in the first intestinal region (Fig. 8). Their cytoplasm possess a small number of vacuoles with amorphous contents (Fig. 21, va), and is otherwise similar to that of the columnar cells of the crop. Due to the pressure of the numerous mucous cells, the base of the columnar cells becomes thinner and their nuclei lie in the middle or apical third (Figs. 21, 33).

The abundance of mucous cells is the more conspicuous feature of this intestinal region (Table 3, Fig. 33). The mucous cells are mainly of type II (Fig. 21, mc II), but some mucous cells of type I may also be present (Table 4).

Second Intestinal Region—The second intestinal region is characterized by the presence of type I intestinal secreting cells (Fig. 22, ic I). The columnar epithelium is usually lower than in the first intestinal region (Table 1). Ciliated and nonciliated columnar cells occur in the second intestinal region. Both cell types contain rare vacuoles with amorphous contents. The nucleus of the columnar cells is located in the basal or middle third (Fig. 22).

The number of mucous cells is relatively small in the second intestinal region (Table 3). Morphologically, the cells are similar to the type I mucous cells of the oesophagus and crop (Fig. 22, mc I).

The intestinal secreting cells of type I (Figs. 22, 34, ic I) have a claviform shape. The large, oval nucleous is rich in chromatin and has a conspicous nucleolus (Fig. 34, nu). The basal cytoplasm stains violet (MF). The numerous secretory granules are found in the supranuclear cytoplasm (Fig. 22, sgr). The granules stain red with MG and MF.

Third intestinal region—In the third intestinal region, type II intestinal secreting cells are found (Figs. 23, 35). Ciliated cells occur only

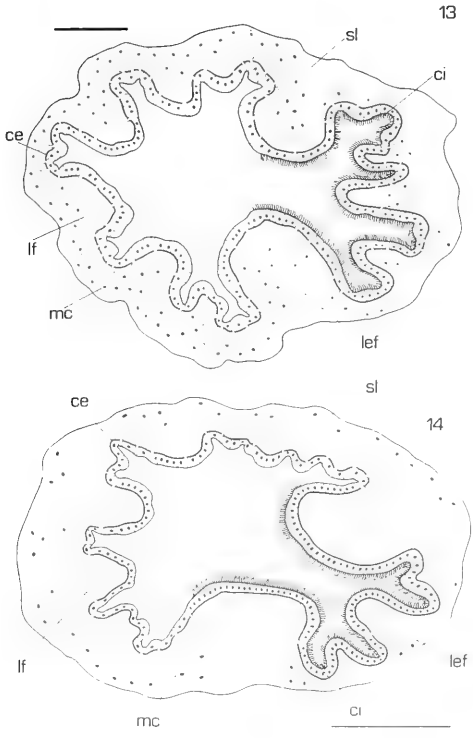


FIG. 13. Cross-section of the rectum of *Tandonia budapestensis*.

FIG. 14. Cross-section of the rectum of *Boettgerilla pallens*. Scale bar 0.1 mm.

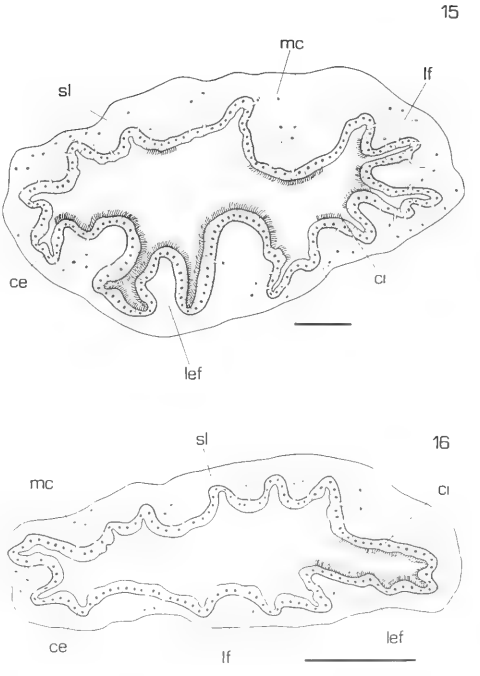


FIG. 15. Cross-section of the rectum of *Deroceras rodnae*.

FIG. 16. Cross-section of the rectum of *Deroceras laeve*. Scale bar 0.1 mm.

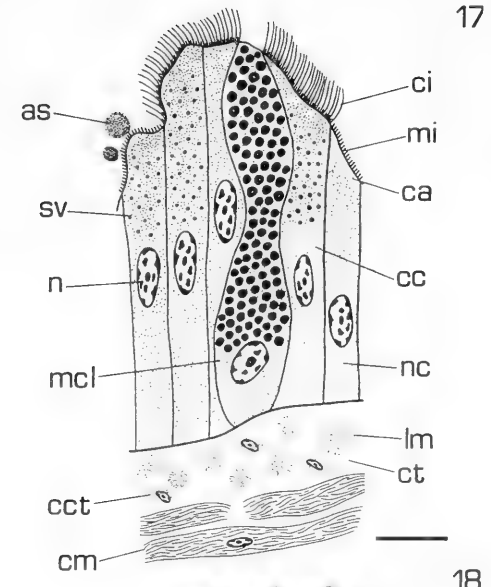
at the crests of the folds (Fig. 10). The remaining cells are nonciliated. The cytoplasm of the columnar cells has a small number of supranuclear vacuoles (Fig. 23, sv).

The intestinal secreting cells of type II (Figs. 23, 35, ic II) have granules, the contents of which were not stained by any of the methods used. The granules are larger than those of the intestinal secreting cells of type I. Other features of type II intestinal secreting cells are similar to those of type I.

The gland cells become more abundant than in the second intestinal region (Table 3). In *L. marginata* and *M. tenellus*, four types of gland cells (mucous cells of type III, IV and V, and cystic cells) occur in the third and fourth intestinal regions, in the intestinal caecum, and in the rectum. In *D. rodnae* and *D. reticulatum*, all four types are also present, but their occurrence is different (Table 4). Only three of these gland cell types can be seen in *D. laeve*, and only two of them in *B. pallens* and *T. budapestensis* (Table 4). These cell types were identified and described with the aid of historesin sections. With the exception of the cystic cells, the gland cells cannot be distinguished on paraffin sections, remaining unstained or staining light green with MG and light blue with AZ.

The mucous cells of type III (Figs. 23, 25, 36, 38, 42, 45, Table 2, mc III) have an acidophilic cytoplasm and granules staining light or dark blue (MF). The cells are usually subepithelial. Their cell body is located in the subepithelial connective tissue or external to the ring muscle layer (Figs. 25, 38).

The mucous cells of type IV (Figs. 25, 38, 39, mc IV) are also usually subepithelial. Their cell body has a smaller diameter than that of type III mucous cells (Table 2). The nucleus, however, is larger than that of type III mucous cells. The acidophilic cytoplasm has numerous small secretory granules that stain red (MF).



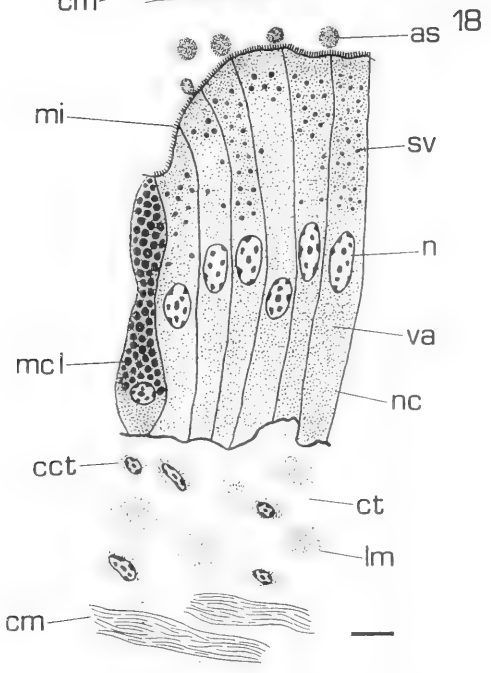


FIG. 17. Semi-schematic drawing of part of a transverse section of the oesophagus of *Lehmannia marginata*.

FIG. 18. Semi-schematic drawing of part of a transverse section of the crop of *Lehmannia marginata*. Scale bar 10 μm.

The mucous cells of type V (Figs. 23, 25, 47, Table 2, mc V) are always subepithelial and are easily distinguishable from the other mucous cells by their shape and by their contents. The cell body has a sacculiform shape, so that its transition to the cell neck is very gradual, and the cell body is small and located close to the epithelium. Most of the cell is filled with elongated secretory granules that stain blue with MF. The granules often coalescens, forming a secretion mass. The nucleus is peripheral and oval to elongated in shape and is surrounded by acidophilic cytoplasm.

The cystic cells (Figs. 23–25, 46, cy), like the mucous cells of type V, are always subepithelial, but have a very large sacculiform cell body (Table 2). The cystic cells are filled with an amorphous secretion that stains pink with MF and red with MG. Basophilic cytoplasm can be seen in a peripheral zone of the cell body. The nucleus is basally located.

Fourth Intestinal Region—The epithelium of the fourth intestinal region is squamous to cuboidal, and two to three times lower than in the third intestinal region (Table 1, Figs. 25, 37–39). The cells are nonciliated, except for the longitudinal ciliated area (Figs. 11, 25, 37). Both nonciliated and ciliated cells have microvilli. The cytoplasm of the cells of the fourth region seldom has supranuclear vacuoles (Fig. 25). The basal part of the cells is highly folded. In the fourth intestinal region of *B. pallens* and *T. budapestensis*, the ciliated area is absent.

The epithelium of the ciliated area is higher and has long cilia (Figs. 25, 37). Apical cells are more acidophilic than the other cells of the region.

The gland cells (Table 4) are usually more abundant than in the third intestinal region (Table 3). In the fourth intestinal region, the gland cells are always subepithelial (Fig. 25). The intestinal secreting cells are absent. The cell body of the mucous cells of type III and IV, as well as that of the cystic cells, is located external to the muscle layers.

Intestinal Caecum—The epithelium of the intestinal caecum is squamous and lower than that of the fourth intestinal region (Table 1, Figs. 24, 40, 41). The cytoplasm of the squamous cells is similar to that of the fourth intestinal region. The number of gland cells is small (Tables 3, 4).

Rectum—The epithelium of the rectum is cuboidal to columnar. The epithelial cells of the leader folds (Figs. 43, 44, 46) are higher and show features similar to those of the

TABLE 1. Epithelial height of the digestive tube of Limacoidea and Milacidae (μm).

	B. pallens	*L. marginata*	*D. laeve*	*D. reticulatum*	*D. rodnae*	*M. tenellus*	*T. budapestensis*
Oesophagus	49.7	47.0	31.5	40.3	46.5	51.3	29.0
Crop	79.8	65.0	55.0	63.0	58.5	72.3	37.8
Stomach	52.5	41.5	53.8	49.0	45.1	58.5	56.3
1. intestinal region	56.0	61.0	58.3	62.5	57.3	61.3	32.5
2. intestinal region	53.8	54.0	42.8	42.0	57.3	67.5	41.8
3. intestinal region	34.0	37.8	33.3	21.3	32.8	30.3	17.5
4. intestinal region	10.5	15.5	15.0	10.1	6.8	9.8	10.0
Caecum	—	13.8	—	6.3	6.0	—	—
Rectum	7.1	10.0	7.5	13.4	11.4	14.0	12.1

leader fold of the fourth intestinal region. The rectum of *L. marginata* and *M. tenellus*, without leader folds, has ciliated cells in the crests of the folds (Figs. 12, 42, 47). In the rectum of *B. pallens*, *T. budapestensis* and *D. laeve* (Figs. 13, 14, 16), ciliated cells occur only in the leader folds, and all other cells are nonciliated. In *D. rodnae* and *D. reticulatum*, ciliated cells occur in the leader folds, as well as in the crest of other folds (Fig. 15).

In the rectum, the gland cells (Table 2) are again fewer than in the fourth intestinal region (Table 3). The gland cells are still fewer in the leader folds (Figs. 13–16, 43, 44). The cell body is located in the subepithelial connective tissue or external to the muscle layers (Fig. 45).

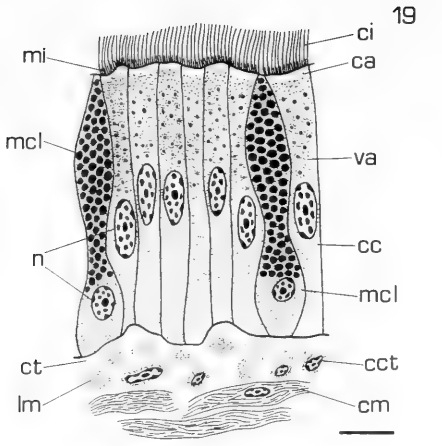
Subepithelial Layers: The epithelium of the alimentary canal is surrounded by a thin layer of connective tissue and two muscle layers, an inner longitudinal layer, and an outer circular layer. The longitudinal muscle layer is rather irregular; some longitudinal muscle bundles were observed within or externally to the circular layer.

The muscle layers are thicker in the oesophagus (Fig. 26), in the third intestinal region (Fig. 36) and in the rectum (Figs. 42, 44). The longitudinal layer is thicker in the oesophagus and in the third intestinal region, whereas in the rectum both muscle layers are well developed.

In the other organs and regions, the muscle layers are thin. In the crop (Fig. 27), in the stomach (Fig. 32), and in the first and second intestinal regions (Figs. 33, 34), the longitudinal layer is the less developed; in the fourth intestinal region (Figs. 37, 38), the circular layer is the least developed. In the intestinal caecum (Figs. 24, 40, 41), the muscle layers are poorly developed and not well demarcated.

DISCUSSION

The light microscope observations demonstrated that the alimentary canal of the Limacoidea and Milacidae is divisible into five morphologically distinct organs: oesophagus, crop, stomach, intestine and rectum. In the intestine of the species studied here, I distinguish four histologically different regions, whereas other pulmonates have only three intestinal regions (Carriker & Bilstad, 1946; Moussa et al., 1983; Roldan & Garcia-Corrales, 1988; Boer & Kits, 1990). Walker, in Runham (1975) subdivided the intestine of *Deroceras reticulatum* into only three intestinal regions. He termed our fourth intestinal region as rectum without mentioning the actual rectum, which is located in the pallial cavity. Another peculiar feature of the intestine of some Limacidae and Agriolimacidae is the presence of an intestinal caecum. This can be long, as in *Lehmannia marginata,* or short, as in *Deroceras reticulatum* and *D. rodnae.* The histological and ultrastructural features of the caecum imply the probably function of this organ. A simple columnar epithelium is present from the oesophagus to the third intestinal region and in the rectum. In the fourth intestinal region and in the intestinal caecum, however, the epithelium is simple squamous, with cells showing distinct ultrastructural features that are characteristic of water- and ion-transporting epithelia (Leal-Zanchet, in preparation b). This would indicate that water and ions are absorbed from the faecal pellets. This was also suggested by Boer & Kits (1990) for



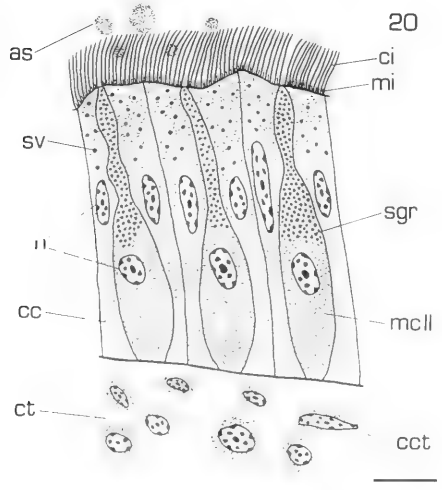
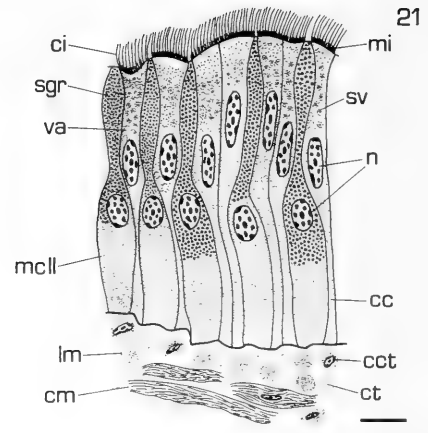


FIG. 19. Semi-schematic drawing of part of a transverse section of the stomach (out of the typhlosoles) of *Lehmannia marginata*.

FIG. 20. Semi-schematic drawing of part of a transverse section of the stomach of *Lehmannia marginata* showing the leader groove. Scale bar 10 μm



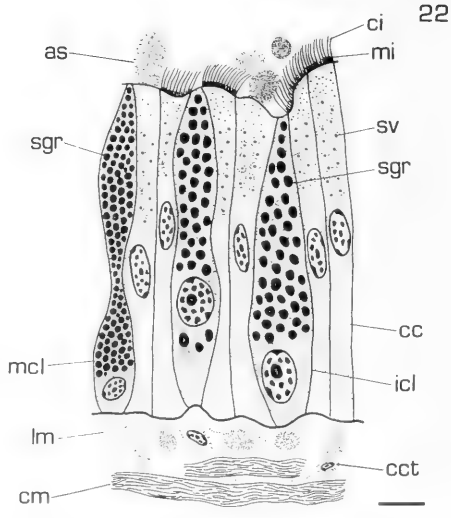


FIG. 21. Semi-schematic drawing of part of a transverse section of the first intestinal region of *Lehmannia marginata*.

FIG. 22. Semi-schematic drawing of part of a transverse section of the second intestinal region of *Lehmannia marginata*. Scale bar 10 μm.

Lymnaea stagnalis and is consistent with the findings of Deyrup-Olsen (1987), who verified that the distal part of the intestine of *Ariolimax columbianus* plays a significant role in osmoregulation.

Most of the epithelium of the alimentary canal shows ciliated supporting cells. The cilia play a role in the transport of the food bolus and the faecal pellets (Roldan & Garcia-Corrales, 1988; Boer & Kits, 1990). In the stomach and in the rectum, however, there are distinct folds with cilia that are longer and very numerous. In addition, ultrastructural data show that these cilia have very long roots and are interconnected by well-developed basal feet on the basal bodies (Leal-Zanchet, in preparation b). In the stomach, such folds are the typhlosoles and the transversal fold, and in the rectum they are termed leader folds. The cilia of the typhlosoles, the trans-

TABLE 2. Length and width of the cell body (CB), of the nucleus (N) and diameter of the secretory cell granules (SGR) of gland cells of the digestive tube of Limacoidea and Milacidae (μm).

		B. pallens	*L. marginata*	*D. laeve*	*D. reticulatum*	*D. rodnae*	*M. tenellus*	*T. budapestensis*
MC I	N	7.5 × 5.3	8.2 × 5.8	8.5 × 5.7	6.8 × 5.1	8.8 × 5.3	10.0 × 7.0	8.6 × 5.5
	SGR	2.0	2.3	2.0	1.8	1.7	2.1	1.7
MC II	N	8.0 × 6.4	7.5 × 5.0	9.5 × 5.0	8.0 × 5.5	11.0 × 6.5	8.8 × 7.3	9.5 × 5.5
	SGR	0.5	0.5	0.5	0.5	0.5	0.5/2.0	0.5
MC III	CB	20.9 × 15.9	19.3 × 15.1	20.6 × 13.2	18.0 × 10.9	21.0 × 15.1	20.3 × 15.8	26.5 × 15.7
	N	7.1 × 5.9	10.4 × 6.8	7.3 × 4.9	8.5 × 4.6	8.0 × 5.0	8.4 × 6.3	8.4 × 6.4
	SGR	1.5	1.4	1.4	1.7	2.1	2.3	1.9
MC IV	CB	20.4 × 13.5	16.5 × 12.0	19.8 × 12.8	16.9 × 10.4	20.2 × 14.9	17.6 × 12.0	19.2 × 14.7
	N	9.3 × 7.6	9.4 × 6.4	10.1 × 6.8	7.6 × 5.6	7.0 × 6.0	8.9 × 7.3	9.4 × 7.6
	SGR	1.2	0.9	0.9	0.9	1.2	0.9	1.2
MC V	CB	—	11.4 × 11.9	—	11.8 × 9.5	14.0 × 6.5	14.0 × 6.5	—
	N	—	6.8 × 3.7	—	5.0 × 3.2	6.5 × 3.8	6.5 × 3.8	—
	SGR	—	—	—	—	—	—	—
CY	CB	—	27.2 × 17.5	—	25.7 × 18.8	23.0 × 17.5	17.7 × 13.5	—
	N	—	12.4 × 6.8	—	14.3 × 6.7	10.2 × 5.3	11.3 × 4.8	—
	SGR	—	—	—	—	—	—	—

TABLE 3. Frequency of mucous cells in the digestive tube of Limacoidea and Milacidae.

	B. pallens	*L. marginata*	*D. laeve*	*D. reticulatum*	*D. rodnae*	*M. tenellus*	*T. budapestensis*
Oesophagus	15%	9%	5%	3%	3%	4%	10%
Crop	8%	9%	5%	9%	10%	8%	8%
Stomach	18%	10%	11%	13%	13%	10%	18%
1. intestinal region	54%	41%	35%	44%	46%	35%	55%
2. intestinal region	15%	8%	15%	4%	6%	13%	4%
3. intestinal region	22%	18%	19%	16%	21%	11%	27%
4. intestinal region	34%	17%	26%	16%	21%	18%	36%
Caecum	—	4%	—	8%	7%	—	—
Rectum	27%	21%	20%	25%	22%	19%	44%

versal fold and the rectal leader folds have a distinct function. This would be consistent with the studies of Walker (1972) on the physiology of the stomach of *Deroceras reticulatum;* he showed that the typhlosoles, the transversal fold, and the leader groove play an important role in the transport of fine material. No data were found about the physiology of the rectum. The rectal leader folds seem to be better suited to aid faeces transport than the usual folds. In the rectum of other pulmonates, the presence of leader folds has not yet been described.

In some regions of the alimentary tract of the Limacoidea and Milacidae, the epithelium is unciliated, namely in the crop, in the fourth intestinal region, and in the intestinal caecum. The absence of cilia in the crop was also observed in other Stylommatophora (Ghose, 1963; Rigby, 1963, 1965; Roldan & Garcia-Corrales, 1988). In the fourth intestinal region of *Boettgerilla* and *Tandonia,* the epithelium is completely unciliated, but in *Malacolimax, Lehmannia* and *Deroceras* a reduced ciliated area is present. According to Runham (1975), the variation in the presence or absence of cilia in the organs of the alimentary canal may reflect the relative importance of cilia and muscles for transport and mixing of food material. In the crop, the muscle layers alone are responsible for mixing food material with the crop juice (Runham, 1975) and also for transport of the food material towards the stomach. In the fourth intestinal region, the ciliated area present in *Malacolimax, Lehmannia* and *Deroceras* seems to aid faeces transport towards the rectum together with the muscle layers.

TABLE 4. Distribution of gland cells in the intestine, caecum and rectum of Limacoidea and Milacidae.

	B. pallens	*L. marginata*	*D. laeve*	*D. reticulatum*	*D. rodnae*	*M. tenellus*	*T. budapestensis*
1. intestinal region	MC II	MC II MC I	MC II MC I	MC II MC I	MC II MC I	MC II MC I	MC II
2. intestinal region	IC I MC I	IC I MC I	IC I MC I	IC I MC I	IC I MC I	IC I MC I	IC I MC I
3. intestinal region	IC II MC III MC IV	IC II MC III MC IV MC V CY	IC II MC III MC IV	IC II MC III MC IV	IC II MC III MC IV	IC II MC III MC IV MC V CY	IC II MC III MC IV
4. intestinal region	MC III MC IV	MC III MC IV MC V CY	MC III MC IV	MC III MC IV MC V CY	MC III MC IV CY	MC III MC IV MC V CY	MC III MC IV
Caecum	—	MC III MC IV MC V CY	—	MC III MC IV MC V CY	MC III MC IV CY	—	—
Rectum	MC III MC IV	MC III MC IV MC V CY	MC III MC IV MC V	MC III MC IV MC V CY	MC III MC IV MC V CY	MC III MC IV MC V CY	MC III MC IV

The presence of five mucous cell types is now reported for the alimentary canal of limacids and agriolimacids. Type I mucous cells are present in the proximal regions of the alimentary canal, such as the eosophagus, the crop, in parts of the stomach, and also in the second intestinal region. Type II mucous cells are found in the stomach and in the first intestinal region. The mucous cells of type III, IV and V occur in distal regions of the canal, such as the third and fourth intestinal regions, the intestinal caecum, and the rectum. The mucous cells of type I and II are intraepithelial, whereas the mucous cells of type III, IV and V are subepithelial. The mucous cells of type V are absent in *Boettgerilla pallens* and *Tandonia budapestensis*.

The functional role of the mucus would be the lubrification of the lumen, helping in the transport of food and faeces, the clumping of food particles for the formation of the food bolus, the formation of the faecal string, and the compaction of the faecas (Carriker & Bilstad, 1946; Pereira & Breckenridge, 1981). The mucous cells of type I of the Limacoidea and Milacidae, the occurrence of which is limited to the proximal parts of the tract, must be related to the formation of the food bolus. The formation of the faecal pellets that takes place in the distal part of the stomach (Walker, 1972) should involve the mucous cells of type II. The mucous cells of the distal regions of the alimentary canal—types III, IV and V—should be concerned with the compaction of the faeces.

Gland cells that are termed intestinal secreting cells have been described for the alimentary canal of various pulmonates (Haffner, 1924; Baecker, 1932; Walker, in Runham, 1975; Roldan & Garcia-Corrales, 1988; Leal-Zanchet et al., 1990; Franchini & Ottaviani, 1992). The intestinal secreting cells described by these authors are similar to the type I intestinal secreting cells of the limacoids. We observed also another type of gland cells (i.e., intestinal secreting cells of type II) that are clearly distinguishable from intestinal secreting cells of type I. The secreting cells of type I and II occur in all the species studied in the present investigation. The occurrence of the intestinal secreting cells of type I and II in the second and third intestinal regions, respectively, and their positive reaction to protein (Leal-Zanchet, in preparation a), indicate that the secretion of these cells is probably of an enzymatic nature and may play a role in digestion.

The occurrence of cystic cells in the alimentary canal of gastropods has not yet been described, but similar cells are known in the salivary glands of pulmonates (Blain, 1957; Bani, 1964, Boer et al., 1967). In Limacidae

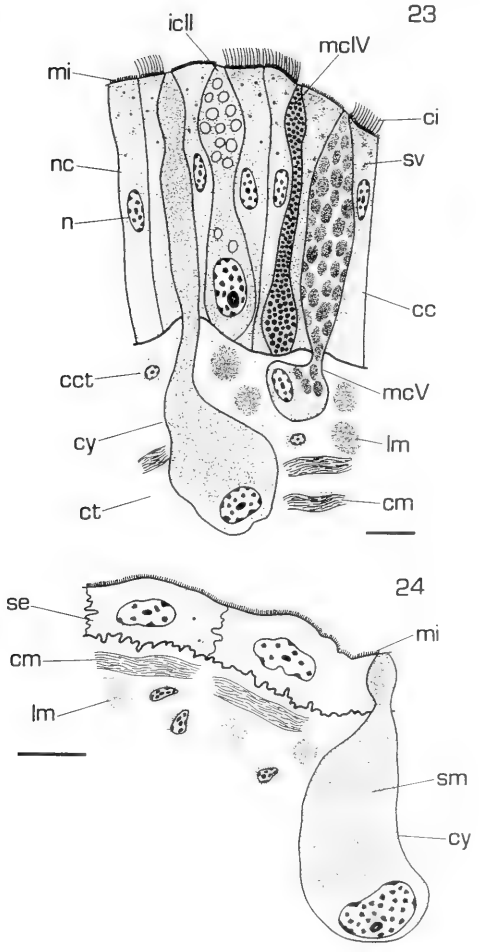


FIG. 23. Semi-schematic drawing of part of a transverse section of the third intestinal region of *Lehmannia marginata.*

FIG. 24. Semi-schematic drawing of part of a transverse section of the intestinal caecum of *Lehmannia marginata.* Scale bar 10 μm.

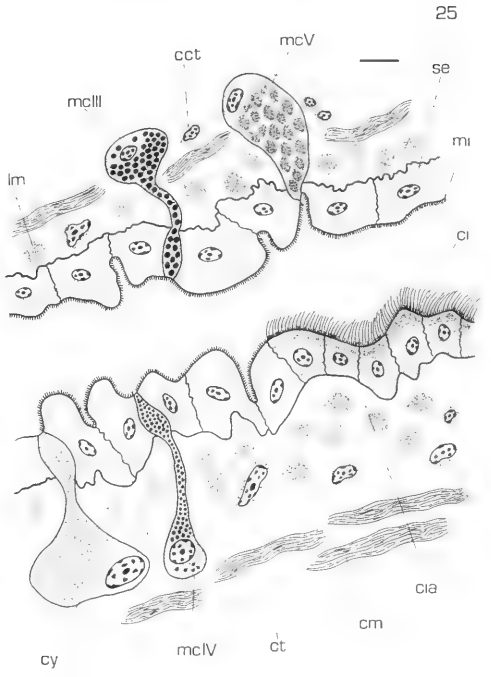


FIG. 25. Semi-schematic drawing of part of a transverse section of the fourth intestinal region of *Lehmannia marginata.* Scale bar 10 μm.

and Agriolimacidae, the secretion of the cystic cells is positive to histochemical reactions for protein (Leal-Zanchet, in preparation a), but the exact role of the cystic cells is still unclear. The cystic cells occur in the distal regions of the alimentary canal of the limacids and agriolimacids, except in *Deroceras laeve,* but are absent in *Boettgerilla* and *Tandonia.*

The alimentary diet of the species studied in the present investigation differs widely. *Deroceras* and *Tandonia* are herbivorous. *Lehmannia* lives on a specialized diet of lichens, whereas *Malacolimax* feed preferently on fungus (Wiktor, 1973). Little is known about the diet of *Boettgerilla pallens,* but some data obtained in laboratory experiments suggest that this species is carnivorous (Leal-Zanchet, in press). Although the animals have a distinct diet, only few distinguishing anatomical and histological features were observed. *Boettgerilla* presents a mere shortening of the intestinal regions, a characteristic observed in others carnivorous slugs, such as *Daudebardia* (Zonitidae) and *Diplompharus* (Rhytididae) (Wiktor, 1983; Tillier, 1989). If *Boettgerilla* is carnivorous, the occurrence of a powerful protease in its digestive system would be expected. At present, having completed anatomical, histological, histochemical (Leal-Zanchet, in preparation a) and ultrastructural (Leal-Zanchet, in preparation b) studies, we still cannot relate the different diets of the Limacoidea and Milacidae to their distinct histological features. An investigation of the enzymes of Limacoidea and Milacidae would clarify many questions.

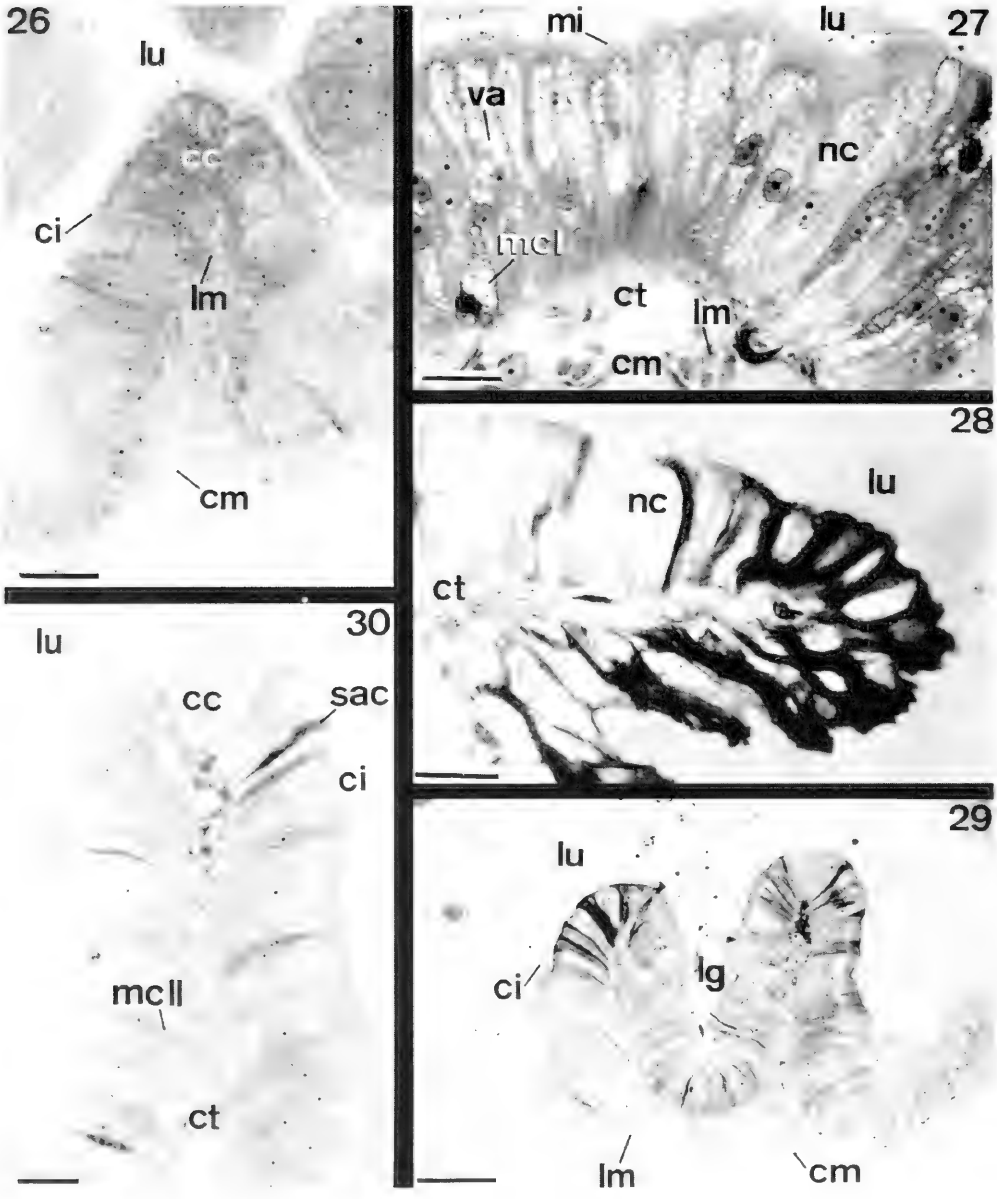


FIG. 26. Cross-section of the oesophagus of *Malacolimax tenellus* showing a fold. Scale bar 50 μm.

FIG. 27. Cross-section of the crop of *Malacolimax tenellus*. Note the abundant vacuoles with amorphous contents. Scale bar 25 μm.

FIG. 28. Cross-section of the crop of *Tandonia budapestensis* showing a fold. Scale bar 25 μm.

FIG. 29. Cross-section of the distal part of the stomach of *Lehmannia marginata* demonstrating the typhlosoles and the leader groove. Scale bar 80 μm.

FIG. 30. Cross-section of the stomach of *Lehmannia marginata* demonstrating the lining epithelium of a typhlosole. Note the strong acidophilic columnar cells and the mucous cells of type II. Scale bar 25 μm.

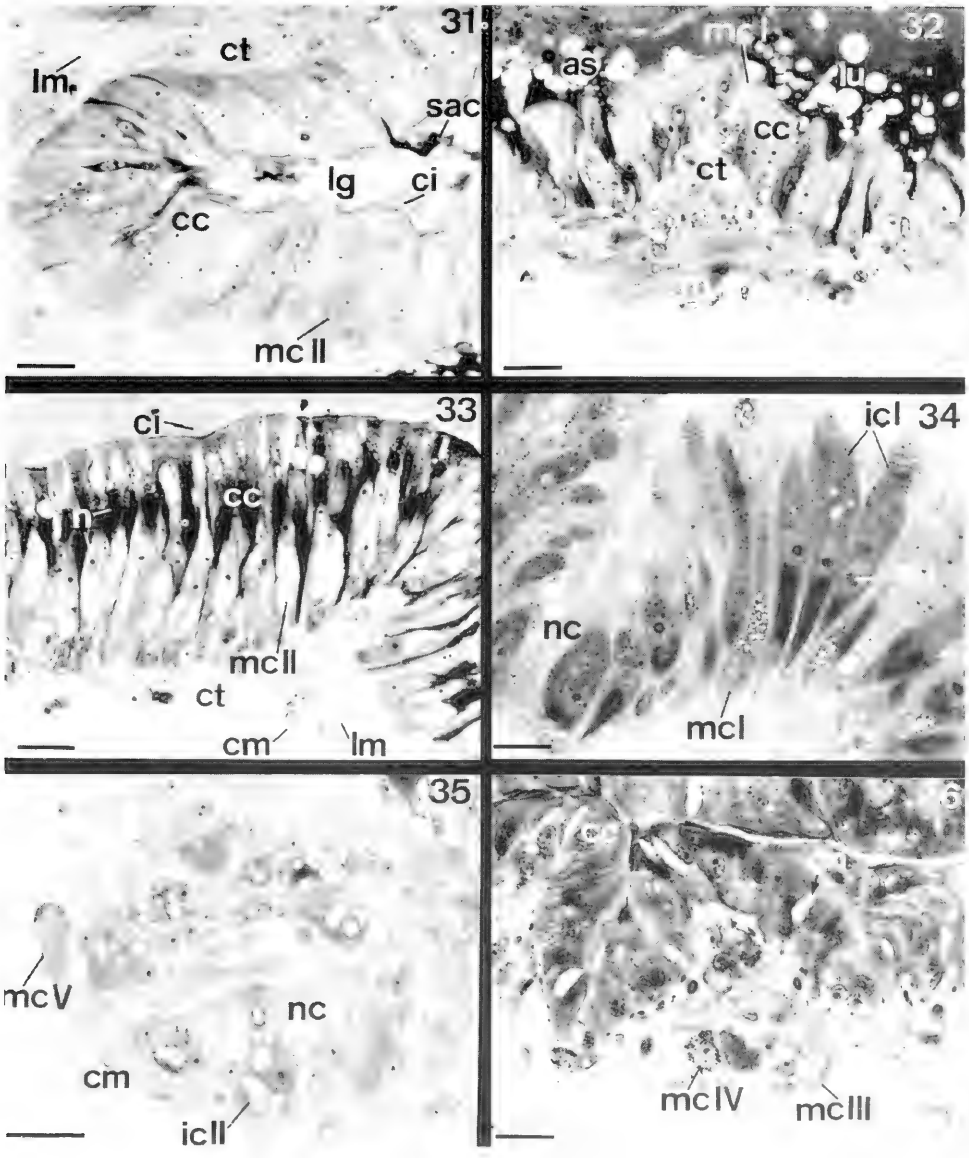


FIG. 31. Cross-section of the stomach of *Lehmannia marginata* showing the lining epithelium of the leader groove. The holocrine secretion of a strong acidophilic columnar cell can be seen. Scale bar 25 μm.

FIG. 32. Cross-section of the stomach wall (out of the typhlosoles) of *Lehmannia marginata*. Note the apocrine secretion of the columnar cells. Scale bar 25 μm.

FIG. 33. Cross-section of the first intestinal region of *Lehmannia marginata* showing the numerous mucous cells of type II. Scale bar 25 μm.

FIG. 34. Cross-section of the second intestinal region of *Malacolimax tenellus* demonstrating the intestinal secreting cells of type I. Scale bar 25 μm.

FIG. 35. Cross-section of the third intestinal region of *Malacolimax tenellus*. The cell body of a mucous cell of type V can be seen. Scale bar 25 μm.

FIG. 36. Cross-section of the third intestinal region of *Boettgerilla pallens*. Scale bar 25 μm.

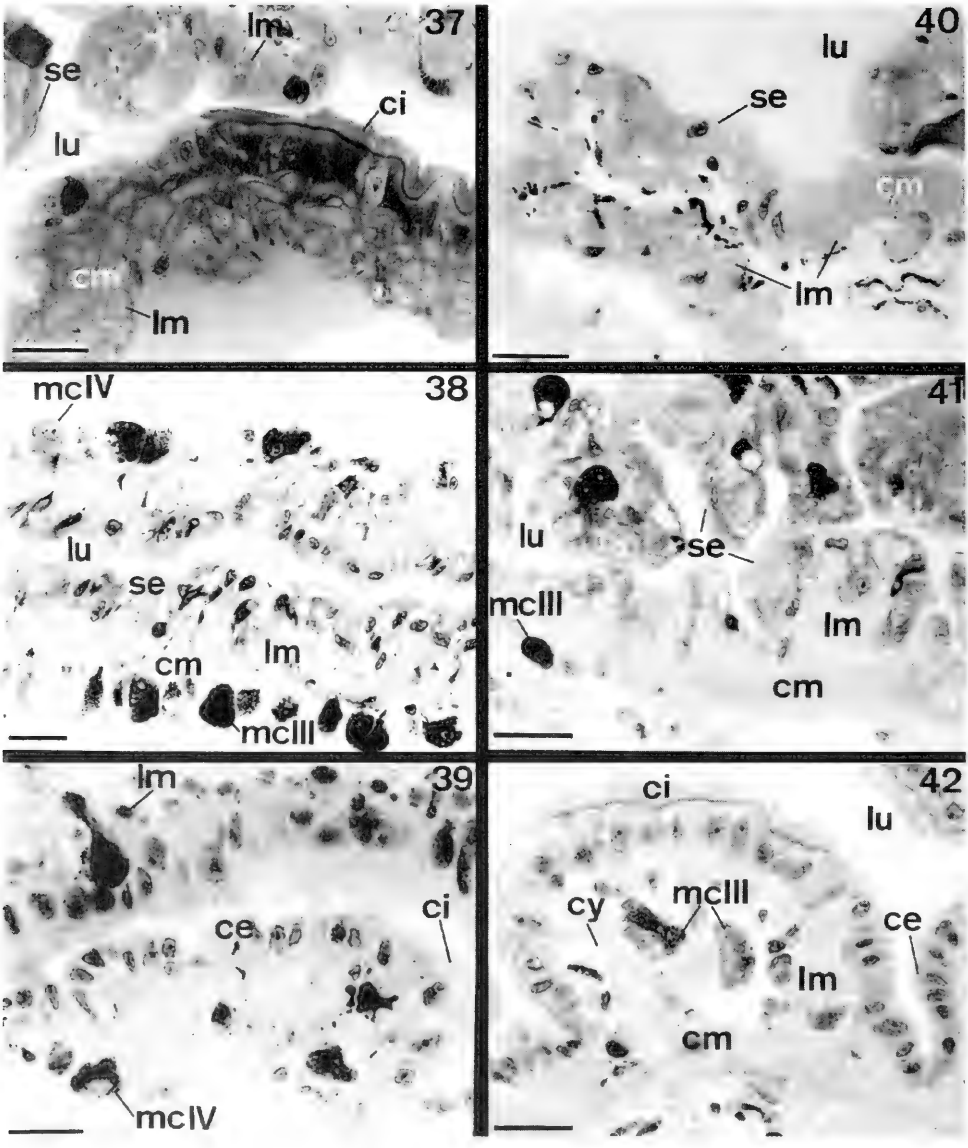


FIG. 37. Cross-section of the fourth intestinal region of *Lehmannia marginata*. Exceptionally, the circular layer lays directly below the epithelium. Note the ciliated area. Scale bar 25 μm.

FIG. 38. Cross-section of the fourth intestinal region of *Boettgerilla pallens*. A ciliated area is absent. Scale bar 25 μm.

FIG. 39. Cross-section of the fourth intestinal region of *Deroceras laeve*. A ciliated area can be seen. Scale bar 25 μm.

FIG. 40. Cross-section of the intestinal caecum of *Lehmannia marginata*. Scale bar 25 μm.

FIG. 41. Cross-section of the intestinal caecum of *Deroceras reticulatum*. Scale bar 25 μm.

FIG. 42. Cross-section of the rectum of *Lehmannia marginata* showing a fold. Scale bar 25 μm.

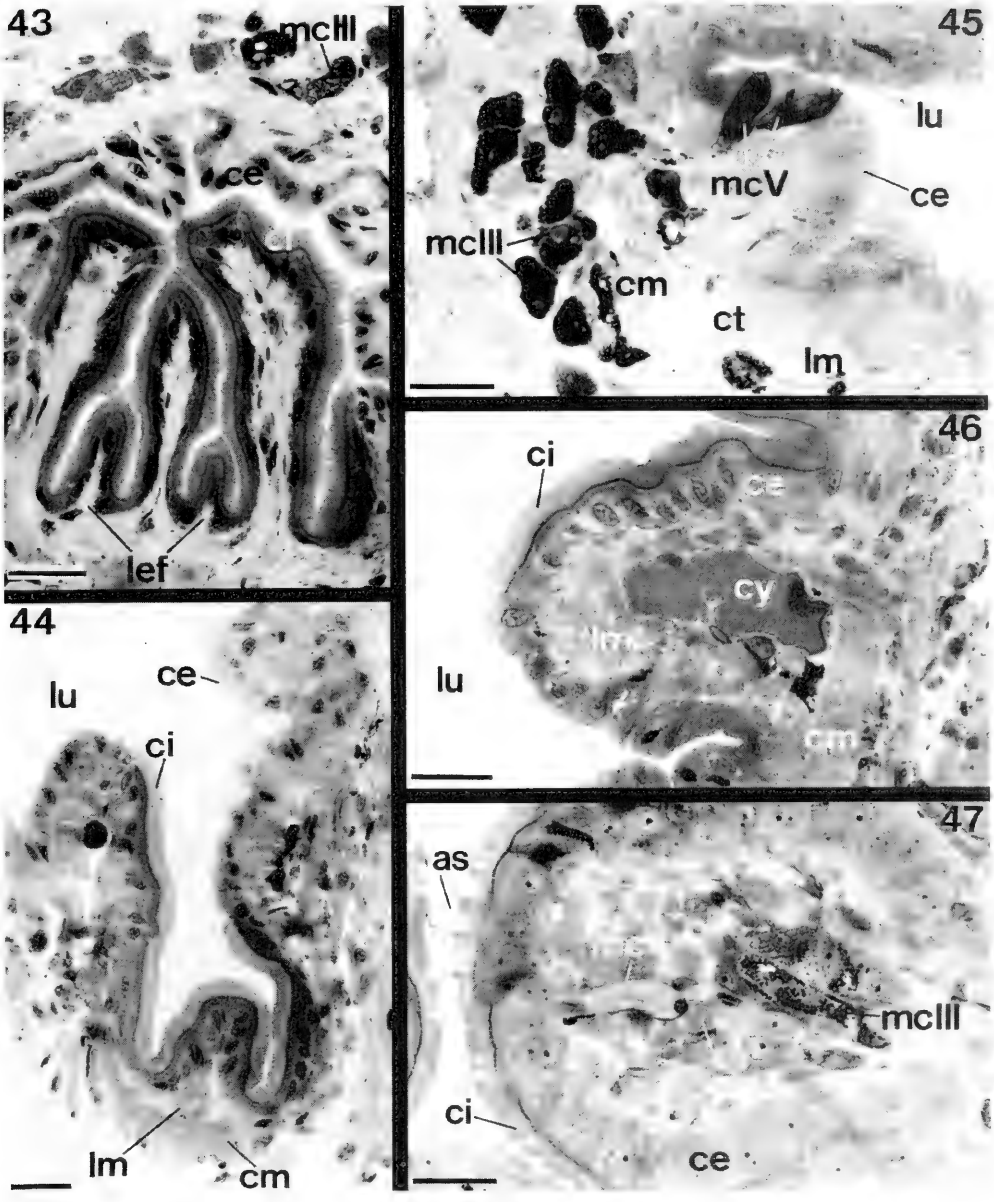


FIG. 43. Cross-section of the rectum of *Boettgerilla pallens* showing the well developed leader folds. Cilia are absent outside the leader folds. Scale bar 25 μm.

FIG. 44. Cross-section of the rectum of *Deroceras laeve*. Small leader folds and well-developed muscle layers can be seen. Scale bar 25 μm.

FIG. 45. Cross-section of the rectum of *Deroceras reticulatum.* Note the numerous mucous cells of type III and two cell necks of mucous cells of type V. Scale bar 25 μm.[

FIG. 46. Cross-section of the rectum of *Deroceras reticulatum* demonstrating the large cell body of a cystic cell. Scale bar 25 μm.

FIG. 47. Cross-section of the rectum of *Malacolimax tenellus.* Observe the small cell body of a mucous cells of type V. Scale bar 25 μm.

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RESUMO

Estudo comparativo da anatomia e histologia do tubo digestivo dos Limacoidea e Milacidae (Pulmonata: Stylommatophora)

Descreve-se comparativamente a anatomia e histologia do tubo digestivo de *Deroceras laeve, D. reticulatum, D. rodnae, Lehmannia marginata, Malacolimax tennelus, Boettgerilla pallens* e *Tandonia budapestensis.* O tubo digestivo destes animais é composto pelo esôfago, papo, estômago, intestino e reto. Um ceco intestinal está presente em *D. reticulatum, D. rodnae* e *L. marginata.* O intestino pode ser subdividido em quatro regiões histologicamente distintas. Do esôfago à terceira região intestinal, e no reto, o tubo digestivo é revestido em sua maior parte por um epitélio cilíndrico a cúbico simples. Na quarta região intestinal e no ceco o epitélio apresenta-se pavimentoso a cúbico simples. As células epiteliais de suporte podem ser ciliadas ou não, mas apresentam sempre microvilos. Distinguem-se também oito tipos celulares secretores: células mucosas do tipo I, células mucosas do tipo II, células mucosas do tipo III, células mucosas do tipo IV, células mucosas do tipo V, células císticas, células secretoras intestinais do tipo I e células secretoras intestinais do tipo II. Subepitelialmente, encontram-se uma camada de tecido conjuntivo frouxo e duas camadas musculares, uma longitudinal interna e outra circular externa.

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MORPHOLOGY OF THE WESTERN ATLANTIC HALIOTIDAE (GASTROPODA, VETIGASTROPODA) WITH DESCRIPTION OF A NEW SPECIES FROM BRAZIL

Luiz Ricardo L. Simone

Seção de Moluscos, Museu de Zoologia da Universidade de São Paulo, Caixa Postal 7172, CEP 01064-970, São Paulo, SP, Brazil

ABSTRACT

Haliotis aurantium, new species, is described from the southeastern coast of Brazil and compared morphologically with *Haliotis pourtalesii,* which inhabits the Gulf of Mexico and the Caribbean Sea. These species differ mainly in characters of the head, epipodium, metapodium and digestive system. The species are also compared anatomically with other species of *Haliotis* based on published descriptions and on comparative dissections of *H. lamellosa* and *H. tuberculata.*

INTRODUCTION

The finding of a haliotid in the western Atlantic excited considerable interest (Henderson, 1915; Harry, 1966; Klappenbach, 1968; Sarasua, 1968; Merrill & Petit, 1969; Guince, 1969; Nijssen-Meyer, 1969; Silva & Guerra, 1981; Martinez & Ruiz, 1994), although until now few specimens with soft parts have been found (Titgen & Bright, 1985).

All western Atlantic haliotid specimens have been identified as *Haliotis pourtalesii* Dall, 1881, with apparently in two disjunct populations, one from North Carolina to Cuba and other in Brazil (Rios, 1985; Titgen & Bright, 1985). These identifications were based only on shell characters, because no anatomical information has appeared to date. Photos and a brief description of the head-foot and color pattern of a specimen from the Gulf of Mexico were given by Titgen & Bright (1985).

Specimens from the two regions were carefully compared in their morphology, showing that, despite the similarity in their shells, the specimens from these regions are sufficiently distinct to be regarded as separated species.

Few papers provide anatomical information on haliotids (e.g., Freure, 1905; Crofts, 1929, 1937, 1955; Campbell, 1965; Russell & Evans, 1989). The present paper provides an anatomical description, as a basis for comparison between the two western Atlantic species, as well as for use in a future systematic rearrangement of the family, which has about 70 species in the single genus *Haliotis* Linné, 1758 (Abbott & Dance, 1983).

The complex history of the discovery of *H. pourtalesii* in waters near Florida and the loss of its type specimen have been discussed elsewhere (e.g., Foster, 1946; Titgen & Bright, 1985). Also there are misidentifications based on young specimens of other species (Abbott, 1974) and based on similar species occurring in Pacific waters (*H. dalli* Henderson, 1915; *H. roberti* McLean, 1969), demanding care in literature analysis.

MATERIAL AND METHODS

The specimens from Brazil are in the collection of the Museu de Zoologia da Universidade de São Paulo (MZSP), some of them collected by Instituto Oceanográfico da Universidade de São Paulo (IOUSP) in the project "Monitoramento Ambiental Oceânico da Bacia de Campos." The northern specimens are in the collection of the National Museum of Natural History (USNM) and Marine Invertebrate Museum, Rosenstiel School of Marine and Atmospheric Science, University of Miami (UMML). Those with soft parts are preserved in 70% ethanol.

Two Brazilian and five northern specimens were available with soft parts for dissections, which were made using standard techniques. Some organs, such as the buccal mass and pallial organs, were dehydrated in ethanol series, stained with carmine, fixed and cleared in creosote. All drawings were made with the aid of a camera lucida. Shells, radulae and jaws were also examined using SEM in the Laboratório de Microscopia Electrônica do Insti-

tuto de Biociências da Universidade de São Paulo. The shells were not coated with gold. Odontophoral muscles were examined by direct dissection, although the jugal muscles were not seen in detail. The nomenclature of buccal musculature follows Fretter & Graham (1962).

Anatomical comparison with other Haliotidae is based on the literature (Fleure, 1905; Crofts, 1929, 1937, 1955; Fretter & Graham, 1962; Campbell, 1965, digestive system; Russell & Evans, 1989, circulatory system) and on comparative examination of two lots of the MZSP collection: MZSP 13340, *Haliotis tuberculata* Linné, 1758, 5 specimens in 70% ETOH from Trieste, Italy; MZSP 28202, *Haliotis lamellosa* Lamarck, 1822, 1 specimen in 70% ETOH from Trieste, Italy.

In the figures the following abbreviations are used: ac: anterior cartilages; af: accessory oesophageal fold; al: aperture of left oesophageal pouch; an: anus; ar: aperture of right oesophageal pouch; cm: main (right) columellar muscle; da: direct anterior radular tensors; dg: digestive gland; dr: direct radular tensor muscle; ef: efferent gill vessel; ep: epipodium; ff: dorsal epipodial flap; gc: gastric caecum; go: gonad; hz: horizontal muscle; if: intermediary epipodial flap; im: intertentacular membrane; in: intestine; ja: jaws; la: left auricle; lc: left columellar muscle; lg: left gill; lh: left hypobranchial gland; lk: left kidney; lm: lateral protractor muscle; lp: left oesophageal pouch; ma: main epipodial tentacle; mb: mantle border; mt: metapodium; nr: nerve ring; oa: outer approximator muscle of cartilages; od: odontophore; oe: oesophagus; om: ommathophore; os: osphradium; pc: posterior cartilage; pr: pigmented region of dorsal epipodial flap; pv: posterior ventral radular tensor muscle; ra: right auricle; rd: radula; rg: right gill; rh: right hypobranchial gland; rk: right kidney; rp: right oesophageal pouch; rs: radular sac; rt: rectum; sa: sorting area; sf: pigmented multipapillate tentacles surrounding ma; sl: pallial slit; sn: snout; sr: subradular membrane; st: stomach; tc: metapodial tentacle covered with long cilia; te: cephalic tentacle; tm: metapodial tentacle; ts: slit pallial tentacle; ty: gastric typhlosole; vp: ventral buccal protractor muscle; ve: ventricle; vf: ventral epipodial flap.

Abbreviations of institutions: MNRJ: Museu Nacional do Rio de Janeiro; MORG: Museu Oceanográfico da Fundação Universidade de Rio Grande; MZSP: Museu de Zoologia da Universidade de São Paulo; UMML: Marine Invertebrate Museum, Rosenstiel School of Marine and Atmospheric Science, University of Miami; USNM: National Museum of Natural History, Smithsonian Institution.

SYSTEMATICS

Haliotis aurantium, new species (Figs. 3–9, 11–13, 18–35)

Haliotis pourtalesii: Klappenbach, 1968: 1–2; Rios, 1970: 16, pl. 1; Silva & Guerra, 1971: 49–50, figs. 1–4; Rios, 1975: 11, pl. 1, fig. 4; Rios, 1985: 10, pl. 5, fig. 35; Rios, 1994: 22, pl. 5, fig. 39 (*non* Dall, 1881).

Types: Holotype, MZSP 28201, from type locality; paratypes: MZSP 18482, 1 shell, off Ubatuba, São Paulo, 24°07′S 44°06′W, 150 m depth; MZSP 19569, 2 shells, 22°27′6″S 40°30′W, off Cabo de São Tomé, Rio de Janeiro, Brazil, 95 m depth (11/ii/1969); MZSP 28391, 1 specimen, 21°05′S 41°19′W, east of Ponta do Ubú, Espirito Santo, Brazil, 48 m depth (E. C. Oliveira Fo. col., 1986).

Type Locality: Brazil, Rio de Janeiro, off Campos Bay (sta. 21), 22°06′06″S 40°08′38″W, 95 m depth (R. V. Astrogaroupa, 22/vii/1991).

Diagnosis

Minute southwest Atlantic species with unpigmented head-foot and mantle; two tentacles in mantle slit; epipodial tentacles randomly arranged; pair of large epipodial tentacles posteriorly; pair of metapodial tentacles sometimes present; lobed snout border; left pouch of buccal mass covering ventral surface of odontophore; several pairs of lateral radular protractor muscle.

Description

Shell (Figs. 3–7). Auriform, fairly thin, subelliptical, up to 15 mm in length, few more than three whorls (Fig. 4). Color of exposed areas from homogeneous vivid reddish orange in living specimens to pale yellow in eroded specimens. Protoconch (Fig. 5) of two whorls, low, sculptured by several minute, uniform, spiral threads. Spire small, low, submarginal, situated on posterior fourth of shell (Figs. 4, 7). Aperture subelliptical, nacreous. Base of shell concave with some lateral torsion. Columella with a sulcus inside raised parietal margin of aperture (Fig. 6). Three to four oval

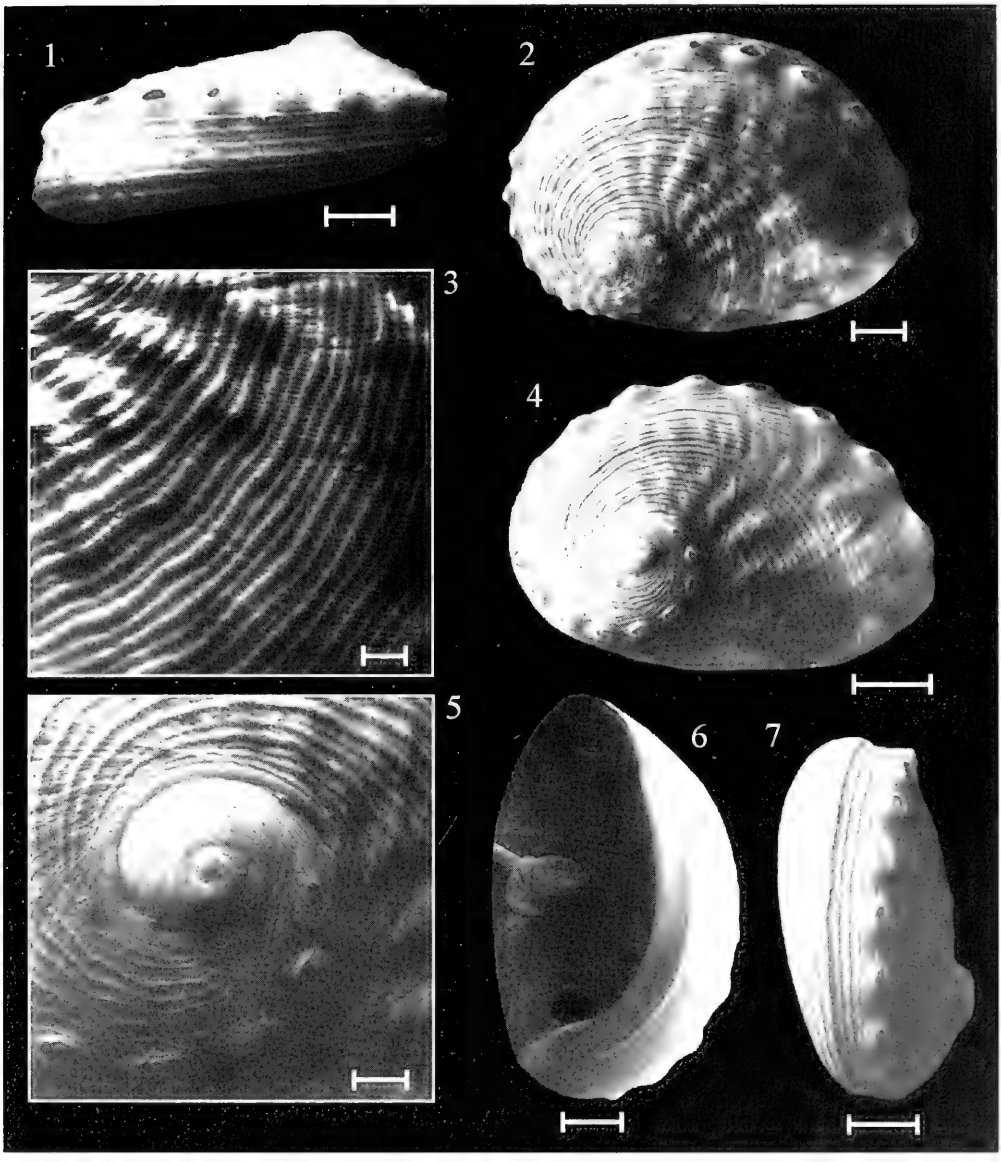


FIG. 1–7; shells in SEM. (1) and (2) left and dorsal view of *Haliotis pourtalesii* USNM 833627, scales = 2 mm; (3) detail of middle-outer region of body whorl of *Haliotis aurantium*, MZSP 18482, paratype, scale = 0.5 mm; (4) to (7) *Haliotis aurantium* holotype: (4) dorsal view, scale = 2 mm; (5) detail of protoconch, scale = 0.5 mm; (6) ventral view, scale = 2 mm; (7) left view, scale = 2 mm.

shell pores (tremata) open, preceded by several closed, all of them oval, with elevated margins (Figs. 4, 6, 7). Spiral sculpture of sharp, rather widely shaped cords, between which finer threads are occasionally intercalated (Fig. 3). About 30 cords and threads in area between suture and outer margin of body whorl. Lateral portion of body whorl with three cords followed by strong, angular, peripheral ridge or carina (Fig. 7). Immediately below this, three or four more cords present (Figs. 6, 7). Axial sculpture consisting of radiating lamellae, which roughly correspond to pores in their position, considerably variable,

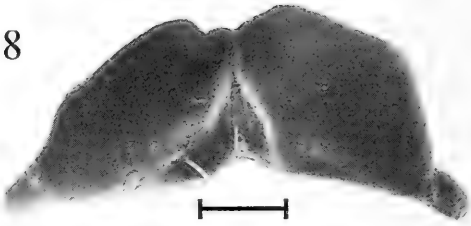
sometimes missing. Minute uniform axial cords between spiral cords occasionally present. Surface with very fine growth lines. No periostracum apparent.

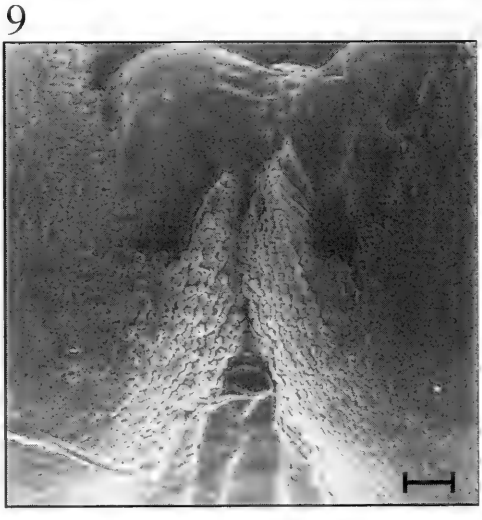
Head-Foot. Head somewhat protruding (Fig. 21). Tentacles stubby, short, broad, covered with long cilia, pigmented by regular pale brown, successive transverse bands (Fig. 21). All other structures without pigment. Ommatophore well developed, in outer basal region of tentacles (Figs. 18, 19, 21), with dark, vesicular, opened eyes. Intertentacular membrane a semi-transparent, thin flap (Figs. 21, 26, 27) between the two cephalic tentacles, covering anterior region of snout and inner region of tentacles (Fig. 21). Snout well developed (Figs. 21, 25), cylindrical, broad, with irregular ventral margin. Foot large, about same size as shell aperture (Fig. 19), without pigment. Epipodium with many lobed tentacles (Figs. 19, 22, 23), without pigment, uniform in size, some of them covered with long cilia, apparently without special organization (Fig. 22); in posterior extremity of epipodium two epipodial tentacles larger and longer (Fig. 23), and a median area without tentacles (Fig. 23). In the holotype, a pair of long metapodial tentacles present on posterior border; dissected paratype without this structure. Main (right) columellar muscle very large, circular in section (Fig. 18). Secondary (left) columellar muscle very small (Fig. 25: lc).

Mantle border. Trifolded and simple, without pigment. Slit deep (Figs. 18, 20), with two tentacles covered with long cilia, one on the left-anterior border and other on the right-posterior border of slit (Figs. 18, 20).

Pallial cavity. Short, about half of body whorl (Fig. 20). Gills short, bipectinate, right gill shorter than left (Fig. 20). Afferent gill vessel in base of gill's insertion. Efferent vessel between two flaps of each gill leaflet, inserting in gill sub-terminally, anterior to posterior extremity of gill (Fig. 20). Hypobranchial glands present, left larger, with several transverse, uniform furrows; right much smaller, with three oblique furrows. Both hypobranchial glands situated at left of slit (Figs. 18, 20, 24). Rectum between both hypobranchial glands, slightly free in posterior half of pallial cavity; anus papillated near posterior extremity of slit (Figs. 20, 24).

Circulatory and excretory systems. Kidneys and pericardium situated ventrally, in mid-left side of animal just behind pallial cavity (Fig. 24). Left kidney short, broad, with a short





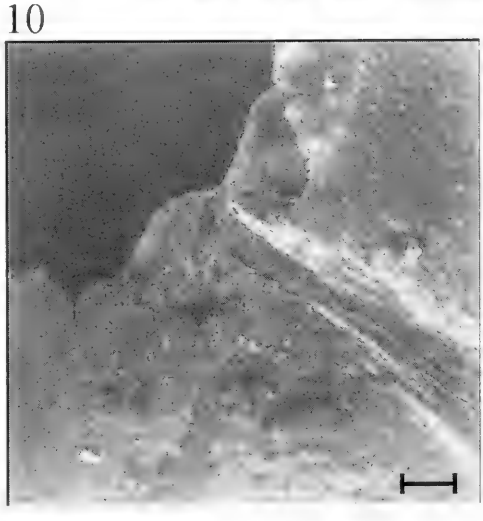
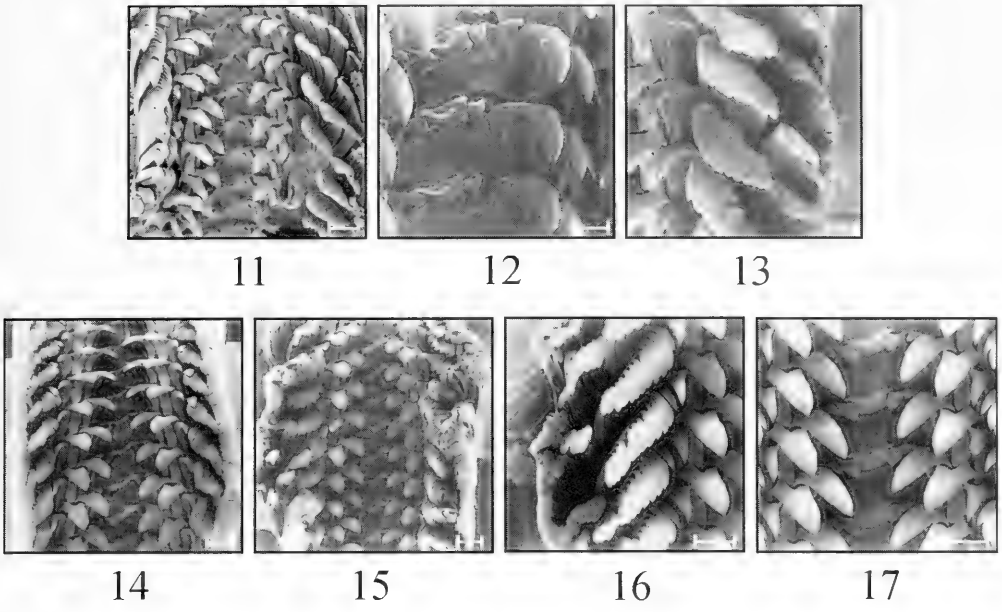


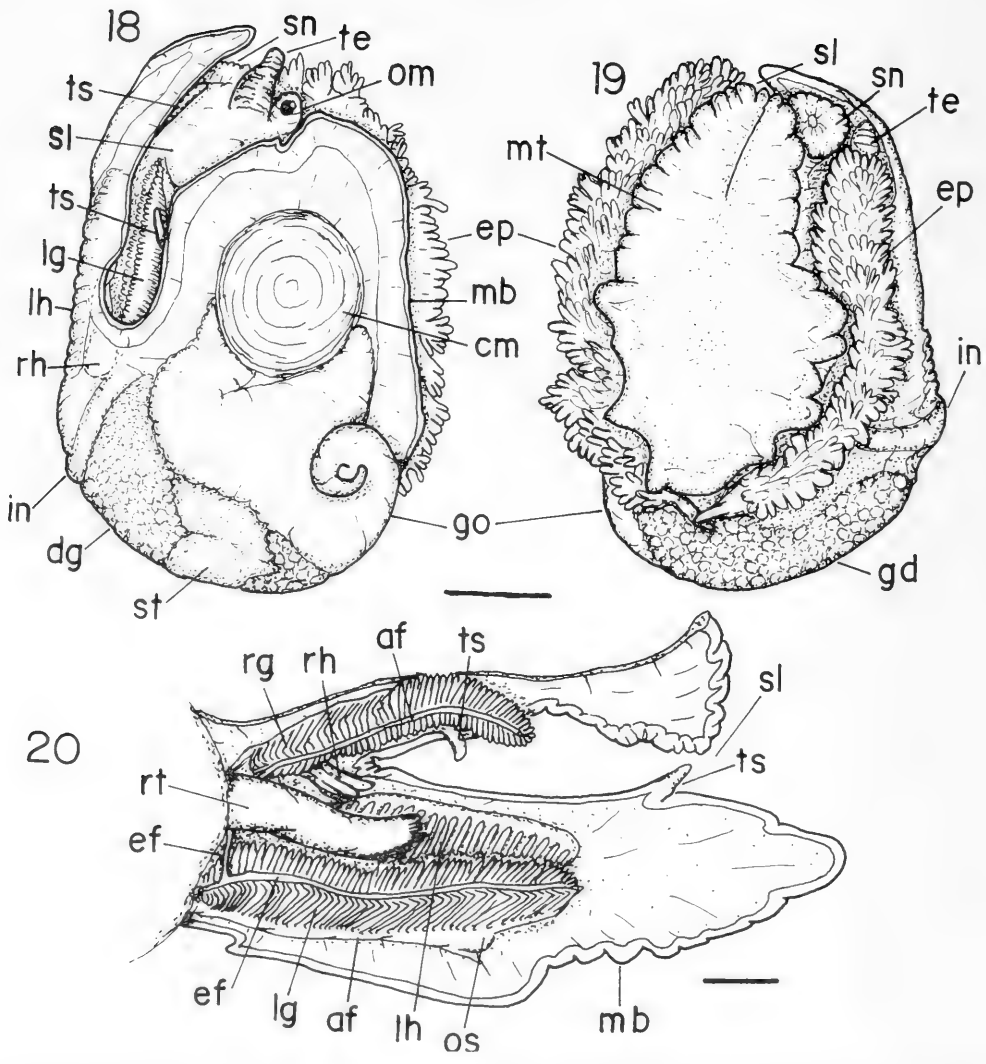
FIG. 8–13. Jaws and radula in SEM: (8) jaws of *Haliotis aurantium,* scale = 200 μm; (9) detail of same, scale = 20 μm (10) detail of central region of jaws of *Haliotis pourtalesii,* scale = 50 μm; (11) radula of *Haliotis aurantium,* scale = 100 μm; (12) detail of same, central region, scale = 50 μm; (13) detail of Fig. 11, marginal region, scale = 50 μm.



FIGS 14–17. Radulae of *Haliotis pourtalesii* in SEM: (14) USNM 833627, scale = 100 μm; (15) UMML 30–8376, scale = 100 μm; (16) detail of Fig. 14, marginal region, scale = 100 μm; (17) the same, central region, scale = 100 μm.

papillated nephrostome in ventral base of rectum. Right kidney long, thin, lying right margin of pallial cavity (Fig. 24), its nephrostome a longitudinal slit in its anterior extremity. Ventricle large, surrounding intestine; left auricle anterior to ventricle and right auricle ventral to it.

Digestive system. Mouth in snout, covered internally by pavement-shaped papillae (Fig. 28). Jaws two small plates (Figs. 8, 28), with rounded borders, situated in mid-dorsal region of mouth; median and anterior region of each plate with a small, sharp pointed projection (Figs. 8, 9). Buccal mass very large, complex; odontophore surrounded by two glandular oesophageal pouches (Figs. 25–27), both with inner surface covered by many tall villiform papillae (Fig. 28). Left pouch broad, short, covering ventral and lateral-left surfaces of odontophore. Right pouch narrow, long, beginning at right and running obliquely (Figs. 26, 27). Both pouches open in ventral-anterior region of oesophagus in two separated, tall, ring-like folds; a third short accessory fold also at this position (Fig. 28: af). Odontophore short, with very long radular sac, extending behind buccal mass and terminating near stomach (Fig. 25). Radula (Figs. 11–13): rachidian teeth broad, short, each with a large curved terminal cusp and two lateral bolsters (Fig. 12); first lateral tooth with triangular base and small rounded cusp; second lateral tooth long, with a somewhat rectangular base and a lateral-terminal, hook-like cusp; third lateral tooth the largest, with a long, irregular, curved base, and a large, long, sharp cusp, of almost the same length as base; fourth and fifth lateral teeth similar to third, but narrow; fifth narrowest, sharply pointed. About 32 pairs of marginal teeth per row (Fig. 13), with a long stalk; main cusp rounded, curved, spoon-like, flanged on each side by two small, sharp secondary cusps; marginal teeth gradually decrease in size laterally. Odontophore muscles (Figs. 31–35) consisting of: pair of ventral buccal protractor muscles, with their origin in ventral-lateral inner surface of peribuccal wall, and their insertion in ventral lateral region of posterior cartilages (Figs. 31, 32: vb); pair of direct radular tensor muscles, their origin in mid-ventral region of posterior cartilages and insertion on lateral angles of ventral edge of radula (Figs. 31, 33, 34: dr); pair of muscles as outer approximator of cartilages, their origin in anterior surface of posterior cartilages and insertion in outer lateral surface of anterior cartilages



FIGS. 18 to 20. *Haliotis aurantium* n. sp. anatomy: (18) topography of the holotype specimen in dorsal view; (19) the same in ventral view, scale = 2 mm; (20) pallial organs, mantle deflected, inner-ventral view, scale = 1 mm.

(Figs. 33–35: oa); pair of small posterior ventral radular tensor muscles, their origin in ventral inner surface of peribuccal wall and insertion in mid-ventral region of radular sac (Figs. 31, 32: pv); several pairs of small lateral protractor muscles, their origin in dorsal inner surface of peribuccal wall and insertion in dorsal-mid surface of radula (Fig. 32; lm); pair of direct anterior radular tensors, their origin in ventral-dorsal surface of posterior cartilages and insertion in lateral borders of sub-radular membrane and ventral surface of radula up to mid line, in a "M" shape (Fig. 33: da); and horizontal muscle, uniting ventral edge of both anterior cartilages (Figs. 31, 35: hz). Anterior odontophoral cartilages long, flattened, anteriorly sharp, posteriorly broad, with rounded borders (Fig. 35). Posterior odontophoral cartilages very short, elliptic, situated in outer posterior extremity of anterior catilages (Fig. 35).

Oesophagus short, flattened tube (Figs. 25, 26), with about eight internal longitudinal folds (Fig. 28). Stomach very large, U-shaped, near mid line in posterior region of animal (Fig. 18).

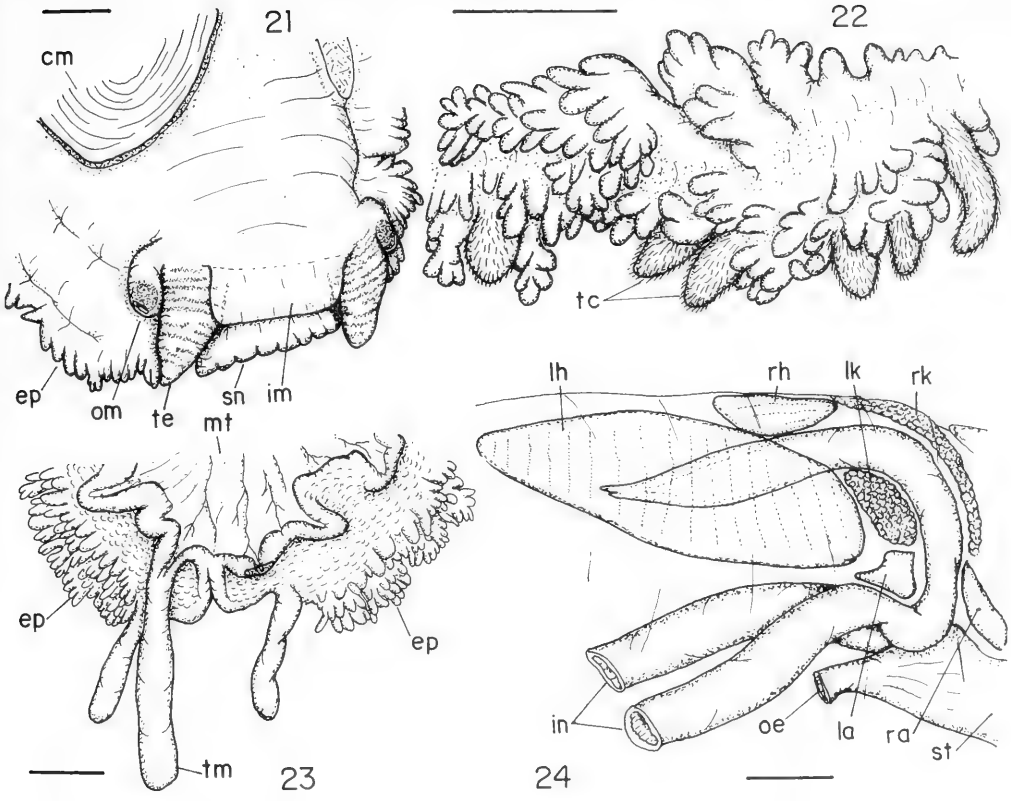


FIG. 21 to 24. *Haliotis aurantium* n. sp. anatomy: (21) detail of head, frontal view, mantle removed, scale = 1 mm; (22) detail of left-posterior fourth of epipodium, scale = 1 mm; (23) detail of posterior extremity of foot, ventral view, scale = 1 mm; (24) semi-diagrammatic drawn of cleared pericardial and nearby structures, ventral-right view, scale = 1 mm.

Oesophageal branch of stomach ventral, conical, with a very small caecum (Fig. 26). Internally oesophagial branch of stomach with mosaic of low, irregular folds near oesophagus opening (Fig. 30), where three longitudinal folds begin, two of them contouring posterior extremity of stomach, becoming weaker posteriorly; the third fold becoming larger and running to intestinal branch of stomach where it becomes weaker (Fig. 30: ty). Some radial muscle fibers in stomach wall originating between oesophageal and intestinal branches of stomach (Fig. 30: lc) and inserting in small left columellar muscle (Fig. 25: lc). Intestinal branch of stomach dorsal, conical, larger than oesophageal branch (Fig. 26); two typhlosoles running alongside gastric intestinal branch from caecum into intestine, one of them presenting in its mid region a series of oblique folds, differentiating a small sorting area (Fig. 29: sa). Other regions of stomach inner surface smooth, covered by thin greenish cuticle (Figs. 29, 30). Intestine long, with thin transparent walls, running near right side of head, when it twists and returns to posterior region near stomach (Figs. 25, 27); in this posterior region, it is sigmoid, running through pericardium (Fig. 24) and exiting into pallial cavity (Figs. 20, 25, 26). Intestine and stomach full of gravel.

Digestive glands large, green, with mosaic of irregular brown spots on its surface (Figs. 18, 25) and occupying visceral mass ventral to gonad, surrounding stomach (Fig. 25).

Genital system. Very large ovary occupying all of spire and part of body whorl (Figs. 18, 25: go), pale cream in color. Ovary with three lobes (Fig. 18), one within spire, one posterior to main columellar muscle, the third in left side of this muscle (Fig. 18). Oviduct, which probably runs within right kidney, not seen. Ventral limit of gonad at the digestive gland and stomach (Fig. 25). Male not examined.

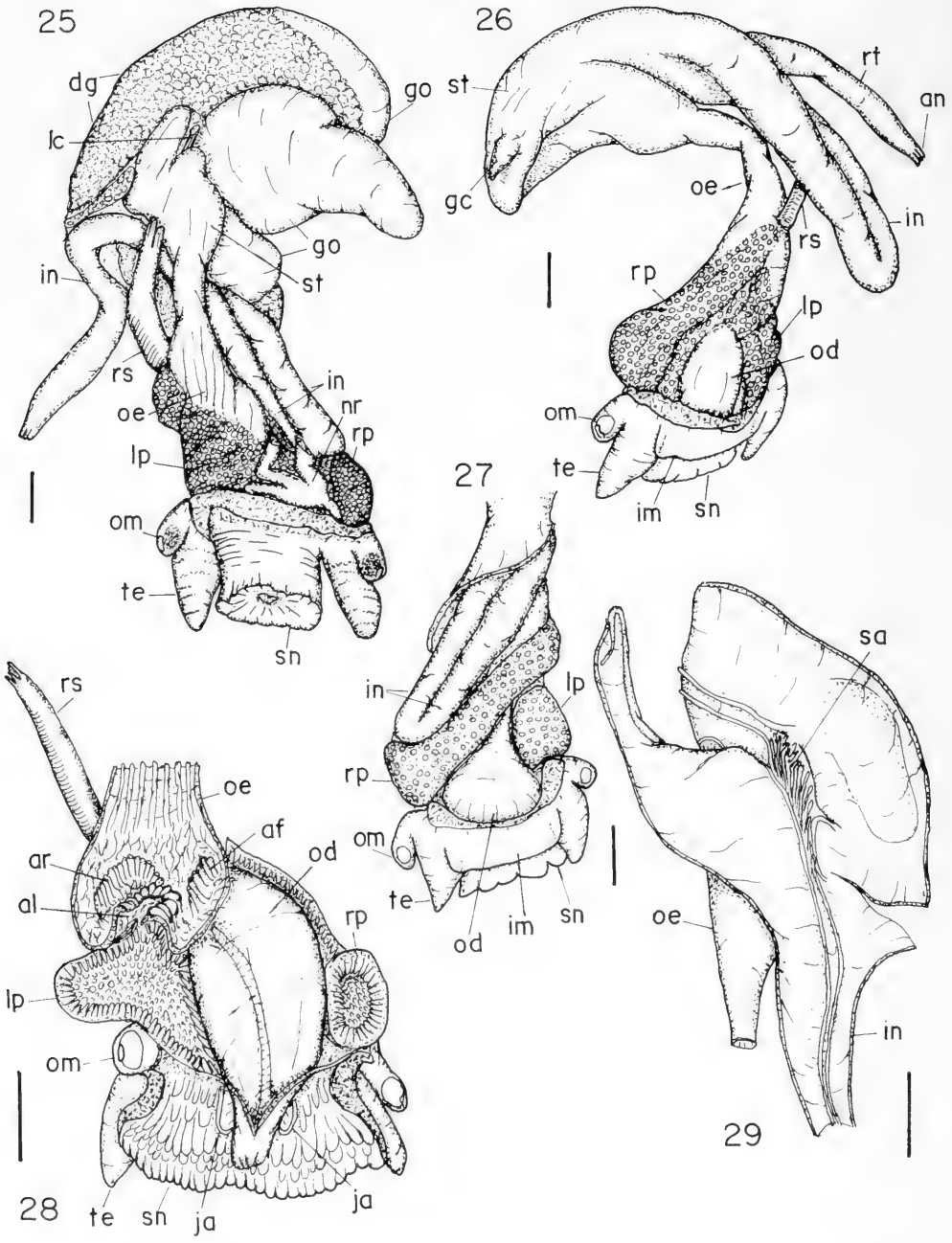


FIG. 25 to 29. *Haliotis aurantium* n. sp. anatomy: (25) cephalic organs and visceral mass, ventral view, foot and mantle removed; (26) extracted head and digestive ducts, right-dorsal view; (27) detail of anterior region of digestive system, dorsal view, head tegument partially removed; (28) snout, buccal mass and esophagus opened longitudinally, ventral view; (29) stomach opened longitudinally in intestinal branch. Scales = 1 mm.

Nervous system. Only circum-oesophageal region examined, agreeing closely with that described by Fleure (1905) and Crofts (1929) for *Haliotis tuberculata*.

Measurements (respectively length in mm, width in mm, number of whorls, of opened pores, of closed pores). Holotype, MZSP 28201: 12.9 by 8.7, 3.2, 4, 14; Paratypes: MZSP 18482: 15.0 by 10.5, 3.2, 3, 16; MZSP 19569: 13.2 by 9.0, 3.0, 4, 13 and 7.2 by 5.2, 2.1, 4, 8.

Habitat. From 77 to 150 m depth, on gravel, generally associated with *Laminaria* sp.

Etymology. The specific name refers to the orange color of the external shell surface (Latin, *aurantium*).

Material Examined. Types.

Material Available. BRAZIL; *Amapá;* MORG 15000, 1 shell, off Cabo Orange, 100 m (R. V. Almirante Saldanha, 30/xi/1988). *Espirito Santo;* MNRJ-HSL 6603, 2 specimens, off Vitoria (lost); MORG 15930, 1 shell, off Vitória, 87 m (R. V. A. Saldanha, 24/iv/1969); MNRJ 3577, 20 shells, 20°37′05″S 39°59′00″W, 87 m (R. V. A. Saldanha, sta. DHN 2027, 24/ix/1971). *Rio de Janeiro;* MNRJ 3554, 14 shells, 21°56′05″S 40°07′00″W, 77 m (R. V. A. Saldanha, sta DHN 2012, 11/ix/1969); MNRJ 3578, 4 shells, off Cabo Frio, 23°05′00″S 40°05′00″W, 111 m (R. V. A. Saldanha, sta. DHM 2168); MORG 15931, 1 shell, off Cabo Frio, 90 m (R. V. A. Saldanha, 9/ix/1969); MORG 26226, 1 shell, off Cabo Frio (R. V. A. Saldanha, x/1986, on *Laminaria*); MORG 15929, 1 shell, off Cabo de São Tomé, 77 m (R. V. A. Saldanha, 11/iv/1969). *Rio Grande do Sul;* MORG 17467, 1 shell, off Conceição, 126 m (R. V. Mestre Jerônimo, 2/x/1972).

Haliotis pourtalesii Dall, 1881 (Figs. 1, 2, 10, 14–17, 36–45)

Haliotis (Padollus) Pourtalesii Dall, 1881: 79 [Gulf Stream near Florida Reefs, 180 m (31/iii/1869)] (described from memory).

Haliotis pourtalesii: Dall, 1889: 168; Henderson, 1911: 81 [neotype]; Cooke, 1914: 103; Henderson, 1915: 660, pls. 45, 46; Smith, 1937: 78, pl. 29, fig. 3; Foster, 1946: 38–40, pl. 22, figs. 1, 2; Parker, 1960; Harry, 1966: 207–208, pl. 30; Jung, 1968; Sarasua, 1968: 1–8, figs. 1, 2; Merrill & Petit, 1969: 117; McLean, 1969: 115; Guice, 1969: 140; Abbott, 1974: 18, fig. 30; Titgen & Bright, 1985: 147–152 figs. 1, 2; Abbott & Dance, 1983: 19, fig.; Ode, 1986: 69–73; Martinez & Ruiz, 1994: 63–64, figs. 1-2.

Type: Neotype USNM 271601 [3 miles off Sand Key, Florida, 49 m, 1913]

Diagnosis

Minute northwest Atlantic species with pigmented epipodium and metapodium; three tentacles in mantle slit; epipodial tentacles arranged in three layers around well-developed (main) tentacles; without large epipodial tentacles posteriorly; without metapodial tentacles; snout border papillated; ventral surface of odontophore free of pouches; only one pair of lateral radular protractor muscles.

Description

Shell. Figs. 1, 2.

Head-foot. Head somewhat protruding (Fig. 39). Tentacles long, narrow, covered with long cilia; pigmented by regular dark brown, successive, well-spaced transverse bands and a mid longitudinal band (Fig. 39). Dark brown spots abundant in dorsal and ventral epipodium faces and dorsal face of metapodium, scanty in metapodial sole. Ommatophore well developed, on outer basal region of tentacles, with dark, vesicular, open eyes (Figs. 36, 37, 39). Intertentacular membrane semitransparent, thin, covering anterior region of snout and inner region of cephalic tentacles (Fig. 39). Snout well developed, cylindrical, broad, with regular small, abundant papillae on its ventral border (Fig. 39). Foot large, about same size as shell aperture (Fig. 37). Epipodium with many tentacles (Fig. 37) arranged as follows (Fig. 41):(1) a dorsal flap (ff) fringed by flattened, polytomic tentacles in a uniform zigzag pattern; regions nearest foot with dark pigment in dorsal and ventral faces (pr), other regions white; (2) intermediary flap (if) with conspicuous, large, white, sharp tentacles (called "main" tentacles), covered with long cilia (ma); bases of these main tentacles, which are ventral to pigmented region of superior flap (pr), surrounded by two (one on each side) large, multipapillated, dark-brown colored tentacles (sf); between these structures, many other short tentacles, with rounded tips, without pigment nor evident cilia; (3) ventral flap (vf) extremely rich in tentacles, some of them longer

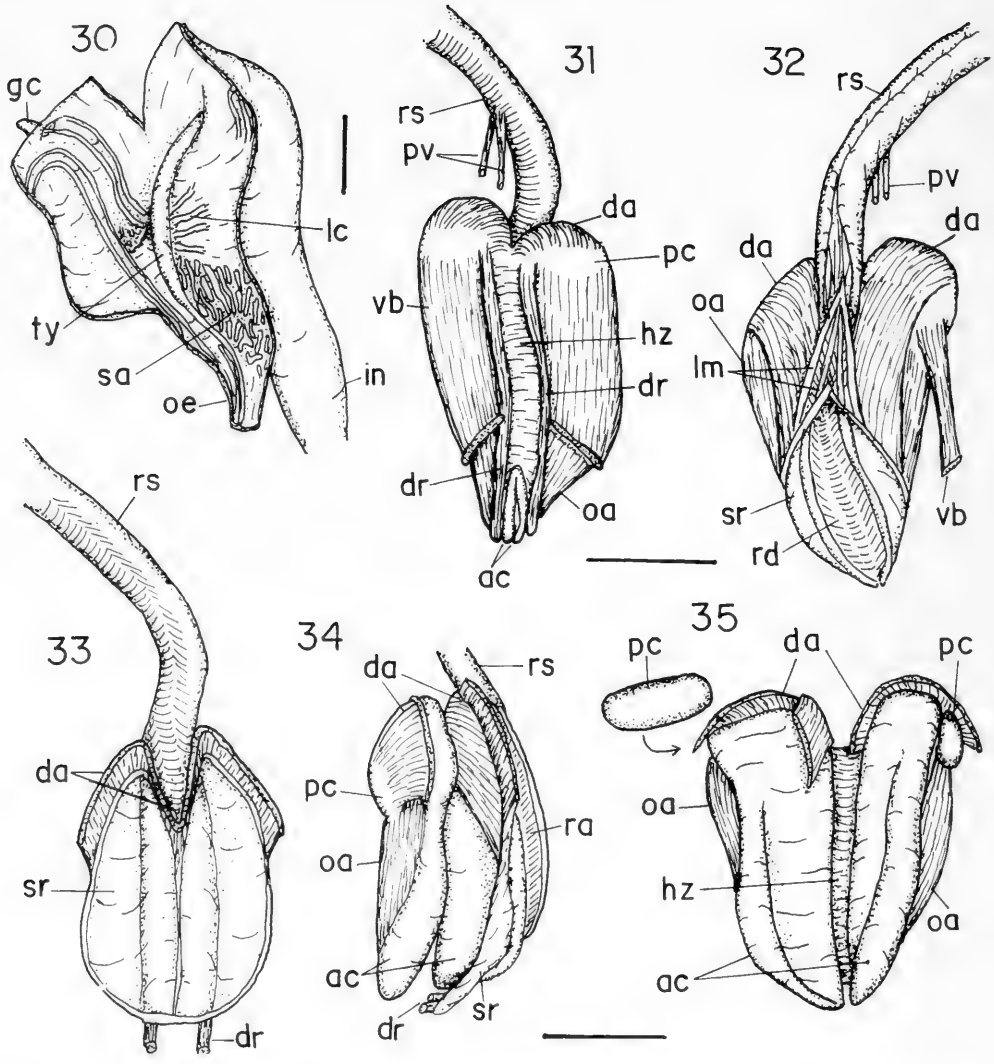


FIG. 30 to 35. *Haliotis aurantium* n. sp. anatomy: (30) oesophageal branch of stomach opened longitudinally; (31) ventral view of odontophore; (32) dorsal view of same; (33) ventral view of radular ribbon and subradular membrane showing the "M" in shape insertion of "da"; (34) lateral-right view of odontophore, direct anterior radular tensor muscle (da) partially sectioned; (35) dorsal view of odontophore with part of its muscles removed, right anterior cartilage deflected. Scales = 1 mm.

(tc), sharp, covered with long cilia, similar to but smaller than main tentacles; other tentacles short, without pigment, with rounded tips without evident cilia. Epipodium on each side begining near snout and ending at posterior extremity of foot, where it unites with metapodial sole (Fig. 40); practically no region without tentacles. Number of main epipodium tentacles in each side from 10 to 12. Main columellar muscle very large, circular in section (Fig. 36). Secondary (left) columellar muscle very small, with some fibers attached to mid wall of stomach (Fig. 42: lc).

Mantle border. Trifolded, simple, depigmented. Slit deep, with three sharp tentacles covered with long cilia, two of them about mid region of the slit (one in each side), and the third in posterior extremity of slit (Figs. 36, 38).

Pallial cavity. Short, about half of body whorl (Figs. 36, 38). Gills somewhat long, bipecti-

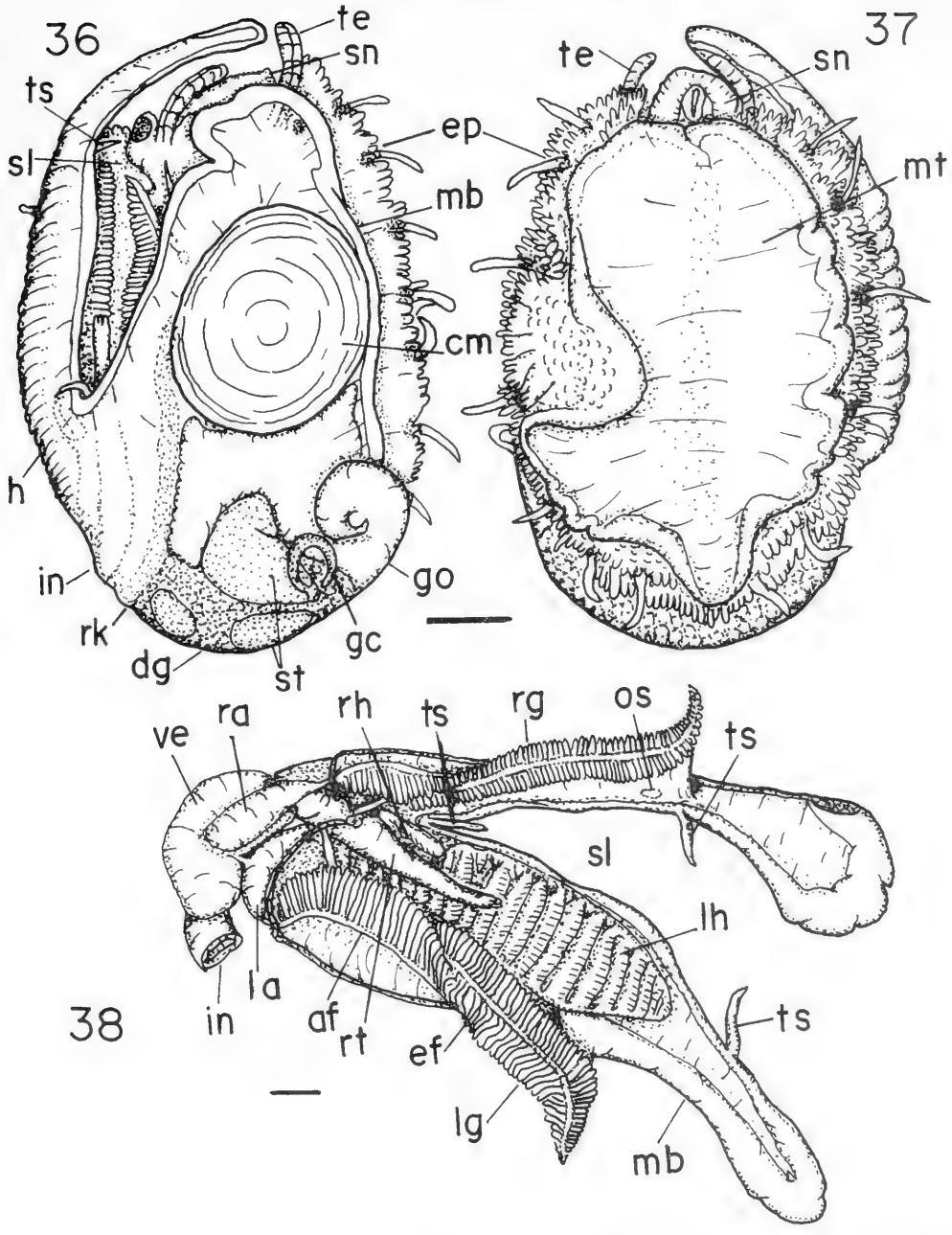


FIG. 36 to 38. *Haliotis pourtalesii* anatomy: (36) topography of the specimen USNM 833627 in dorsal view, scale = 2 mm; (37) same in ventral view, scale = 2 mm; (38) pallial organs, mantle deflected, inner-ventral view, scale = 1 mm.

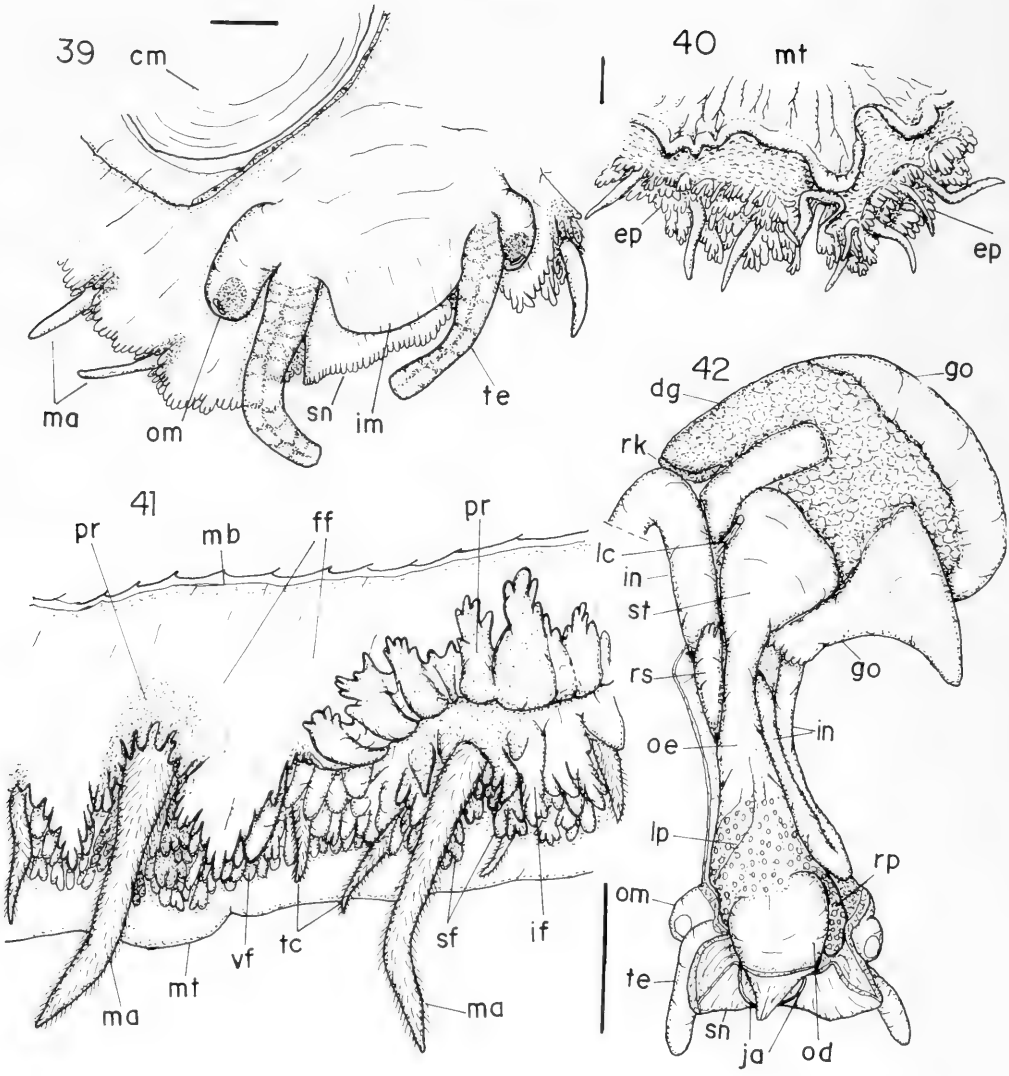


FIG. 39 to 42. *Haliotis pourtalesii* anatomy: (39) detail of head, frontal view, mantle removed, scale = 1 mm; (40) detail of posterior extremity of foot, ventral view, scale = 1 mm; (41) detail of left-posterior fourth of epipodium, scale = 1 mm; (42) cephalic organs and visceral mass, ventral view, snout opened longitudinally, scale = 1 mm.

nate; right gill somewhat shorter than left (Fig. 38). Afferent gill vessel in base of gill's insertion. Efferent vessel between two flaps of each gill leaflet, inserting in gill sub-terminally, anterior to posterior extremity of gill. Hypobranchial glands present, left very large (Fig. 38), with about 18 transverse, successive, uniform folds; right smaller, with two oblique, somewhat curved folds (Figs. 36, 38). Both hypobranchial glands at left of slit (Fig. 38). Rectum lying between hypobranchial glands, slightly free in posterior half of pallial cavity; anus papillated, opening in posterior third of slit (Fig. 38).

Circulatory-excretory systems. Kidneys and pericardium in posterior left side of animal, just behind pallial cavity. These structures are very similar to those of the preceding species (Figs. 24, 38).

Digestive system. Mouth in snout, covered

internally by smooth walls (Fig. 42). Jaws two somewhat large plates (Fig. 42), brown, in mid-dorsal region of buccal cavity, lateral and anterior border with a sharp edge, median-anterior region with a sharp projection (Fig. 10). Buccal mass large, complex; odontophore (Fig. 42) surrounded, except in its ventral region, by two glandular pouches, both with inner surface covered by many tall, villiform papillae (Fig. 43). Left pouch broad, short, covering left side of odontophore. Right pouch narrow, long, running obliquely from right side of odontophore. Both pouches opening in ventral-anterior region of oesophagus in separate, tall, ring-like folds, that of left chamber more anteriorly (Fig. 43). Radular sac very long, running close to and attached to dorsal region of buccal mass and oesophagus (Fig. 42). Radula (Figs. 14–17): similar to that of preceding species, except for the marginal teeth, which have a much longer, sharp central cusp (Fig. 16); and in being more abundant, with about 40 pairs per row (Figs. 14, 15). Odontophore similar to that of preceding species (Figs. 44, 45), including cartilages and intrinsic muscles; except lateral protractor muscle of radula (Fig. 45: lp), which in *H. pourtalesii* is a single, larger pair.

Oesophagus short, flat (Fig. 42), with about ten internal longitudinal folds (Fig. 43). Stomach very large, U-shaped; walls irregular, with two ventral (oesophageal) and three dorsal (intestinal) chambers and a well-developed, narrow spiral caecum in right dorsal region of stomach, with about one whorl (Fig. 36: gc). Stomach and intestine of studied specimen with a large quantity of clear gravel and organic materials including foraminiferan shells and unidentified bristles.

Intestine, rectum and digestive gland characters similar to those of preceding species (Fig. 42).

Genital system. Gonad with only two lobes: a lobe within spire and another just posterior to main columellar muscle (Fig. 36). Ventrally, this gland terminates at the digestive gland and stomach (Fig. 42).

Measurements (respectively length in mm, width in mm, number of whorls, of opened pores, of closed pores). USNM 833627: 17.8 by 12.5, 3.2, 6, 18. UMML 30–8376: a) 20.1 by 13.0, 3.1, 5, 18; b) 17.6 by 11.3, 3.0, 5, 17; c) 9.9 by 7.0, 2.5, 5, 12; d) 21.6 by 15.0, 3.2, 5, 19; e) 19.6 by 13.0, 3.0, 5, 17; f) 13.7 by 9.9, 3.0, 6, 16; g) 12.9 by 9.1, 2.3, 5, 14.

Habitat. Depth from 50 to 160 m. In the literature, the habitat is referred to as bottoms with rocks, stones sand and shell debris, or reef (Nijssen-Meyer, 1969).

Material examined: USNM 833627, 1 specimen, U.S.A., Gulf of Mexico, off Florida, 25°16′55″N, 83°37′47″W, 74 m depth (15/viii/1984). UMML 30–8376, 4 specimens + 5 shells, off VENEZUELA, 11°03′N 65°59′W, 69–155 m (R. V. Pillsbury sta. P-736, 22/vii/1968).

DISCUSSION

Haliotis pourtalesii and *H. aurantium* are atypical Haliotidae. Generally, haliotids are large gastropods, over 150 mm in length, whereas both these species are less than 25 mm. Haliotids generally are common and occur in shallow waters, whereas these species are rare and found in deep water (slope). However, both have the same general anatomical characters of the family, modified due to miniaturization.

Haliotis aurantium differs from *H. pourtalesii* in having: (1) a smaller size; (2) only two tentacles on the mantle slit (not three); (3) only transverse bands in the cephalic tentacles (without a longitudinal band); (4) foot without pigment; (5) epipodial tentacles of a simpler structure (see below); (6) a pair of larger tentacles on the posterior extremity of the epipodium and a proportionally large area with out tentacles posteriorly; (7) a pair of large tentacles on the posterior extremity of the metapodium sometimes present (absent in paratype) (Fig. 23: tm); (8) right gill much shorter than the left (in *H. pourtalesii* both gills are about the same size and are proportionally longer than in *H. aurantium*); (9) left hypobranchial gland with three folds (not two); (10) right hypobranchial gland proportionally smaller and with weaker transverse folds; (11) pericardial structures situated more anteriorly; (12) snout bordered by lobes (without small papillae); (13) marginal teeth with rounded main cusp (those of *H. pourtalesii* are sharp); (14) left pouch of the buccal mass covering the ventral surface of the odontophore (*H. pourtalesii* has this region free); (15) several pairs of small lateral protractor muscles of the radula (not one large pair); and (16) kidneys and pericardium more ventrally placed.

Both species show considerable shell variation, from specimens with well-developed axial ridges to specimens lacking them (e.g., the figures of Sarasua, 1968). There is also variation in the number of spiral ridges, which

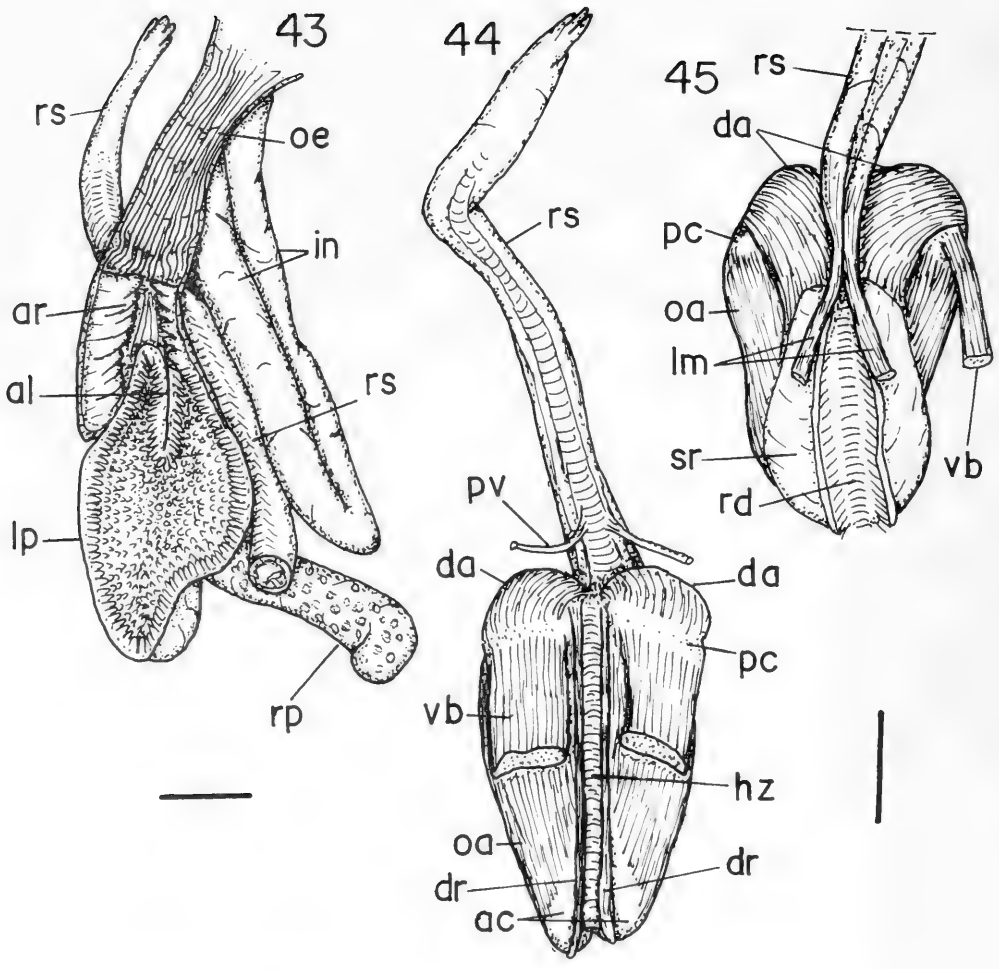


FIG. 43 to 45. *Haliotis pourtalesii* anatomy: (43) anterior region of digestive system, odontophore removed, left pouch (lp) and oesophagus opened longitudinally; (44) ventral view of odontophore; (45) same in dorsal view. Scales = 1 mm.

apparently increases with shell growth. Between the spiral ridges, shells sometimes have delicate axial and uniform threads (Fig. 3). Shared shell characters of these species are small size (up to 25 mm), the reddish-orange color of the exposed areas, and the prominent borders of the tremata. A possible difference between the species is the number of open pores; in specimens of *H. aurantium,* they varied from 3 to 4, whereas *H. pourtalesii* had from 5 to 6 open pores. Analysis of more specimens is necessary to establish whether this is consistent. Comparative examination of available shells, as well as those figured in the literature, no shell character exclusive of a species was found. Thus, it is difficult to separate them using only the shell, although, if a specimen is found from North Carolina to the Caribbean Sea it is probably *H. pourtalesii,* whereas if collected in Brazil (from Espirito Santo to Rio Grande do Sul), it is probably *H. aurantium.* This criterion was used in the synonymic list, but the anatomical study of specimens from all localities was not undertaken and perhaps examination of additional specimens could modify this concept. In both areas, a considerable number of specimens were found. Between these areas shells have been recorded from three localities: off Surinam (Nijssen-Meyer, 1969), off Para

River (Rios, 1975) and off Maranhão (Kempf & Matthews, 1968). Although this could be due to transportation, further sampling in these areas should clarify the distribution of these taxa. Dr. Mello (Museu de Malacologia of the University of Pernambuco), has not obtained haliotids from dredge samples in the northeastern coast of Brazil (pers. comm.).

The epipodial tentacles of *H. aurantium* (Fig. 22) differ from those of *H. pourtalesii* (Fig. 41) in being entirety unpigmented and in having no specific arrangement around the main tentacles. The characters of the epipodial tentacles of *H. pourtalesii* have some similarity to those of the Mediterranean *H. lamellosa* and *H. tuberculata,* (pers. obs), but these differ from *H. pourtalesii* in having only two epipodial tentaculated flaps, the main tentacles inserting dorsally in the dorsal flap; the ventral flap has a similar organization to the dorsal one, but its main tentacles are smaller, ventrally inserted, and situated between the main tentacles of the dorsal flap. *Haliotis tuberculata* has a practically straight epipodium, with three or four small undulations between the main tentacles (Crofts, 1929: pl. I); *H. lamellosa,* in contrast, has two strong undulations between the main tentacles. Epipodial tentacle characters have been used in haliotids by Owen, et al. (1971) for separating seven eastern Pacific abalone species, and even their hybrids. Using the good figures of that paper, it is clear that the species studied herein differ considerably from those taxa.

Haliotis aurantium and *H. pourtalesii* differ anatomically from *H. tuberculata* (Fleure, 1905; Crofts, 1927, 1937, 1955; person. obs.) and from *H. lamellosa* (pers. obs.) in several characters: the cephalic tentacles possess spots (not of uniform color); the intertentacular membrane simple and free in its lateral margins (*H. lamellosa* has minute lobes in lateral margins, and in *H. tuberculata* the lateral regions are attached to omatophores); as well as the other characters of epipodial tentacles (noted above); the epipodium of Atlantic species begins abruptly near the snout (in the Mediterranean species, there is a coiled expansion in each side, which partially covers the snout); gill proportionally shorter and with fewer leaflets; hypobranchial glands proportionally smaller and simpler (those species have strong and tall folds, *H. pourtalesii* has low folds and *H. aurantium* only furrows); rectum only covered by tegument (both Mediterranean species have the posterior region of the rectum covered on both sides by the hypobranchial glands); anus long and papilliform (*H. tuberculata* and *H. lamellosa* have a short, broad anus); stomach shorter with clear delimitation; and gastric spiral caecum much shorter (which also differentiates it from *H. cracherodii,* see Campbell, 1965). With regard to the tentacles of the mantle slit, *H. pourtalesii, H. tuberculata* and *H. lamellosa* are similar in having three tentacles in somewhat the same disposition; *H. aurantium* has only two (there is no tentacle situated just in angulated posterior extremity of slit).

The auriform shell with tremata, the complex tentaculate epipodium and the lack of an operculum, are known synapomorphic characters of the Haliotidae within the Vetigastropoda. At least three additional synapomorphies are offered here: (1) the insertion in the stomach of some fibers of the right retractor muscle, (2) the insertion in a "M" shape of the direct radular anterior muscle, and (3) the intertentacular membrane of the head ("head pleat" of Crofts, 1927).

Schremp (1981: 1125, pl., fig. 1) called a Pliocene haliotid found in the Imperial Formation of California *Haliotis pourtalesii.* Because this identification is based only on the shell, this specimen might instead be the Pacific *Haliotis roberti* McLean, 1969, considered a synonym by that author.

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HISTOCHEMICAL AND ULTRASTRUCTURAL IDENTIFICATION OF BIPHASIC GRANULES IN THE ALBUMEN SECRETORY CELLS OF *ARION SUBFUSCUS* (GASTROPODA, PULMONATA)

BENJAMIN J. GOMEZ[1], ANA M. ZUBIAGA[2], M. TERESA SERRANO[1] & EDUARDO ANGULO[1]

ABSTRACT

Two types of globules, differing in size, structure, and nature, are demonstrated within each secretory cell of the albumen gland of *Arion subfuscus*. Large secretory globules, with a homogeneous granular content, are rich in proteins and carbohydrates, with the protein-reacting groups probably partially masked by intimate junction with the polysaccharides. Smaller secretory globules lie inside the large ones and are more electron-lucent and foam-like in substructure, containing mostly glycosaminoglycans but also proteins. Lectin-sugar specificities show that the carbohydrates synthesized by the gland are mainly galactogen, but other neutral polysaccharides, such as glycogen, are also present in the secretory vesicles. The reactions of each type of globule to the lectins are also very different.

Key words: albumen gland, lectin histochemistry, ultrastructure, *Arion subfuscus*.

INTRODUCTION

The albumen gland is a large female accessory gland that surrounds the distal part of the ovotestis duct, enclosing the carrefour system and the most proximal portion of the spermoviduct in stylommatophorans. It is composed of a large number of secretory branched tubuies, which all open into the slit-like central lumen of the gland (Tompa, 1984). This gland synthesizes the perivitelline fluids of the molluscan egg, which is composed mainly of galactogen (Bayne, 1967; Meenakshi & Scheer, 1968), a polysaccharide of galactose (Duncan, 1975). Besides galactogen, some proteins mineral salts, and monosaccharides are present in the albumen gland secretions (Runham, 1988).

The tubules of the albumen gland in gastropods contain two cell types — the large glandular cells and the small centrotubular cells (Jong-Brink, 1969; Breckenridge & Fallil, 1973; Els, 1978).

It is the aim of this study to investigate in detail the nature of the albumen gland secretions of the arionid species *Arion subfuscus* (Draparnaud, 1805), by means of general and lectin histochemical methods. The ultrastructure of secretory granules is also described.

MATERIALS AND METHODS

For general and lectin histochemical studies the albumen glands were fixed in Carnoy's fluid (Culling, 1974) for six h, dehydrated with alcohol, and embedded in paraffin wax. Sections, 6–8 μm thick, were obtained.

Neutral carbohydrates were detected by the periodic acid Schiff technique (PAS). Acetylation followed by saponification, and diastase treatment were used as controls and also for glycogen demonstration (Martoja & Martoja-Pierson, 1970). Alcian Blue staining (AB) was used at pH = 0.5 to stain strongly sulphated mucosubstances and at pH = 2.5 for carboxylated and weakly sulphated mucosubstances (Bancroft & Stevens, 1982). The combined High Iron Diamine with Alcian Blue (HID-AB) was used to stain sulphated mucosubstances black and non-sulphated acidic mucosubstances blue (Culling, 1974). The Mercuric Bromophenol Blue (BB) with deamination (Chapman, 1975), as well as Chloramine T-Schiff techniques (Martoja & Martoja-Pierson, 1970), were used for detection of proteins. Bock's technique (Bock et al., 1976) was used for demonstrating protein disulphide groups. In order to detect lipid material, glands were fixed in Baker's formol-calcium and sections were stained with Sudan Black B.

[1]Departamento de Biología Celular y Ciencias Morfológicas. Facultad de Ciencias, Universidad del País Vasco, Apdo. 644-48080, Bilbao, Spain
[2]Departamento de Biología Animal y Genética. Facultad de Ciencias, Universidad del País Vasco, Apdo. 644-48080, Bilbao, Spain

TABLE 1. Lectins employed and their major binding specificities: Fuc: fucose; Gal: galactose; GalNac: N-acetylgalactosamine; Glc: glucose; GlcNac: N-acetylglucosamine; Man: mannose; NeuAc: neuraminic acid or sialic acid.

Taxonomic name of the source	Acronym	Carbohydrate binding specificity
Ulex europaeus	UEA-I	L-Fuc; α-1,2 Gal β-1,4 GlcNAc, β-1,6
Canavalia ensiformis	Con A	α-D-Man > α-D-Glc >> α-D-GlcNAc
Limax flavus	LFA	NeuAc α-2,3/6Gal; NeuAc α-2,3/6GalNAc
Arachis hypogaea	PNA	Gal β-1,3 GalNAc > Gal
Ricinus comunis	RCA-I	Gal β-1,4 GlcNAc; Gal
Glycine max	SBA	D-GalNac > D-Gal

Lectin histochemistry was used according to Welsch & Schumacher (1984). Deparaffined sections were incubated for 30 min with lectins coupled to fluorescein isothiocyanate (FITC) : PNA (Peanut agglutinin); SBA (Soybean agglutinin); LFA (*Limax flavus* aggl.); UEA I (*Ulex europeus* I aggl.); RCA I (*Ricinus communis* aggl.); Con A (Concanavalin A)(for lectin specificities, see Table 1). After incubation, the sections were rinsed in phosphate buffered saline (pH = 7.4) for 2 h. Preparations were studied by fluorescence microscopy. Sections not treated with FITC-lectins were used as controls to observe the autofluorescence of the tissue. The specificity of the staining (Goldstein & Hayes, 1978) was tested by preincubating the lectin in a solution of the appropriate reactive sugar (Zubiaga et al., 1990).

For transmission electron microscopy, tissues were fixed for 2 h in 25% Karnovsky in 0.1M cacodylate buffer at pH 7.3 (Glauert, 1981), postfixed for 1 h in 1% osmium tetroxide in the same buffer and embedded in Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate. The grids were observed in a Zeiss EM 9 and Philips EM 300 electron microscopes.

RESULTS

Ultrastructure

In the secretory cells of the albumen gland of *Arion subfuscus,* the organelles involved in the synthesis of secretory materials are abundant and include flattened and parallely arranged rough endoplasmic reticulum cisternae and Golgi stacks with many vesicles budding from them (Fig. 1). The middle and apical portions of the cells are filled by secretory granules (large secretory globules, 2–3 μm in diameter) with a matrix of finely granulated material, homogeneously distributed (Fig. 1). Inside these large globules, there are small aggregates of irregular lumps (small secretory globules, 0.3–0.4 μm in diameter), which contain a fine granulation (Fig. 1).

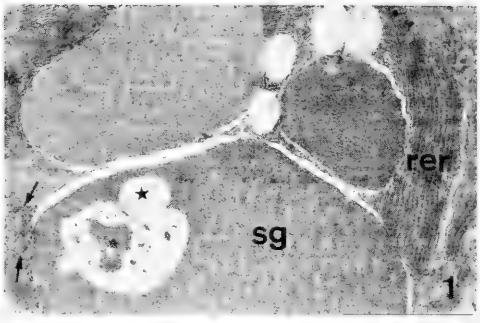

FIG. 1. TEM micrograph showing the matrix of large secretory globules (sg) containing small globules (*). Rough endoplasmic reticulum cisternae are flattened and abundant (rer). Arrows indicate Golgi system. ×15000.

Histochemistry

The general histochemical tests show that the composition of the albumen gland secretion is complex. The small globules show a reaction distinct from that of the general matrix of the large globules in which they are inmersed. Within each large globule there are one or two small ones. The results of the histochemical tests carried out are shown in Tables 2 and 3.

The small globules react strongly to the AB technique at both pH levels, and they stain black with the HID-AB technique indicating that they are composed mostly of sulfomucins. Moreover, these small globules are

TABLE 2. Results of the histochemical tests of the albumen gland tubules of *Arion subfuscus*. Three portions of the secretory cells have been distinguished: 1, cytoplasm; 2, large secretory globules; 3, small secretory globules; –, negative reaction; +, weak reaction; ++, moderate reaction; +++, strong reaction.

	Secretory cells			
Staining technique	1	2	3	Centrotubular cells
AB pH = 0.5	–	–	+++	–
AB pH = 2.5	+	–	+++	–
HID-AB	+	–	+++	–
	blue		black	–
PAS	++	+++	–	++
PAS-diastase	+	++	–	+
PAS-acet	–	++	–	+
PAS-acet.-sap	++	+++	–	++
Deamination-PAS	++	+++	–	+
Bromophenol blue	++	+	+	+
Chloramine T-Schiff	++	++	+	+
Bock	–	–	++	–
Sudan Black	–	–	–	–

TABLE 3. Lectin specificities of the different portions of the secretory cells of the *Arion subfuscus* albumen gland. –, negative reaction; +, weak reaction; ++, moderate reaction; +++, strong reaction.

Lectins	PNA	SBA	LFA	UEAI	RCAI	ConA
Cytoplasm	–	++	–	–	+	+
Large globules	+++	+++	–	–	+++	+
Small globules	+++	–	+++	+++	–	–

PAS-negative, whereas exhibiting a strong reaction to the Bock technique for protein SH groups.

The large globules show a strong reaction to the PAS technique, even after treatment with diastase or after acetylation followed by saponification. They also show moderate reactions to tests for protein.

The reactions of each type of globule to the lectins are different. The large granules bind specifically to PNA, SBA, RCA I, and Con A (Fig. 2), whereas the small granules bind to PNA, LFA, and UEA I (Fig. 3).

DISCUSSION

Previous histochemical and biochemical studies have shown that the albumen gland of gastropods produces a galactogen and protein-rich nutritive fluid for the developing embryos (Grainger & Shilliote, 1952; Bayne, 1967; Meenakshi & Scheer, 1968; Okotore et al., 1981; Dictus & Jong-Brink, 1987). The protein percentage varies from one species to another. Thus, in the pulmonates *Biomphalaria glabrata* (see Jong-Brink, 1969) and *Achatina fulica* (see Ramasubramaniam, 1979) the protein content is high, whereas in *Deroceras laeve* only a small amount of protein has been demonstrated (Els, 1978). We have found that protein is present in the secretory globules of the albumen gland of *Arion subfuscus,* as well as in the cytoplasm of the secretory cells, but the reaction to protein tests is only moderate. Nevertheless, the rough endoplasmic reticulum is very abundant. The protein-reacting groups could be intimately joined with polysaccharides and thus masked. In this sense, Bayne (1967) could not separate the proteins from sugars by electrophoretic studies, but he found great amounts of free amino-acids in albumen gland homogenates. This fact, together with the near absence of digestion of secretory granules by pronase (Kress & Schmekel, 1992), indicates that there is a very close union between both components.

The presence of galactogen in the albumen gland has been shown using different methodologies (Bolognani-Fantin & Vigo, 1968; Varute & Nanaware, 1972). In this work, we have used lectin histochemical methods to detect galactogen. The secretory granules of *Arion subfuscus* show a strong reaction with

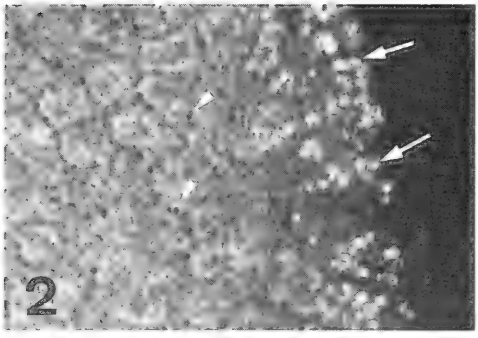

FIG. 2. Lectin histochemistry. RCA I binding to large secretory globules (arrows). Arrow heads indicate the nuclei of cells. ×40.

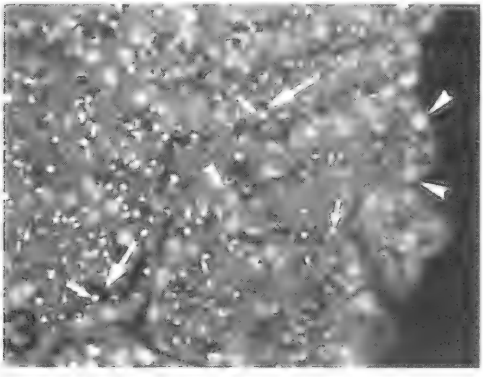

FIG. 3. Lectin histochemistry. LFA binding to small secretory globules (small arrows) which are present inside the large secretory globules (arrows). Arrow heads indicate the nuclei of cells that show a weak reaction to LFA. ×65.

PNA, SBA, and RCA I. These three lectins bind specifically to galactose residues (Goldstein & Hayes, 1978; Zubiaga et al., 1990). According to Okotore et al. (1981) and Okotore & Uhlenbruck (1982), these residues are probably D-Gal β1, 3D-Gal.

Moreover, the secretory granules of *A. subfuscus* as well as the cytoplasm of secretory cells also react with Con A. This indicates the additional presence of glucose or mannose sugar residues (Goldstein & Hayes, 1978) free in the cytoplasm as well as inside secretory vesicles. The strong positive reaction of the cytoplasm and secretory granules of the albumen gland of this species with the PAS technique decreases after acetylation and diastase treatment. Thus, the general histochemical tests also indicate the presence of glycogen and other neutral carbohydrates (Culling, 1974; Bancroft & Stevens, 1982) different from galactogen (Rangarao, 1963; Bolognani-Fantin & Vigo, 1968) inside secretory globules.

On the other hand, the strong reaction with AB at pH 0.5 together with the black-staining reaction with HID-AB, indicates the presence of sulphated acidic mucosubstances in the small granules contained in the large ones of the albumen gland secretion of *Arion subfuscus.* We conclude that the small and foamy-looking globules contain mainly glycosaminoglycans. Acidic groups have also been reported in the albumen gland of other gastropods (Rangarao, 1963; Bayne, 1967), but the secretory granules have always been described as ultrastructurally homogeneous without smaller clear granules inside (Nieland & Goudsmit, 1969).

As with other stylommatophorans (Bayne, 1967; Ramasubramaniam, 1979), no lipid has been detected in albumen secretions of *Arion subfuscus.*

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ESTRATEGIAS DE DEPREDACIÓN DEL GASTRÓPODO PERFORADOR *TROPHON GEVERSIANUS* (PALLAS) (MURICOIDEA: TROPHONIDAE)

Sandra Gordillo y Sandra N. Amuchástegui

Centro Austral de Investigaciones Científicas (CADIC, CONICET), C.C. 92 (9410) Ushuaia, Tierra del Fuego, Argentina

RESUMEN

Se analizaron las diferentes estrategias de depredación utilizadas por *Trophon geversianus* (Trophonidae) sobre cuatro especies de bivalvos típicos de la región fueguina: *Mytilus chilensis, Aulacomya atra, Hiatella solida* y *Tawera gayi.*

Los datos fueron obtenidos utilizando tres metodologías: análisis de valvas perforadas transportadas sobre la playa actual, estudio de la depredación bajo condiciones de laboratorio, y observación de la depredación en poblaciones naturales durante la bajamar.

Se concluye que (1) no todas las perforaciones producidas por los murícidos *sensu lato* son cilíndricas; (2) en las distintas presas hay variación en la forma y sitio de las perforaciones; y (3) la intensidad de depredación es baja a moderada.

Palabras clave: depredación, gastrópodos, perforaciones, Tierra del Fuego.

ABSTRACT

Predation strategies of *Trophon geversianus* (Trophonidae) on four bivalve species: *Mytilus chilensis, Aulacomya atra, Hiatella solida* and *Tawera gayi,* from the fuegian region were analyzed.

Data were obtained by three methods: analysis of bored shells transported on modern beachs, study of predation under laboratory conditions, and observation of predation on populations in the field.

We conclude that (1) not all muricid holes are cylindrical; (2) there is variation among prey species in the shapes of holes and in the sites of drilling; and (3) predation intensity is low to moderate.

Key words: predation, gastropods, drillholes, Tierra del Fuego.

INTRODUCCIÓN

Una característica notable de la mayoría de los moluscos es su capacidad para secretar un exoesqueleto calcáreo de protección. Otra, irónicamente, es la habilidad de algunos de estos moluscos de excavar o perforar dichos exoesqueletos mediante un proceso mecánico-químico, descripto por Carriker & Williams (1978) y Carriker (1981), entre otros.

Es ampliamente conocido que los principales responsables de dichas perforaciones son distintas especies que pertenecen a dos grupos dentro de Gastropoda. Por un lado, y entre los mesogastrópodos, figuran los natícidos (Naticidae). Por otro lado, y entre los neogastrópodos, la superfamilia Muricoidea comprende los murícidos *sensu stricto* (Muricidae), táididos (Thaididae) y trofónidos (Trophonidae) con especies perforadoras de valvas.

Los bivalvos epifaunales son atacados particularmente por los murícidos *sensu lato*, es decir por representantes de las distintas familias que integran el grupo Muricoidea (Kabat, 1990). Los bivalvos infaunales, en cambio, son comúnmente víctimas de los natícidos (Kabat, 1990; Anderson et al., 1991), aunque también pueden ser presas de algunos Muricoidea como lo mencionan Vermeij (1980) y Gordillo (1994) para distintas especies.

La morfología de la perforación ha sido utilizada por distintos autores para diferenciar familias. La perforación de los murícidos *sensu lato* es cilíndrica con bordes casi rectos; mientras que la perforación de los natícidos, en contraste, tiene una forma más paraboloide y bordes biselados (Carriker, 1981; Aitken & Risk, 1988; Kabat, 1990; Anderson et al., 1991). Un caso atípico dentro de Muricoidea lo constituyen los trofónidos, ca-

paces de realizar perforaciones que varían entre cilíndrica y cono-truncada, según la presa (Gordillo, 1994, 1995a).

Para Tierra del Fuego, trabajos anteriores realizados por Gordillo (1994, 1995a) y Amuchástegui (1995) describen la depredación de *Trophon geversianus* en distintas especies que viven en el litoral fueguino. El objectivo de este trabajo ha sido reunir y sintetizar toda la información recabada sobre la depredación de los trofónidos que caracterizan esta región.

UBICACIÓN Y CARACTERIZACIÓN DEL ÁREA DE ESTUDIO

El Canal Beagle se ubica entre la Isla Grande de Tierra del Fuego, la Isla Navarino y la Isla Hoste a los 54°LS y 68°LO. Este canal es de aguas tranquilas, con una profundidad media de aproximadamente 150–200 m y un litoral rocoso con playas de rodados. El canal posee un régimen de mareas de desigualdades diurnas con una amplitud media de 1 metro.

La temperatura media anual es de 5.5°C con una amplitud térmica moderada. La salinidad superficial del agua varía entre 27 y 31 gramos/litro (Iturraspe et al., 1989). La temperatura estival del agua oscila en 11–12°C, mientras que en invierno es de 3–4°C (Iturraspe et al., 1989).

MATERIAL Y MÉTODO

El principal depredador considerado fue el trofónido *Trophon geversianus* y, en segunda instancia, *Xymenopsis muriciformis*. Las presas estudiadas fueron cuatro especies de bivalvos típicos de la región: *Mytilus chilensis, Aulacomya atra, Hiatella solida* y *Tawera gayi*. El Apéndice presenta la posición taxonómica de las especies tratadas según Gordillo (1995b). El material estudiado procede de distintos sitios de las zonas mesolitoral e infralitoral del Canal Beagle. Los especímenes se obtuvieron por recolección manual durante la bajamar o con buceo autónomo a profundidades de hasta 3 metros.

Los datos sobre los que posteriormente se aplicaron los distintos parámetros fueron obtenidos utilizando tres metodologías:

Análisis de valvas perforadas transportadas sobre la playa actual: el material procede de distintas playas indicadas en la Figura 1. En cada sitio, y dentro del supralitoral, se tomó como unidad de muestreo un cuadrante de 0.5 × 0.5 m con un número de réplicas predeterminado. En cada cuadrante se contabilizaron todas las valvas de las cuatro especies que aparecieron en sus respectivas superficies.

Estudio de la depredación bajo condiciones de laboratorio: se utilizaron acuarios de vidrio de distinta capacidad, según la experiencia, variando la cantidad de depredadores y presas (Tabla 1). Los ejemplares fueron recolectados del mesolitoral e infralitoral de distintas localidades del Canal Beagle (Fig. 1). Los acuarios se mantuvieron en la cámara fría del CADIC a una temperatura de 2 a 9°C. Diariamente se realizaron observaciones sobre el comportamiento de los depredadores y se retiraron las valvas vacías.

Observación de la depredación en condiciones naturales durante la bajamar: se analizó la presencia de *Trophon geversianus* en una superficie que varió, según las características del sitio elegido, entre 2.5 y 50 m^2. Estos muestreos se realizaron mensualmente durante la bajamar (con un nivel menor a 0.45 m) en las localidades indicadas en la Figura 1. Se midió la longitud de los ejemplares hallados, consignando si depredaban o no, particularmente sobre *Mytilus chilensis* por ser la presa más abundante del mesolitoral.

Los parámetros analizados fueron los siguientes:

Morfología de la perforación: para analizar este parámetro se midieron los diámetros externos e internos (mm) de la perforación, utilizando una lupa binocular con retículo.

Selectividad por el sitio a perforar: para determinar la preferencia con respecto al sitio de perforación en las distintas especies, la superficie de la valva fue dividida en regiones según muestra la Figura 2. Para analizar este parámetro se sometieron los datos a la prueba estadística de Chi-cuadrado (X^2). Para este y otros parámetros de selectividad se hace referencia a "selectivo" para expresar la preferencia o comportamiento estereotípico y "no selectivo" para denotar un comportamiento aleatorio.

Selectividad por la valva derecha o izquierda: para analizar la preferencia por alguna de las dos valvas opuestas, los valores obtenidos también se sometieron a una prueba de Chi-cuadrado (X^2).

Selectividad por un tamaño de la presa: en valvas transportadas, para determinar la existencia de selectividad por un tamaño de valva

TABLA 1. Experiencias de laboratorio. Se incluye el tamaño y el número o peso de las presas y de los depredadores y la capacidad de los acuarios.

ACUARIOS	PRESAS	DEPREDADORES	CAPACIDAD
Acuario A	*Mytilus chilensis* 100 gr. (x < 30 mm) 100 gr. (30 mm < x < 50 mm) 100 gr. (50 mm < x < 70 mm)	6 *Trophon geversianus* (23 mm ≤ x ≤ 45.6 mm)	19 litros
Acuario B	igual al Acuario A	6 *Trophon geversianus* (22.7 mm ≤ x ≤ 43.3 mm)	19 litros
Acuario C	*Aulacomya atra* 100 gr. (x < 30 mm) 100 gr. (30 mm < x < 50 mm) 100 gr. (50 mm < x < 70 mm)	6 *Trophon geversianus* (18.5 mm ≤ x ≤ 52.3 mm)	19 litros
Acuario D	igual al Acuario C	6 *Trophon geversianus* (22.7 mm ≤ x ≤ 45.3 mm)	19 litros
Acuario E	*Mytilus chilensis* n = 25 (24 mm ≤ x ≤ 65 mm) *Tawera gayi* n = 3 (31 mm; 31.7 mm y 33 mm)	3 *Trophon geversianus* (30 mm; 48 mm y 54 mm)	4 ½ litros
Acuario F	*Tawera gayi* n = 20 (20 mm ≤ x ≤ 36 mm) *Hiatella solida* n = 5 (20 mm ≤ x ≤ 45.4 mm)	6 *Trophon geversianus* (33.0 mm ≤ x ≤ 51.5 mm)	4 ½ litros

en particular, se relacionó la distribución de frecuencias por tamaño (longitud), considerando separadamente las valvas no perforadas, y las valvas perforadas. Con los datos obtenidos en laboratorio se calculó la relación entre la cantidad de presas ofrecidas y las presas consumidas según su tamaño. Para analizar este parámetro, y en ambos casos, se aplicó la prueba de Chi-cuadrado (X^2).

Correlación entre el tamaño del depredador y el tamaño de la presa: se calculó en forma directa mediante la medición de los especímenes que fueron observados depredando y de sus presas. Se realizó bajo condiciones de laboratorio y en poblaciones naturales en el caso de *Mytilus chilensis.* Para expresar la correlación se calculó el coeficiente de correlación (r).

Éxito-fracaso de la depredación (drilling success; Tull & Bohning-Gaese, 1993): se partió del supuesto de que una perforación incompleta constituye un evento fallido de depredación. Este parámetro expresa el porcentaje de éxito y resulta de dividir el número de perforaciones completas por el número total de intentos, dado por la suma de las perforaciones incompletas y completas. Los porcentajes de depredación obtenidos se expresaron según la siguiente escala: 0–20%: muy poco exitosa; 20–40%: poco exitosa; 40–60%: moderadamente exitosa; 60–80%: exitosa y 80–100%: muy exitosa.

Índice de depredación (predation rate, Vermeij, 1980): la proporción de depredación se calculó tomando la proporción de valvas perforadas en relación al número total de valvas. Los valores obtenidos se expresaron según la siguiente escala: 0.0–0.25: bajo; 0.25–0.50: moderado; 0.50–0.75: elevado y 0.75–1.0: muy elevado.

Tiempo de depredación: un ciclo de depredación está dado por el período de alimentación y el de descanso hasta que se inicia un nuevo período de alimentación. Para determinar el tiempo de depredación, y siempre bajo condiciones de laboratorio, se calculó el tiempo transcurrido (en días) desde que el depredador se apoyó sobre la presa hasta que la abandonó. Luego se tomó, también en días, el período de descanso transcurrido hasta que un mismo depredador atacó a otra presa. El tiempo de depredación se expresó arbitrariamente en: "variable" cuando el desvío resultó mayor o igual a la media aritmética dividida 2 y "estable" cuando el desvío fue menor a dicho cociente.

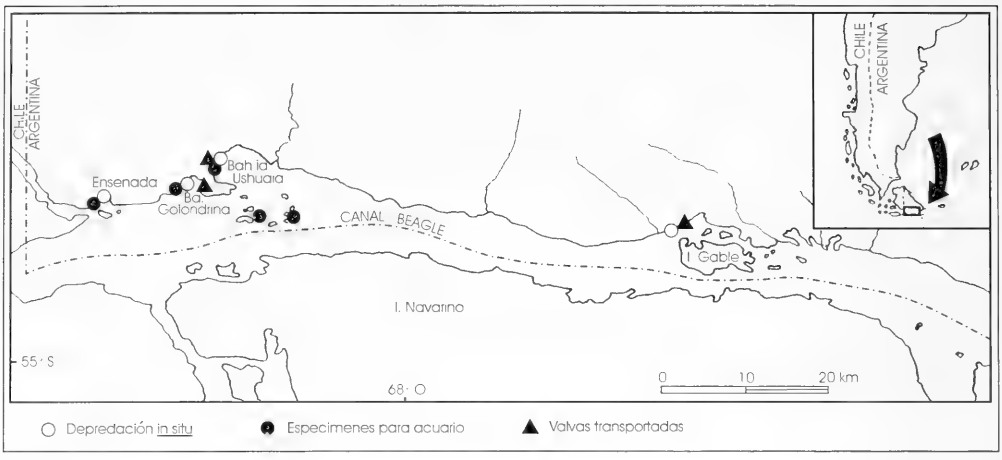

FIG. 1. Mapa de Ubicación. Los símbolos señalan los sitios de procedencia de las muestras.

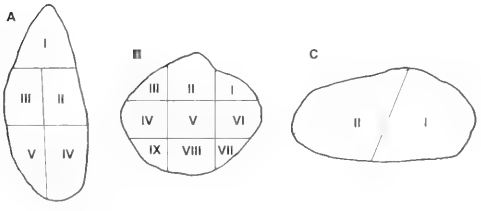

FIG. 2. Sectores en que se subdividieron las valvas de las presas para analizar la selectividad por el sitio a perforar. A, *Mytilus chilensis.* B, *Tawera gayi.* C, *Hiatella solida.*

RESULTADOS

Morfología de la Perforación

Las perforaciones producidas por *Trophon geversianus* mostraron algunas diferencias morfológicas en las distintas presas (Tabla 2, A; Fig. 3).

En *Hiatella solida,* Gordillo (1994) observó que *Trophon geversianus* realiza perforaciones cono-truncadas, tal como se esquematiza en la Figura 3, A.

Las perforaciones excavadas por este mismo depredador en valvas de *Mytilus chilensis* también son de tipo conotruncado, similares a las descriptas previamente por Gordillo (1994, 1995a) para *Tawera gayi* (Fig. 3,B).

Sobre *Aulacomya atra,* las perforaciones producidas por *Trophon geversianus* se diferencian de las anteriores en que se asemejan más a un cilindro simple (Fig. 3, C).

En relación al depredador *Xymenopsis muriciformis* se constató que bajo condiciones de laboratorio, éste produce en *Mytilus chilensis* perforaciones que se asemejan más a la morfología cilíndrica, por las menores diferencias entre los diámetros externo e interno; i.e. sobre un total de 19 ejemplares de esta especie, los diámetros externo e interno fueron respectivamente 1.79 ± 0.2 mm y 1.22 ± 0.16 mm. En *Tawera gayi* y *Hiatella solida,* bajo condiciones de laboratorio, solamente fueron observadas perforaciones de este tipo en un caso para cada especie. Algunas valvas transportadas de *Tawera gayi* (n = 14) presentaron una morfología similar, razón por la cual fueron atribuidas a este depredador. En estas perforaciones se obtuvo un diámetro único de 1.81 ± 0.30 mm, ya que no se notó diferencias entre los dos diámetros.

Finalmente, se observó que las perforaciones incompletas dejadas por *Trophon geversianus* y *Xymenopsis muriciformis* en las distintas presas coincidían en tener el fondo plano.

Sitio de Perforación

Las zonas elegidas por los depredadores variaron según las distintas presas consideradas (Tabla 2, B).

En las valvas de *Mytilus chilensis* se observó que alrededor del 50% de las valvas (53% en el primer caso y 59% en el segundo) estaban perforadas en los sectores II y III, que corresponden a la zona media ventral y dorsal respectivamente, por lo que se consideró que hay preferencia por la zona media.

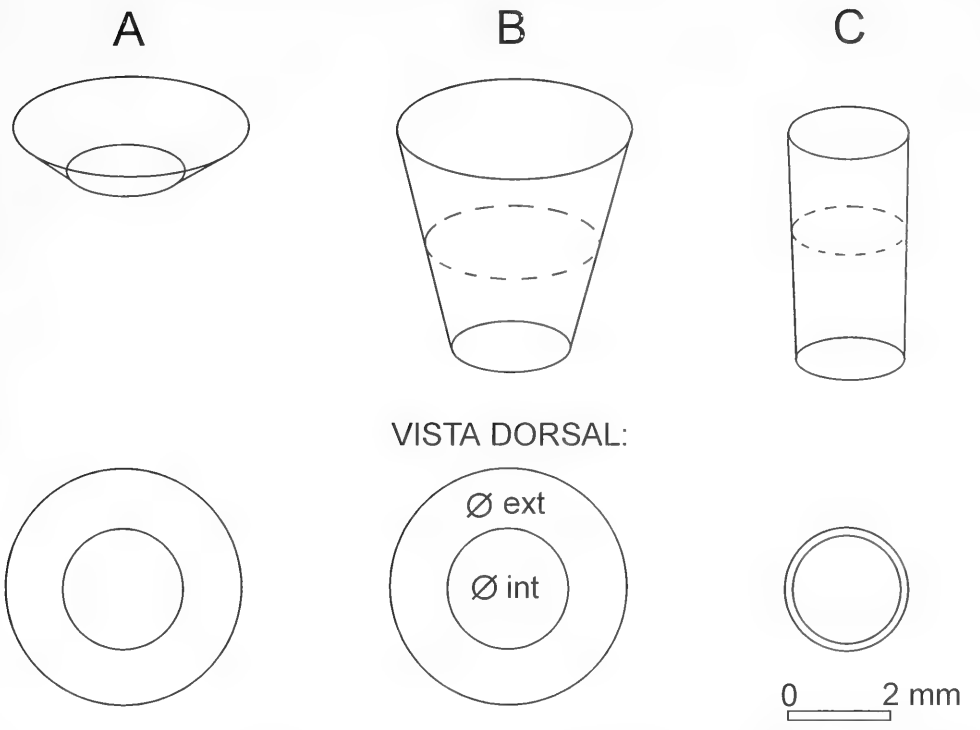

FIG. 3. Morfología de la perforaciones producidas por *Trophon geversianus* en A, *Hiatella solida*. B, *Mytilus chilensis* y *Tawera gayi*. C, *Aulacomya atra* Referencias: o_{ext}, diámetro externo. o_{int}, diámetro interno [Tomado de Gordillo (1994)].

Con respecto al mitílido *Aulacomya atra,* y sólo bajo condiciones de laboratorio, se pudo constatar que las perforaciones se encontraron en el 100% de los casos en proximidades del borde de las valvas, lo que marcó una gran diferencia con el sitio que *Trophon geversianus* elige para perforar las valvas de *Mytilus chilensis.*

En las valvas de *Tawera gayi* hubo preferencia por el sector V o medio central donde se concentraron el 50% de las perforaciones (Gordillo, 1994).

En *Hiatella solida* el 77% de las perforaciones se encontraron en el sector II o posterior en torno a una cóstula radial característica de la especie (línea punteada de la Fig. 2, C), descripto con anterioridad por Gordillo (1994). Una proporción de las mismas (61%) se halló dentro de la zona de inserción del músculo posterior.

Respecto a este parámetro, también se obtuvo que el segundo depredador considerado, *Xymenopsis muriciformis,* eligió el sector umbonal en valvas de *Tawera gayi* en el 100% de los casos (n = 14). Sobre *Mytilus chilensis* (n = 19) no se halló selectividad por un sitio particular, estando las perforaciones distribuidas en toda la superficie de la valva. En *Aulacomya atra* se produjo un único caso de perforación por *Xymenopsis muriciformis* en que la misma se ubicó en el borde de la valva, similar a *Trophon geversianus.*

Preferencia por la Valva Derecha o Izquierda

Para las distintas presas se observó que no hubo preferencia por una de las valvas, ya sea derecha o izquierda (Tabla 2, C). Este parámetro no pudo calcularse para *Aulacomya atra* ya que las perforaciones ubicadas en el borde abarcaron en la mayoría de los casos las dos valvas opuestas.

Selectividad por un Tamaño

Hubo variaciones en los resultados obtenidos en relación a este parámetro (Tabla 2, D).

Con los datos de laboratorio se obtuvo la

proporción de presas ofrecidas y consumidas por *Mytilus chilensis* y *Aulacomya atra.*

Para el caso de *Mytilus chilensis* se observó una marcada preferencia de *Trophon geversianus* por las presas más grandes (50–70 mm). En *Aulacomya atra* los ejemplares seleccionados comprendieron un rango más amplio, ya que el depredador prefirió los ejemplares medianos (30–50 mm) y los grandes (50–70 mm).

En valvas transportadas de estas dos especies, y en base a la distribución de frecuencias de valvas perforadas y no perforadas también se observó preferencia por las valvas más grandes.

En cambio, con la misma metodología, en *Tawera gayi* y *Hiatella solida,* no se observó selectividad por un tamaño particular. En estas dos especies, la mayor proporción de perforaciones sobre valvas con un largo promedio de 31 mm se relaciona directamente con la mayor cantidad de valvas de dicho tamaño y no con una selección del mismo.

Relación Entre los Tamaños de la Presa y del Depredador

Según la especie y la metodología empleada, los resultados variaron entre sin correlación hasta una ligera correlación positiva (Tabla 2, E).

En *Mytilus chilensis,* y bajo condiciones naturales, este parámetro mostró una ligera correlación positiva. Sin embargo, los resultados de laboratorio indicaron ausencia de correlación.

En *Aulacomya atra* con los datos de laboratorio se obtuvo una correlación positiva entre los tamaños del depredador y de sus presas. Bajo condiciones naturales, solamente en cuatro oportunidades se observó a *Trophon geversianus* depredando sobre ejemplares chicos (menores a 30 mm) de *Aulacomya atra.*

Finalmente, con los datos de laboratorio de *Tawera gayi* tampoco se encontró correlación entre estas variables.

Perforaciones Incompletas: Éxito-Fracaso de la Depredación

Los resultados mostraron (Tabla 2, F) que la depredación de *Trophon geversianus* varió entre muy exitosa en *Aulacomya atra* (98.7–100%); muy exitosa a exitosa en *Hiatella solida* (63.7–100%); y exitosa en *Tawera gayi* (64.4–74.1%) y en *Mytilus chilensis* (79–93.3%).

Para estas especies las valvas presentaron una sola perforación, ya sea completa o incompleta. Una excepción la constituye la depredación en *Tawera gayi* bajo condiciones de laboratorio, en que algunas presas fueron atacadas simultáneamente por dos depredadores distintos, resultando perforadas las dos valvas en un 25% de los casos.

Proporción de Valvas Perforadas: Índice de Depredación

El índice de depredación resultó bajo para *Mytilus chilensis, Aulacomya atra* y *Tawera gayi,* mientras que fue moderado en el caso de *Hiatella solida* (Tabla 2, G).

Tiempo de Depredación

Se desconoce aún el tiempo que emplea el depredador en excavar la perforación; sin embargo, los resultados (Tabla 2, H) hacen referencia al tiempo promedio transcurrido desde que el depredador se apoyó sobre la presa hasta que la abandonó.

Los tiempos de depredación y de descanso fueron muy variables en *Mytilus chilensis* y *Aulacomya atra,* y relativamente más estables en *Tawera gayi* y *Hiatella solida,* aunque el número de observaciones en estas dos especies fue menor.

DISCUSIÓN

En relación a la morfología de la perforación es importante destacar que la perforación cono-truncada que *Trophon geversianus* excava en valvas de *Mytilus chilensis, Tawera gayi* y *Hiatella solida* se asemeja más a la perforación típica de los natícidos que a la perforación producida por los murícidos *sensu lato.*

Sin embargo, una forma de diferenciar estas perforaciones de aquellas producidas por los natícidos es a través de las perforaciones incompletas; ya que en los natícidos éstas presentan un fondo deprimido con una prominencia o giba central (Aitken & Risk, 1988; Kabat, 1990), mientras que la perforación incompleta realizada por los dos trofónidos *Trophon geversianus* y *Xymenopsis muriciformis* se caracteriza por tener el

TABLA 2. Cuadro comparativo de las principales características observadas en las cuatro presas en relación a los distintos parámetros considerados

PRESAS	A. Morfología de la perforacion	B. Selectividad del sitio	C. Selectividad por la valva (der/izq)	D. Selectividad por tamaño	E. Correlación de tamaño	F. Éxito/fracaso de la depredación (%)	G. Índice de depredacíon	H. Tiempo de depredación	REFERENCIAS
Mytilus chilensis (MYTILIDAE)	cono-truncada $Ø_{ext}$ = 2.36 ± 0.52 mm $Ø_{int}$ = 1.38 ± 0.21 mm n = 65 (2)	Selectivo 50% en sectores II y III (31% y 22% en sectores II y III resp.) n = 295 (1) (40% y19% en sectores III y II resp.) n = 85 (2) X^2 Signif. al 95%	No selectivo 150 : 145 n = 295 (1) 41 : 44 n = 85 (2) X^2 NS al 95%	Selectivo Selectividad por valvas x > 60 mm n = 897 rango = 20–85 mm GL: 9 (1) ofrecido:consumido (2) X^2 Signif. al 95%	Sin correlación a ligera correlación positiva r = 0.06 GL: 86 (2) NS al 95% r = 0.17 GL: 132 (3) Signif. al 95%	Exitosa 79% n = 391 (1) 93.3% n = 87 (2)	Bajo 0.16 n = 3969 (1)	Variable 9.09 ± 5.2 días de depredación, n = 64; 13.81± 12.9 dias de descanso n = 63 (2)	Amuchástegui (1995); este trabajo.
Aulacomya atra (MYTILIDAE)	cilindro simple $Ø_{ext}$ = 1.76 ± 0.41 mm $Ø_{int}$ = 1.63 ± 0.38 mm n = 69 (2)	Selectivo 100% en el borde (el 79% en sectores IV y V) n = 76 (2) X^2 Signif. al 95%	— 	Selectivo Selectividad por valvas x > 30 mm ofrecido:consumido (2) X^2 Signif. al 95%	Correlación positiva r = 0.54 GL: 76 (2) Signif. al 95%	Muy exitosa 100% n = 16 (1) 98.7% n = 76 (2)	Bajo 0.12 n = 273 (1)	Variable 10.03 ± 6.9 días de depredación, n = 66; 13.84 ± 2.9 días de descanso, n = 54 (2)	Amuchástegui (1995); este trabajo.
Tawera gayi (VENERIDAE)	cono-truncada $Ø_{ext}$ = 3.7 ± 0.65 mm $Ø_{int}$ = 2.2 ± 0.49 mm n = 69 (1) $Ø_{ext}$ = 3.22 ± 0.60 mm $Ø_{int}$ = 1.6 ± 0.27 mm n = 20 (2)	Selectivo 51.6% sector V 22% sector II el % restante repartido en 7 sectores (1) n = 240 X^2 Signif. al 95%	No selectivo 165 : 194 n = 359 (1)(2) X^2 NS al 95%	No selectivo n = 543 rango = 16–40 mm GL: 4 (1) X^2 NS al 95%	No hay correlación r = 0.42 GL: 16 (2) NS al 95%	Exitosa 64.4% n = 183 (1) 74.1% n = 16 (2)	Bajo 0.22 n = 543 (1)	Estable 8.06 ± 2.8 días de depredación, n = 17; 7.29 ± 5.6 días de descanso n = 7 (2)	Gordillo (1994; 1995); este trabajo.
Hiatella solida (SAXICAVIDAE)	cono-truncada $Ø_{int}$ = 1.93 ± 0.64 mm n = 33 (1) $Ø_{int}$ = 2.0 ± 0 mm n = 5 (2)	Selectivo 77% sector II n = 49 (1) X^2 Signif. al 95%	No selectivo 27:35 n = 62 (1)(2) X^2 NS al 95%	No selectivo n = 67 r ango = 24–38 mm GL: 2 (1) X^2 NS al 95%	s/d	Exitosa a Muy exitosa 100% n = 33 (1) 63.7% n = 5 (2)	Moderado 0.42 n = 79 (1)	Estable 6.3 3 ± 0.5 días de depredación n = 3; 14 días de descanso (sólo 1 valor) (2)	Gordillo (1994; 1995); este trabajo.

(1) valvas transportadas; (2) experiencias en laboratorio; (3) depredación *in situ;* NS: no significativo; GL: grados de libertad; s/d: sin datos; .$Ø_{ext}$: diametro externo; diametro interno: $Ø_{int}$

fondo plano, y sin ninguna prominencia central.

En *Aulacomya atra* la perforación dejada por *Trophon geversianus* es relativamente más cilíndrica, es decir con menor diferencia entre diámetros externo e interno. Este tipo de perforación se asemeja más a la perforación producida por el otro depredador *Xymenopsis muriciformis* sobre valvas de *Mytilus chilensis, Tawera gayi* y *Hiatella solida.*

Sólo en una oportunidad se observó una perforación producida por *Xymenopsis muriciformis* en una valva de *Aulacomya atra,* y si se compara su morfología con la perforación de *Trophon geversianus* sobre la misma presa, no se detectan diferencias morfológicas, incluso tampoco por su ubicación ya que ambos depredadores dejan sus marcas en el borde.

También cabe mencionar en relación a *Mytilus chilensis* y *Aulacomya atra* que ejemplares previamente perforados por *Trophon geversianus* fueron capaces de reparar su valva en la zona interna exactamente opuesta al sitio de la perforación. Este mecanismo de reparación también fue observado por Griffiths & Blaine (1994) en *Mytilus galloprovincialis* del hemisferio norte.

Otro aspecto que merece ser destacado es la selectividad por un sitio particular de la valva. El registro fósil muestra que en los murícidos *sensu lato* las perforaciones pueden estar distribuídas al azar (Aitken & Risk, 1988; Tull & Bohning-Gaese, 1993) o ser selectivas (Vermeij et al., 1989; Guerrero & Reyment, 1988). Las observaciones de este trabajo, y las obtenidas previamente por Gordillo (1994) y Amuchástegui (1995), también indican selectividad en relación a este parámetro, en coincidencia con lo señalado por Vermeij et al., (1989) y Guerrero & Reyment (1988) para otras especies de este grupo. El sitio elegido por *Trophon geversianus* para realizar su perforación varía en las distintas presas, y estaría condicionado por la morfología de la presa en relación a su hábitat.

En relación a la selectividad por una valva en particular, los resultados muestran que no existe selectividad o preferencia por una de las valvas ya sea derecha o izquierda, lo que se atribuye a que las presas exponen indistintamente las dos valvas al depredador.

Las diferencias registradas al analizar la selectividad por un tamaño particular de las presas quizás se relacione con otros aspectos no considerados en este trabajo como la existencia de otros depredadores, o con los tamaños relativos que alcanzan las presas. La selectividad observada en *Mytilus chilensis* coincide con la selectividad registrada por Zaixso & Bala (1995) en poblaciones naturales de esta especie a una menor latitud, en Santa Cruz (Argentina). La ausencia de selectividad por tamaño en *Tawera gayi* y *Hiatella solida* concuerda con los resultados obtenidos por Aitken & Risk (1988) para la especie antes mencionada del hemisferio norte.

El éxito de depredación con un rango variable entre un 63.7–100% entre las distintas presas permiten considerar a *Trophon geversianus* como un depredador eficiente. Esta característica podría constituir una ventaja evolutiva si se considera que una perforación incompleta representa un gasto energético inútil, tal como lo considera Kabat (1990) para los natícidos.

Los índices de depredación que variaron entre bajos a moderados indicarían que *Trophon geversianus* es responsable de la mortalidad de una proporción baja a moderada de las poblaciones de las distintas presas.

Finalmente, para interpretar las variaciones de los tiempos de depredación de *Trophon geversianus* sobre las distintas especies, además del tamaño de la presa habría que analizar otros aspectos no considerados en este trabajo, como el grosor de la valva y/o su naturaleza mineralógica.

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APÉNDICE

Ubicación sistemática de las especies consideradas

Clase Gastropoda
- Subclase Prosobranchia
  - Orden Neogastropoda
    - Superfamilia Muricoidea
      - Familia Trophonidae

Trophon geversianus (Pallas)
Xymenopsis muriciformis (King)

Clase Bivalvia
- Subclase Pteriomorpha
  - Orden Mytiloida
    - Familia Mytilidae

Mytilus chilensis (Hupé)
Aulacomya atra (Molina)
- Subclase Heterodonta
  - Orden Veneroida

Familia Veneridae

Tawera gayi (Hupé)
  - Orden Myoida
    - Familia Hiatellidae

Hiatella solida (Sowerby)

MALACOLOGIA, 1998, 39(1-2): 93-111

CLADISTIC ANALYSIS OF THE XANTHONYCHIDAE (= HELMINTHOGLYPTIDAE) (GASTROPODA: STYLOMMATOPHORA: HELICOIDEA)

Maria Gabriela Cuezzo

Facultad de Ciencias Naturales e Instituto Miguel Lillo, Universidad Nacional de Tucumán-CONICET, Miguel Lillo 205, 4000 Tucumán, Argentina

ABSTRACT

A cladistic analysis of the family Xanthonychidae (= Helminthoglyptidae) was carried out. The data set consisted of 34 characters and 25 terminal taxa (including the four outgroups: *Oreohelix, Neohelix, Helix,* and *Bradybaena*). Two different analyses were performed using the program Hennig86. The preferred hypothesis is represented by one of the two trees obtained after successive weighting in the first analysis, which has the same topology of one of the original set of 50. The conclusions of this study suggest that: (1) the family Xanthonychidae as defined by Pilsbry (1939) and Zilch (1959–1960) is a paraphyletic group, (2) Bradybaenidae, Helicidae and Xanthonychidae form a monophyletic group and therefore should be analyzed together as a unit, (3) based upon the preferred hypothesis, three monophyletic groups can be defined, although taxonomic changes will have to wait until a new analysis of the Bradybaenidae-Xanthonychidae-Helicidae group is performed.

Key Words: Stylommatophora, Helicoidea, Xanthonychidae, cladistics, phylogeny, characters.

INTRODUCTION

The Xanthonychidae (= Helminthoglyptidae) are a large group of land snail and semislug genera distributed along the Americas. Pilsbry (1939), while describing the land snail fauna of North America, established the family Helminthoglyptidae, composed of all the "American dart-bearing helices." In this work, he named only five (Helminthoglyptinae, Sonorellinae, Humboldtianinae, Cepolinae and Epiphragmophorinae) out of eight subfamilies he included in the Helminthoglyptidae. The remaining three subfamilies are probably the Central American groups (Pilsbry, 1900, 1927): Lysinoinae, Xanthonychinae, and Metostracinae. Zilch (1959–1960) gave a complete list of the subfamilies and genera included in the Helminthoglyptidae. Richardson (1982) catalogued the Helminthoglyptidae at species level.

Baker (1943, 1959) pointed out that the name Xanthonychidae Strebel & Pfeiffer, 1879, has priority over Helminthoglyptidae Pilsbry, 1939. *Xanthonyx* was also the first genus of an American helicoid described (Fischer & Crosse, 1872). However, only a few authors adopted Xanthonychidae as the correct name for the family (Nordsieck, 1987). Helminthoglyptidae continued to be used to refer to the North American helicoids (Roth, 1996), while Xanthonychidae was employed for the Central and South American helicoids (Emberton, 1991; Miller & Naranjo-Garcia, 1991; Schileyko, 1991). Other authors, however, continued using the name Helminthoglyptidae for all the American helicoids (North, Central and South) (Zilch, 1959–1960; Solem, 1978; Richardson, 1982). For a complete chronologic review of previous studies and tendencies, see Roth (1996). As a result, whenever the names Helminthoglyptidae or Xanthonychidae are used, it is not clear to which genera the authors are referring, unless they state which convention they are following.

For years many authors (Pilsbry, 1939; Nordsieck, 1987; Miller & Naranjo-Garcia, 1991; Schileyko, 1991) have searched for a coherent definition of the family. Pilsbry's (1939) was the concept that has lasted longest. His definition of the Xanthonychidae (= Helminthoglyptidae) is a combination of character states, none of which represents true synapomorphies for the group. The geographic distribution became important in the identification of the genera. Similarly, the absence of clearly defined synapomorphies lead to poor definitions of the other families included in Helicoidea (Scott, 1996). Recent

studies have appeared reviewing the Helicoidea (Nordsieck, 1987; Schileyko, 1991), but they failed to propose their hypotheses in a testable way. The only two studies using cladistic analysis to formulate phylogenetic hypotheses within Helminthoglyptidae are Pearce (1990) and Roth (1996).

The anatomy of several components of the Xanthonychidae is still poorly known. Although several papers have been published with anatomical data on the Central American (Binney, 1879; Pilsbry, 1894, 1900; Baker, 1942; Thompson, 1959; Miller, 1987; Tillier, 1989; Cuezzo, 1996) and South American taxa (Hesse, 1930; Fernandez & Rumi, 1984; Tillier, 1989; Cuezzo, 1997), they are still the least known components of the Xanthonychidae.

Three different problems occurred in the published literature of the Xanthonychidae: (1) Monographic revisions are scarce, and most of the existing studies focus on the description of single species upon which phylogenetic hypotheses are built (Pilsbry, 1900; Pilsbry & Cockerell, 1937; Hylton Scott, 1951, 1962; Parodiz, 1955; Hass, 1962; Miller, 1970, 1971, 1976a, b, 1981, 1985). (2) The taxonomic differences among the genera included in the Xanthonychidae are mainly based on two organs: mucous glands and dart sac (= dart complex), with different combinations of their character states (generally shape and number). In this way, phylogenetic assumptions and classifications (Miller & Naranjo-Garcia, 1991; Schileyko, 1991) had been carried out on single characters or at least single-organ systems (generally the genital system) (Bieler, 1992). (3) Phylogenetic relationships of the genera are established on arbitrarily narrative character transformations (Nordsieck, 1987; Miller & Naranjo-Garcia, 1991; Schileyko, 1991). As stated by Nixon & Carpenter (1993), there is no clear way to establish whether a character is "primitive" or "derived" prior to cladistic analysis, and actually there is no need for the creation of "evolutive scenarios" to explain the possible direction of character transformation.

This study was undertaken (1) to test the monophyly of the Xanthonychidae (= Helmintoglyptidae) (2) to carry out a cladistic analysis in order to hypothesize the relationships among the components of the family in a testable way, and (3) to review the current classification of the Xanthonychidae based on the cladograms obtained.

MATERIALS AND METHODS

The adult alcohol preserved snails used for this study belong to the following Institutions:

ANSP = Academy of Natural Sciences of Philadelphia, Pennsylvania, USA

INBIO = Instituto de Biodiversidad, Costa Rica.

UF = Florida Museum of Natural Sciences, Gainesville, Florida, USA

FMNH = Field Museum of Natural History, Chicago, Illinois, USA

FML = Fundacion Miguel Lillo, Tucumán, Argentina

In the case of the material from ANSP, the first number cited after the institution corresponds to dry lots consulted every time that a verification of the species determination was necessary. A number preceded by the letter "A" (in the case of material from ANSP or FML) corresponds to alcohol-preserved material used in this study. All catalogue numbers from other institutions correspond to alcohol-preserved material. A complete list of the taxa studied is documented in Appendix 1.

The characters used in this study (except characters 30 and 31, which are based on published literature) are based on a selection made after a study of the anatomy of the pallial, digestive, reproductive and nervous systems, and external morphology of the type species of each genus (Appendix 1). Specimens were dissected under a Wild M3C microscope. Illustrations were made with the aid of a camera lucida. Illustrations and a brief description of the characters are included only when there is no agreement with the literature or a clarification of a specific character is needed. Shell characters have not been considered for this analysis under the assumption that they are inadequate for reconstructing phylogenetic history (Nordsieck, 1986; Emberton, 1995). However, the study of the sculpture, apertural barriers and composition of the shell could lead in the future to informative characters (Solem, 1978; Emberton, 1995).

Cladistic analyses were carried out using the computer program Henning86 (Farris, 1988). The program DADA (Nixon, 1992) was used for the construction of the data matrix and CLADOS (Nixon, 1992) for the analysis of the character distribution on the trees. Jackknife, a statistical test for homoplasy, was performed with the program NONA (Goloboff, 1993). FQ, a program provided by P. Goloboff,

integrated jackknife results from 50 replicates. FQ reads a "tread" statement (resulting trees in parenthetical notation) and calculates the majority rule consensus tree showing the frequencies above 50% (frequencies of 100% are not indicated).

The frequency index is:

$$\frac{\sum fi}{(T-2)}$$

where fi is frequency of group i of consensus tree, and T is the number of terminals, note that these depend on the cutoff value.

The data set includes 34 characters and 25 taxa (including the outgroups) (Table 1). Thirteen characters are multistate and were coded as non-additive so that any state could transform into any alternative state at an equal cost. Character polarity is derived from the analysis rather than being an a priori assumption (Nixon & Carpenter, 1993). The assignment of "missing character" ("?") to some taxa is not mentioned in the character description but indicated in the data matrix. Some apomorphies, although not informative for constructing phylogenies, were included among the characters because they are usfull for the characterization of certain terminals. Cladistic analysis was initially carried out with Hennig86 using the commands "mh*;bb*;". Successive weighting (Farris, 1969; Carpenter, 1988) was used after the initial runs. From the cladograms obtained after successive weighting, one cladogram was selected. When more than one tree was obtained, they were sumarized in a Nelson consensus tree.

RESULTS

Selection of the Ingroup and Outgroup Taxa

The xanthonychid (= helminthoglyptid) ingroup taxa considered in this analysis are those included in the family by Zilch (1959–

TABLE 1. Data set containing thirty four characters and twenty five taxa used in analysis #1. Two additional characters (#35–36) were used in analysis #2.

	10	20	30		
Outgroups	123456789	123456789	123456789	1234	56
Oreohelix	–000000100	3011101000	0000000001	01100	00
Neohelix	–000001002	1011101000	0000000001	?0010	00
Helix	–000000001	0011101120	1000000000	10010	11
Bradybaena	–000000011	0000101021	0000000100	10011	24
Xanthonychidae					
Cepolis	–000000011	0021131111	0100001200	10011	23
Polymita	–000002011	1011131111	0100001200	10011	23
Dialeuca	–00000200?	0000130011	0100001200	10021	23
Helminthoglypta	–000000011	0000111111	0010002100	10021	22
Epiphragmophora	–000000011	0100101111	0000002200	10011	24
Monadenia	–000002012	1021111111	0000003000	10021	21
Sonorella	–000002012	0022101100	0000000000	10021	00
Eremarionta	–000000010	1021101021	0021000100	10021	22
Micrarionta	–000000010	?021101121	0001000?00	10021	22
Humboldtiana	–000002012	1021101120	1000000000	10021	13
Charodotes	–000000011	0000121111	0010002100	10021	22
Plesarionta	–000002011	1021101121	0001000000	10011	22
Bunnya	–010010011	2012101120	1000113010	?0011	13
Tryonigens	–100000010	0021001000	0000000000	10020	00
Trichodiscina	–0000000?0	0022101121	0000000000	10021	24
Cryptostrakon	–001110010	0011201121	0000000000	?0012	21
Lysinoe	–100001010	1021101120	1000000100	10010	13
Leptarionta	–10000?01?	20?2101121	0000000000	10021	24
Metostracon	–001110010	0011101121	0000023000	?0010	24
Xanthonyx	–010010010	2021101120	100001?000	?0010	14
Xerarionta	–000002010	1000101121	1001000000	10011	32

1960), with the exception of *Averellia* Ancey, *Dinotropis* Pilsbry & Cockerell, *Sonorelix* Berry, and *Setipelis* Pilsbry, for which no alcohol-preserved material was available. For the purpose of the analysis, subgenera as well as genera (*sensu* Zilch, 1959–1960) have been used as taxonomic units when material was available. This is the case for *Plesarionta* Pilsbry, *Xerarionta* Pilsbry, and *Eremarionta* Pilsbry, which were considered by Zilch to be subgenera of *Micrarionta* Ancey. *Charodotes* Pilsbry, considered a subgenus of *Helminthoglypta* Ancey by Zilch, and *Trichodiscina* Martens, considered a subgenus of *Averellia,* are also treated as separate terminals.

For each genus, the type species, when available, was used as representative of the group. The only exceptions are *Xanthonyx* Crosse & Fischer, *Eremarionta* Pilsbry, *Leptarionta* Fischer & Crosse, and *Xerarionta* Forbes. Consequently, other species were selected as representative of those genera.

For the selection of outgroups, a previous cladistic analysis was used (Nixon & Carpenter, 1993). Based on Emberton (1991), the Bradybaenidae, Helicidae, Oreohelicidae, and Polygyridae were chosen as outgroups. Although the use of more than one outgroup is not necessary, this option was prefered, with the idea that the cladistic inferences might be better founded. Outgroups were treated as all the other terminals in order to test the monophyly of the ingroup. The Bradybaenidae were traditionally assumed to be the sister group of the Helminthoglyptidae (Schileyko, 1978; Roth, 1996). In each case, the nominal genus was used as representative of its family, except in the case of Polygyridae.

My results are presented in Figures 1–3 and are discussed below.

Character Descriptions

External Morphology:

Character 1: Tail keeled (Figs. 4, 6):

Longitudinal row of plaques in mid-dorsal tail. Character states: ("0") absent; ("1") present.

Character 2: Tail horn (Figs. 5, 6):

Projection on the end of the tail with the appearance of a horn. Character states: ("0") absent; ("1") present.

Pallial System:

Character 3: Lobes of kidney:

This character is generally associated with the reduction in space due to the limacization process (Tillier, 1984). Character states: ("0") absent; ("1") present.

Character 4: Position of the heart relative to the kidney (Fig. 7):

The common position of the heart in Stylommatophora is to the left of the kidney with the roof of the lung observed ventrally. However, the heart is partially surrounded by the kidney in *Cryptostrakon* and *Metostracon.* Although this character has usually been associated with the morphology of a semislug, in the other two semislug genera of the Xanthonychidae, the position of the pericardium is to the left of the kidney, as in most of snails. Character states: ("0") to the left of the kidney; ("1") partially surrounded by the kidney.

Character 5: Relation of the mantle to the shell:

Character states: ("0") the mantle does not enclose the shell; ("1") mantle entirely encloses the shell.

Character 6: Diaphragm:

The diaphragm or lung floor forms the base of the pulmonary cavity. Character states: ("0") diaphragm thin, transparent, and membranous; ("1") diaphragm thick, muscular, not transparent; ("2") diaphragm thin but not transparent showing some muscular strands.

Character 7: Anus position (figure and description in Emberton, 1991). Character states: ("0") near mantle collar; ("1") recessed from collar.

Reproductive System:

Character 8: Fertilization Pouch-Spermathecal complex (FPSC):

Character states: ("0") the FPSC is completely free of the albumen gland; ("1") FPSC totally or partially embedded in the base of the albumen gland.

Character 9: Penial sheath:

Character states: ("0") penial sheath absent; ("1") sheath thin, membranous; ("2") sheath thick, muscular.

Character 10: Internal penial structure:

Character states: ("0") with longitudinal ridges; ("1") with one or two pilasters; ("2") smooth or with wrinkles; ("3") basal portions with several pilasters and upper portion with pustules.

Character 11: Penial muscular band: (described and illustrated in Cuezzo, 1997). Character states: ("0") absent; ("1") present.

Character 12: Verge (Figs. 8, 9):

Character states: ("0") verge absent; ("1") verge present, with thin projections or termi-

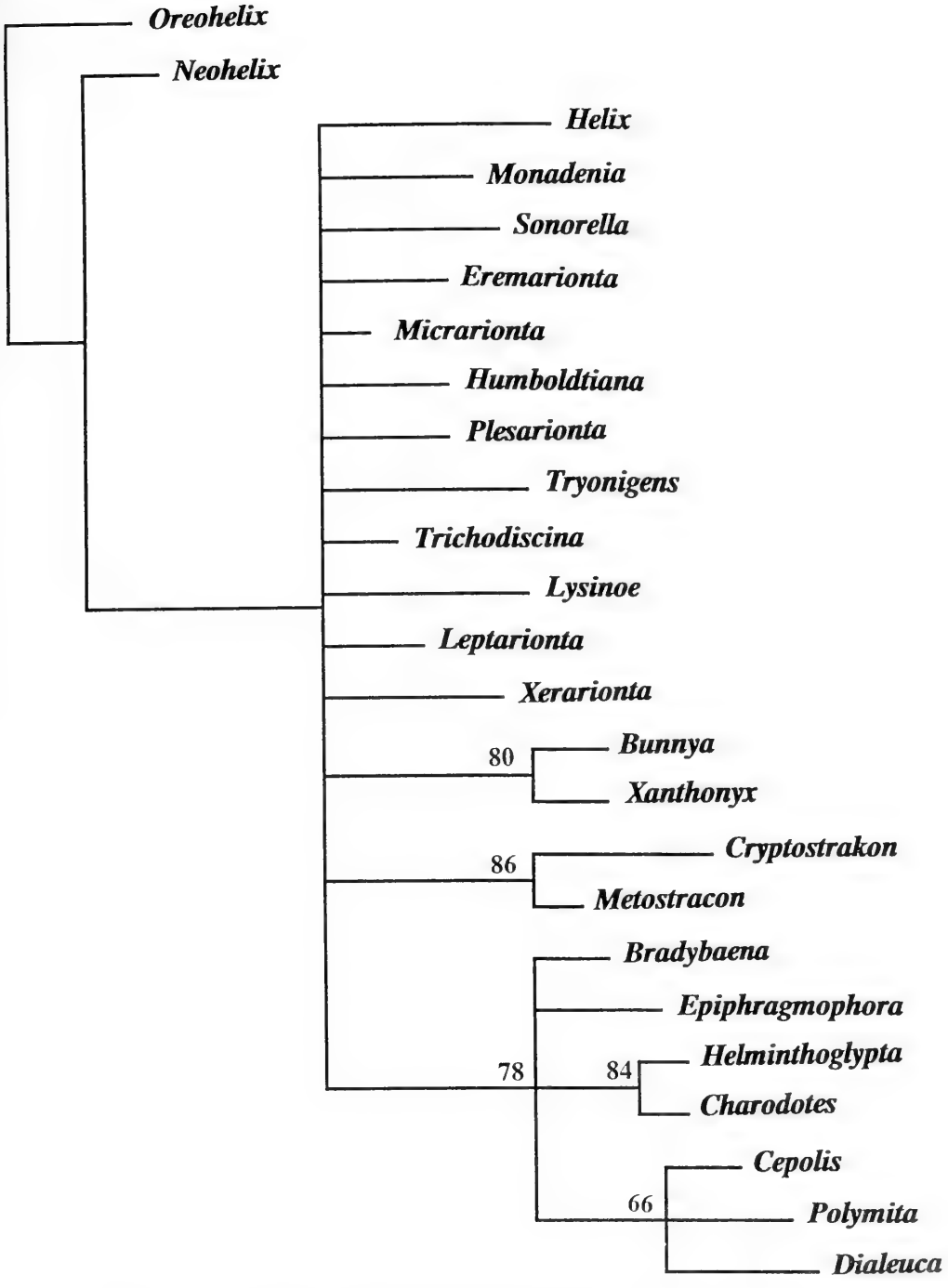

FIG. 1. Consensus tree generated from 50 trees. Numbers in the nodes are the frequencies above 50% obtained with FQ after Jackknife (frequencies of 100% are not indicated).

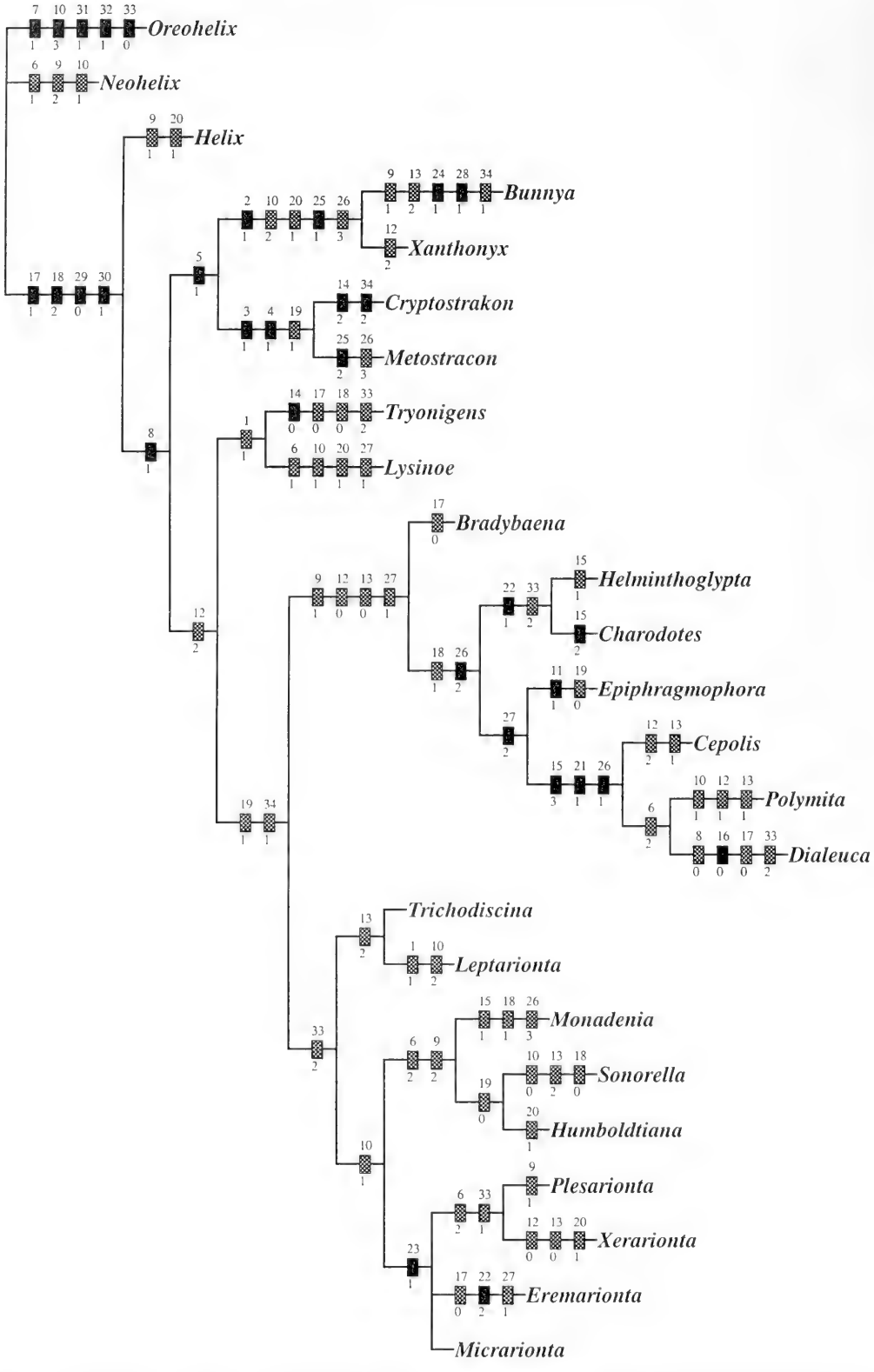

FIG. 1a. Prefered phylogenetic hypothesis for Xanthonychidae, generated by Henig86 and constructed using CLADOS, from data in Table 1. Filled dash marks represents synapomorphies, gray dash marks represents homoplasies. There are no reversals.

FIG. 2. Cladogram #2 for Xanthonychidae, generated by Henig86 and constructed using CLADOS. Alternative hypothesis adding characters 35 and 36. Filled dash marks are synapomorphies, gray dash marks are homoplasies.

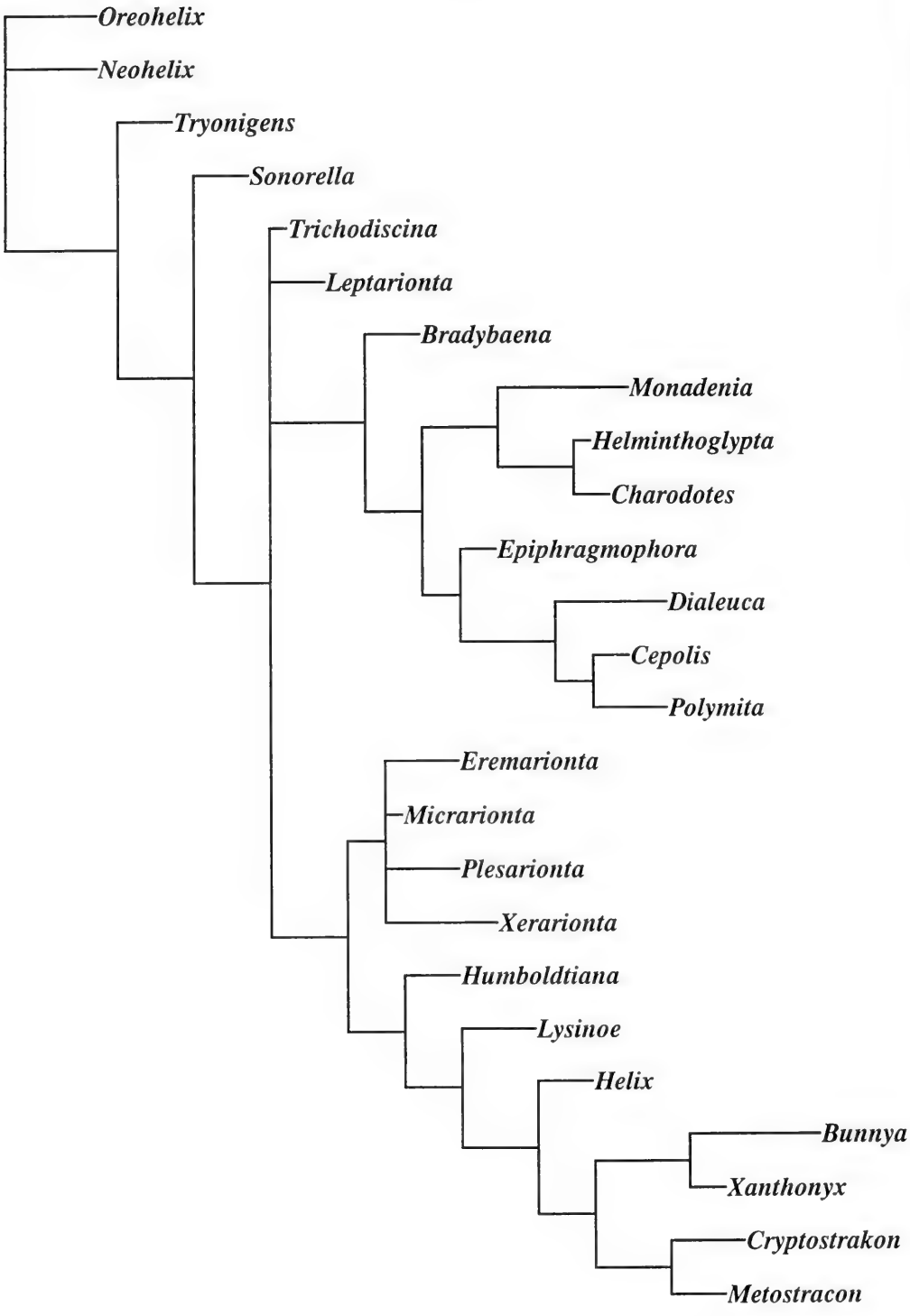

FIG. 3. Consensus of 22 trees, second analysis, generated by Henig86 and constructed by CLADOS.

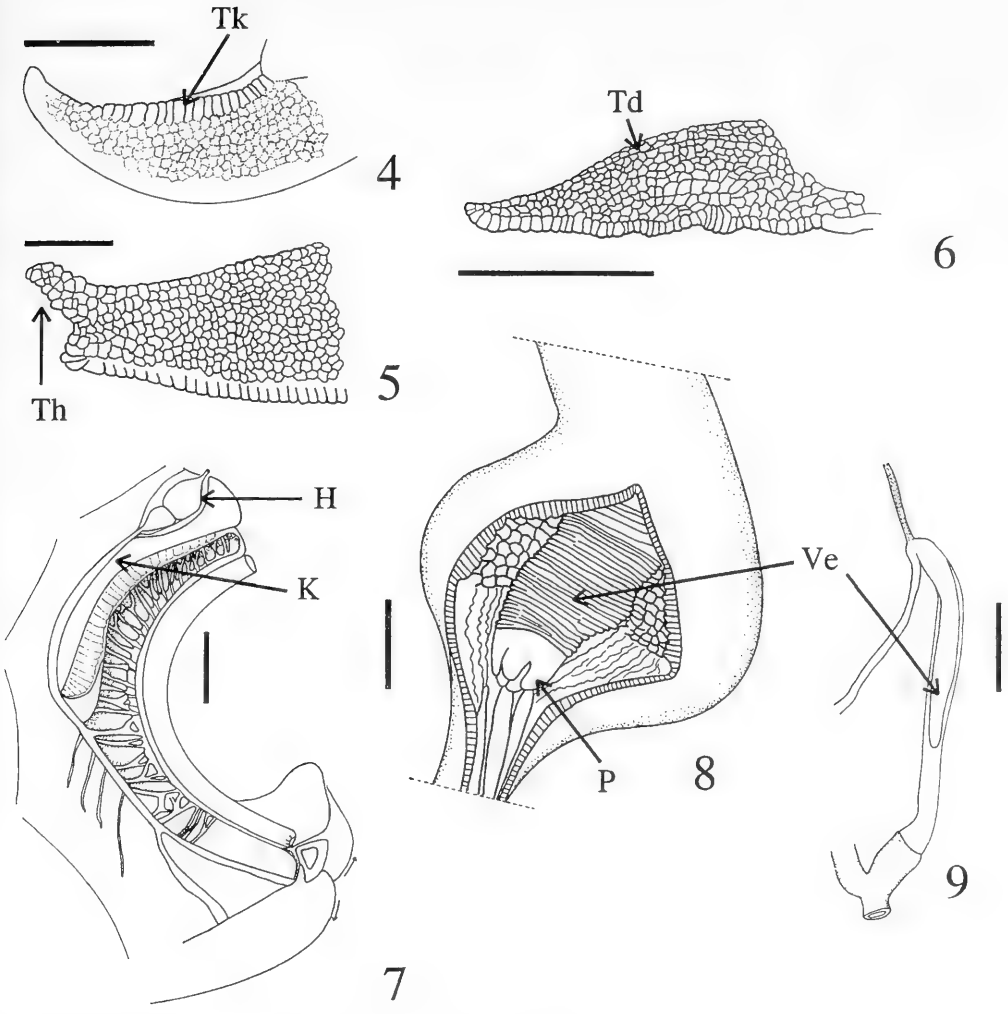

FIG. 4. *Tryonigens:* Tail keeled (Tk), character 1(1). Bar = 0.1 cm.
FIG. 5. *Bunnya:* Tail horn (Th), character 2(1). Bar = 3 mm.
FIG. 6. *Epiphragmophora:* Tail morphology, character 1(0), 2(0). Td = tail middorsal groove. Bar = 1 cm.
FIG. 7. *Bradybaena:* Position of heart (H) respect to the kidney (K), character 4(0). Bar = 3 mm.
FIG. 8. *Polymita:* Portion of the penial complex showing small verge (Ve), character 13(1), with terminal thin projections (P), character 12(1). Bar = 3 mm.
FIG. 9. *Sonorella:* Penial complex showing verge (Ve) without terminal projections, character 12(2), occupying half of the penial sac, character 13(2). Bar = 3 mm.

nal papillae; ("2") verge present, but without terminal papillae in which case it is stout.

Character 13: Relative size of the verge with respect to penial sac (Figs. 8, 9):

Character states: ("0") absent; ("1") present, small to medium sized verge, generally located in the upper portion of the penial sac; ("2") present, large verge, occupying half or more of the penial sac.

Character 14: Atrium:

Character states: ("0") absent; ("1") present, with longitudinal folds; ("2") present, with transversal thin folds.

Character 15: Atrial sac (Figs. 10, 11):

The atrial sac is a projection or prolongation of the atrium. In general, both the atrium and the atrial sac presents the same internal sculpture. Character states: ("0") absent; ("1") present, with internal sculpture consisting of thin folds; ("2") present, with a wide pilaster in the internal wall; ("3") present, with small irregular pustules in internal wall.

Character 16: Epiphallus:

The epiphallus is the portion of the penial complex between the penis and the insertion of the vas deferens. Its delimitation is easy when the penis bears a verge that clearly marks the limit between penis and epiphallus. When the verge is absent, the internal structure of the epiphallus is an important element in determining its limits. The portion of the penial complex termed "penis or preputial chamber" (Gregg & Miller, 1976; Miller, 1981), and "double tube of the upper part of penis" (Miller, 1985) are considered here to be homologous to the lower portion of the epiphallus: Character states: ("0") absent; ("1") present.

Character 17: Flagellum:

In the penial complex, the epiphallus continues as a blind duct that can have different lengths and that decreases in diameter toward the tip. In the type species of the genus *Sonorella,* a reduced flagellum is present, known in the literature as "epiphallic caecum." Because of its position this structure is considered here to be homologous to the flagellum present in the other Xanthonychidae. Character states: ("0") absent; ("1") present.

Character 18: Dart Sac insertion (Figs. 12, 13):

The dart sac is a muscular blind sac usually containing a calcareous dart, which functions in stimulation during copulation. Character states: ("0") absent; ("1") present, one dart sac inserted in the atrium or in the atrial sac, cylindrical to round; ("2") present, one to four dart sacs seated on the vagina.

Character 19: Mucous glands inserted in dart sac (Fig. 14):

The mucous glands that insert in the dart sac are considered homologous because they share the same position and probable function. Character states: ("0") absent; ("1") present, generally bearing one or more ducts.

Character 20: Mucous glands inserted in vagina (Fig. 15):

The mucous glands inserted in the vagina are considered to be homologous because they share the same position and probably the same function. Character states: ("0") absent; ("1") present.

Character 21: Mucous glands inserted in atrial sheath (Fig. 12):

The ducts of these glands are inserted between the folds of the atrial sheath. These glands are not homologous to others in the reproductive system. Character states: ("0") absent; ("1") present.

Character 22: Bulbous reservoirs on mucus gland ducts (Figs. 16, 17):

The reservoirs are swellings in the ducts. Character states: ("0") absent; ("1") present, with glands ending in a common duct and ("2") present, with glands ending in separate ducts.

Character 23: Distal portion of mucous glands (Fig. 17):

Character states: ("0") glands not expanded in their distal portion; ("1") distal portions of mucous glands expanded, flattened and spread upon vagina, dart or base of penis.

Character 24: Vaginal diverticulum:

The vaginal diverticulum is a round, cecil evagination in the ventral side of the vagina under the row of dart sacs. There is no possibility that it is another dart sac, because the internal structure is completely different. Also, no dart was found in the interior. Whereas the dart sacs have two thin dart papillae, the vaginal diverticulum has only longitudinal folds in its interior. Character states: ("0") absent; ("1") present.

Character 25: Albumen gland:

Character states: ("0") albumen gland bean shaped, located in the visceral mass; ("1") albumen gland bilobate, located in the visceral mass; ("2") albumen gland straight, located in the pedal cavity.

Character 26: Vas deferens:

Character states: ("0") vas deferens does not loop around any structure; ("1") vas deferens looped around the penial retractor muscle close to its insertion in the epiphallus; ("2") vas deferens looped around the dart sac; ("3") vas deferens looped around penis-epiphallus.

Character 27: Basal genital sheath:

The observation of this character has been affected in the past by the tendency to clean the genitalia from connective tissue or other membranous tissue before any observations are made. However, many genera of the Xanthonychidae show a conspicuous basal genital sheath that overlaps the basal female and male terminal genitalia. Character states: ("0") absent; ("1") present, formed by membranous tissue; ("2") present, composed of thin muscular tissue.

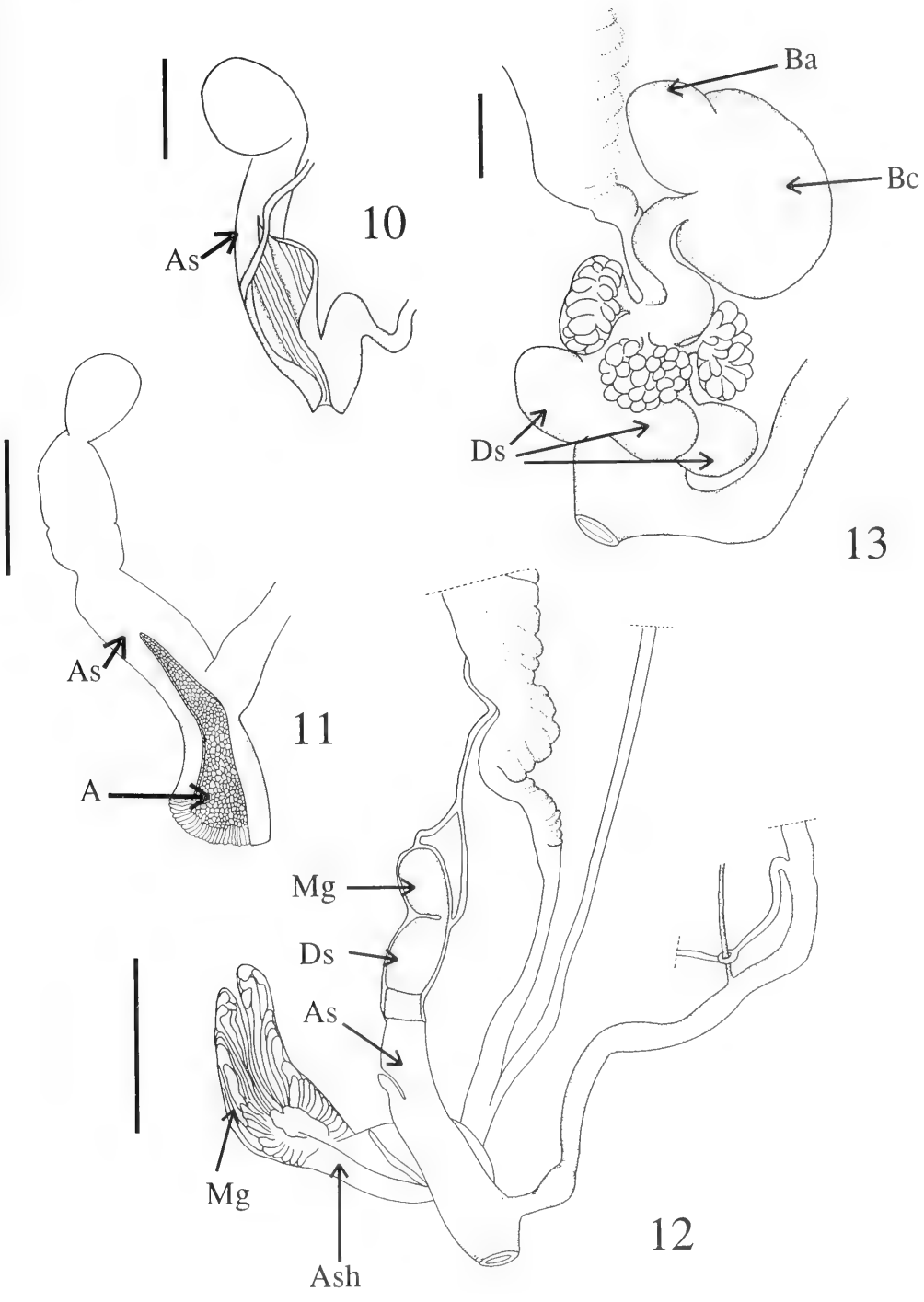

FIG. 10. *Charodotes:* Lower genitalia showing atrial sac (As) with internal wide pilaster, character 15(2). Bar = 3 mm.
FIG. 11. *Dialeuca:* Lower genitalia showing atrium (A) and atrial sac (As) with irregularly distributed pustules in the internal wall, character 15(3). Bar = 3 mm.
FIG. 12. *Cepolis:* Terminal genitalia. Dart sac (Ds) cilindrical inserting in the atrial sac (As), character 18(1). There are another pair of glands (Mg) inserted in the atrial sheath (Ash), character 21(1). Bar = 0.5 cm.
FIG. 13. *Bunnya:* Terminal genitalia. Dart sacs (Ds) seated on the vagina, character 18(2). Ba = bursa copulatrix appendix; Bc = bursa copulatrix. Bar = 2 mm.

Character 28: Bursa copulatrix sac appendix (Fig. 13):

Thick and short appendix in the bursa copulatrix sac. Character states: ("0") absent; ("1") present.

Character 29: Bursa copulatrix duct swollen at the base:

Character states: ("0") absent; ("1") present.

Character 30: Copulation modality:

Character states were selected from the published data (Webb, 1947, 1948, 1959, 1972; Emberton, 1985): ("0") copulation is one sided; ("1") copulation is reciprocal.

Character 31: Reproductive modality:

Character states were selected from published information (Solem, 1978; Tompa, 1984): ("0") oviparous; ("1") ovoviviparous.

Character 32: End of penial retractor muscle:

Character states: ("0") penial retractor inserts without divisions; ("1") penial retractor splits in branches.

Digestive System:

Character 33: Internal structure of the oesophageal crop:

The wall of the oesophageal crop presents different kinds of interior sculpture independent of the thickness of the wall. Character states: ("0") wall with longitudinal ridges that can extend along all the crop length or only portions of it length; ("1") wall with pustules ordered as longitudinal cords or irregularly distributed.

Nervous System:

Character 34: Fusion of the visceral ganglion (illustrated in Emberton & Tillier, 1995):

The visceral ganglion is located in the ventral chain of the nervous system. Although the fusion of the ganglia is traditionally associated with the limacization process, surprisingly non fusion of the visceral ganglion was observed in the case of two semislug genera. This character is discussed by Emberton & Tillier (1995). Character states: ("0") absent; ("1") present, fused with left parietal ganglion; ("2") present, fused with both parietal ganglia.

Characters Added in Analysis #2

Character 35: Position of mucous glands

Character states: ("0") absent; ("1") inserted in vagina; ("2") inserted in dart sac or close to its base; ("3") inserted in vagina and dart sac.

Character 36: Type and shape of mucous glands (according to Miller & Naranjo Garcia, 1991)

Character states: ("0") absent; ("1") tubular; ("2") membranous; ("3") round compact; ("4") vesicular club-shaped.

Cladistic Analysis

Using the program Hennig86, two different analyses were carried out: (1) The initial analysis using the command "mh*; bb*;" produced 50 trees of 99 steps in length, CI = 52, RI = 62. A consensus tree summarizing the 50 original trees obtained is presented in Figure 1. Caution should be used to interpret it, because consensus trees contain less information, being generated from fundamental cladograms instead of original information. After performing succesive weighting, two trees were retained, each with length 372, CI = 79 and RI = 78. One of them (Fig. 1a) is identical to one of the trees of the original set. The other differs only in the position of *Micrarionta.* In one of the trees, its position is resolved, but there are no characters supporting this resolution, whereas in the other, there is no resolution on the position of *Micrarionta.* Consequently, the Nelsen consensus tree (after succesive weighting) has the same topology as the one showing the unresolved trichotomy for (*Plesarionta, Xerarionta*) + *Eremarionta* + *Micrarionta.* There are four synapomorphies (characters 17, 18, 29, 30) supporting the monophyly of the ingroup (Bradybaenidae-Xanthonychidae-Helicidae). *Helix* is the sister group of the Bradybaenidae-Xanthonychidae complex, supported by character 8. The outgroups *Oreohelix* and *Neohelix* are clearly separated from the other genera. Within the ingroup, three monophyletic groups are clearly defined: (a) first, the genera *Bunnya, Xanthonyx, Cryptostrakon* and *Metostracon* with character 5 as synapomorphy; (b) second, *Helminthoglypta, Charodotes, Epiphragmophora, Cepolis, Polymita* and *Dialeuca,* supported by character 26; (c) and third, *Eremarionta, Micrarionta, Plesarionta* and *Xerarionta,* supported by character 23.

(2) A second analysis for comparative purposes has been made by adding to the same matrix two characters: (a) type and shape of mucous glands (character states as defined by Miller & Naranjo-Garcia, 1991; #35 in Table I) and (b) mucous glands insertion (assuming that all the mucous glands are homologous, as it is traditionally considered, with the exception of the glands inserted in the atrial sheath in the Cepoliinae, #36 in Table I). Concurrently, characters 19 and 20 (mucous

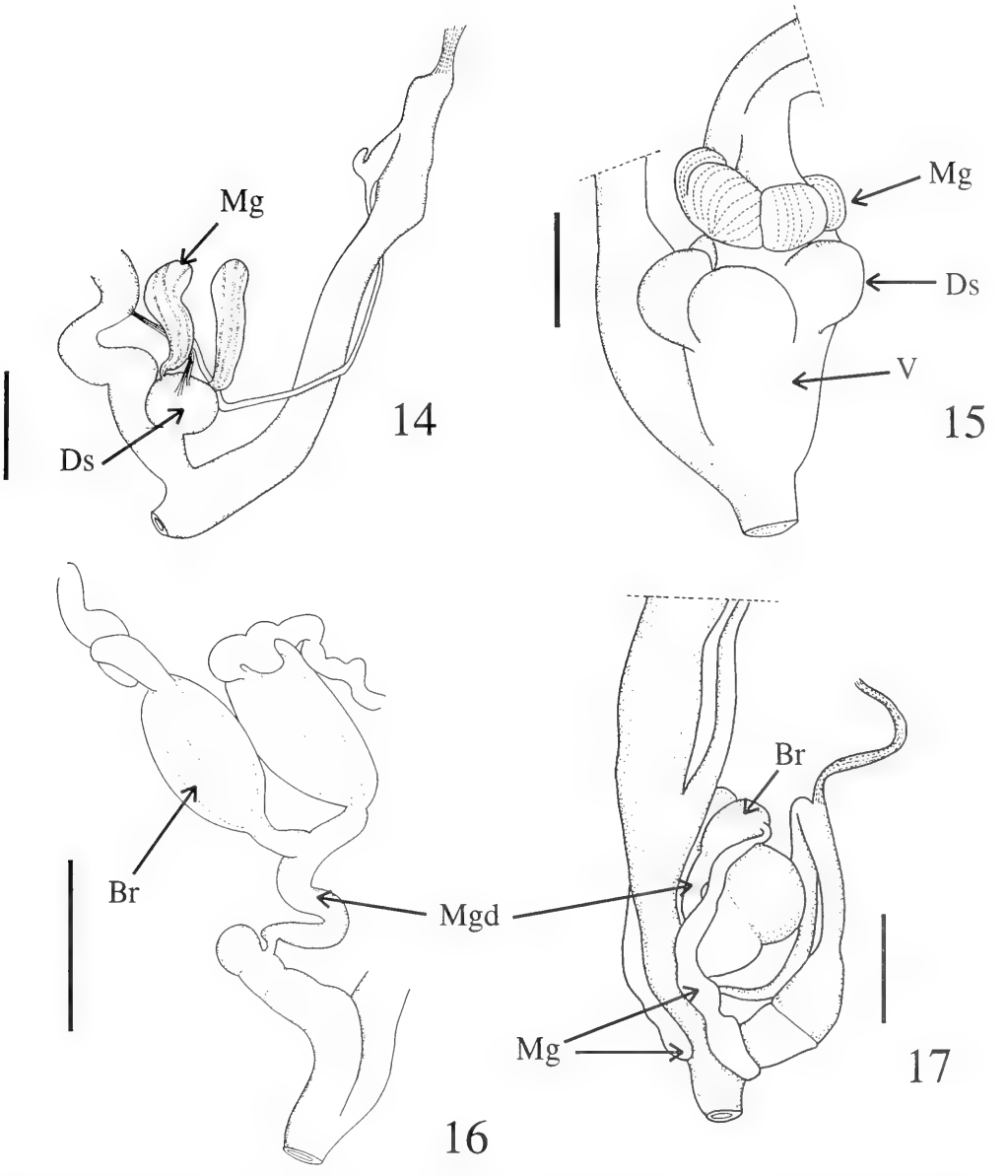

FIG. 14. *Metostracon:* Terminal genitalia. Mucous glands (Mg) inserted in dart sac (Ds), character 19(1). Bar = 0.5 cm.
FIG. 15. *Humboldtiana:* Terminal genitalia. Mucous glands (Mg) inserted in vagina (V), character 20(1). Ds = dart sac. Bar = 0.5 cm.
FIG. 16. *Helminthoglypta:* Terminal genitalia. Bulbous reservoirs (Br) on mucous glands ducts ending in a common duct (Mgd), character 22(1). Bar = 0.5 cm.
FIG. 17. *Eremarionta:* Terminal genitalia. Bulbous reservoirs (Br) on separate mucous glands ducts (Mgd), character 22(2). Distal portion of mucous glands (Mg) expanded and spreaded on vagina and base of penis, character 23(1). Bar = 1 mm.

gland insertion in vagina and in dart sac) were deactivated. This analysis produced initially 40 trees, L = 106, CI = 52 and RI = 62, and after succesive weighting the number was reduced to 22 trees, L = 391, CI = 82, RI = 84. One of them is illustrated in Figure 2. This tree has the same topology as one of the original set and is very similar to the consensus tree (Fig. 3). The differences with the first analysis are: (a) *Sonorella* and *Tryonigens,* the genera that lack a dart complex, are located basally in the cladogram, but they do not form a monophyletic group; (b) excluding *Tryonigens,* the synapomorphy for the rest of the genera is the presence of flagellum (character 17); (c) *Monadenia* is the sister group of *Helminthoglypta-Charodotes,* this relationship supported by character 15 (atrial sac with internal thin folds). The other two monophyletic groups described in analysis #1: *Bunnya* + *Xanthonyx* + *Cryptostrakon* + *Metostracon* (supported by character 5) and *Plesarionta* + *Xerarionta* + *Micrarionta* + *Eremarionta* (supported by character 23), are also clearly delimited in the second analysis; (d) the genus *Helix* representing the Helicidae is internested in the ingroup.

DISCUSSION

Both cladistic analyses suggest that the family Xanthonychidae, or Helminthoglyptidae as it was defined by Pilsbry (1939) and subsequently treated by various authors (Baker, 1959; Zilch, 1959–1960; Schileyko, 1991), is a paraphyletic group. The results obtained (Figs. 1, 1a, 2, 3) support the idea that the families Bradybaenidae and Helicidae should be jointed with Xanthonychidae, because they form a monophyletic group.

Baker's (1959) concept: "Since the sizes of families are matters of convenience or custom, we Americans can leave to the wisdom of our Old World colleagues the advisability of a separate family for the genera of their home lands" had prevailed since 1959, and the Xanthonychidae (American Helicoids) had been maintained as a separate family from the Bradybaenidae (mostly Asian Helicoids) and Helicidae (mainly European) without much justification. Consequently, geographic data become important in the identification of certain genera of the Bradybaenidae-Xanthonychidae-Helicidae group.

The cladogram illustrated in Figure 1a is chosen as the preferred hypothesis, because is the one that best explained the data: (1) the mucous glands are not homologous structures as they are traditionally considered in published literature, because they have different positions and, therefore, should be treated as different characters; (2) this hypothesis (cladogram #1a) shows closest relationship between xanthonychid and bradybaenid snails, with *Helix,* representing the Helicidae, their sister group.

The position of the Helicidae, however, should be carefully reconsidered when more genera of Helicidae and Bradybaenidae can be studied along with the Xanthonychidae.

Four synapomorphies (Fig. 1a) support the Bradybaenidae-Xanthonychidae-Helicidae group (presence of flagellum, dart sac inserted in vagina, bursa copulatrix duct not swollen at the base, and copulation reciprocal). One synapomorphy supports the monophyly of the Bradybaenidae-Xanthonychidae group (fertilization pouch-spermathecal complex embedded in the base of the albumen gland).

Although it is clear that it would be premature to translate this phylogenetic hypothesis (cladogram #1a) into a new classification (too many branches of the cladogram are not strongly supported, and the relationships of the genera could also change when more taxa of the Helicidae and Bradybaenidae are studied), three monophyletic groups are well defined and delimited based upon the analyses performed (cladogram #1a). The first is the semislug group composed of *Cryptostrakon* + *Metostracon* + *Bunnya* and *Xanthonyx.* The monophyly of this group is supported by character 5 (mantle entirely concealing the shell). Other characters, such as shell reduction, kidney size reduction and compactation, rotation of the longitudinal axis of kidney, pulmonary cavity short and reduced, presence of secondary respiratory structures (such as alveoli), detorsion of digestive tract, position of the stomach, oesophageal crop contained in foot cavity, digestive gland invading the pulmonary cavity, presence of air sacs, have been hypothesized by Solem (1978) and Tillier (1983, 1984, 1989) as being correlated with the limacization process. For this reason, none of them were used in the final analysis (#1a) in order to avoid redundant characters (in case Solem and Tillier are correct). However, when these characters were included in preliminary analyses, they appeared as synapomorphies of the semislug group. At this point, it is difficult to test whether Solem and Tillier's hypotheses are

simply evolutionary scenarios because, as proposed, they appear to be based on circular reasoning. Characters 3 and 4, although described also as correlated with the limacization process, were maintained in the analysis, because the lobes of the kidney (character 3) and the position of the heart internested in the kidney (character 4) are present only in *Cryptostrakon* and *Metostracon* but not in the other semislug genera. Similarly, character 5 (mantle entirely enclosing the shell) has been used, because it is not possible to correlate it with a "semislug state." The reason for this is that there are some semislugs that have the shell exposed — *Vitrina:* Vitrinidae; *Binneya* Cooper (Pilsbry, 1939); *Pellicula depressa:* Bulimulidae (Van Mol, 1968, 1971) — others with the shell partially enclosed by the mantle — *Austenia, Parmarion:* Helicarionidae (Solem, 1966) — and still in others the shell is completely concealed by the mantle — *Peltella iheringi:* Bulimulidae (Van Mol, 1968), *Malagarion paenelimax:* Helicarionidae (Tillier, 1979) and the xanthonychid genera studied here. In other words, it is not possible to infer that all semislugs have the shell concealed by the mantle due to the changes in shape (mainly reduction of visceral mass). Also, if the trend towards a "slug stage" is a real phenomenon, all slugs should have the shell completely concealed by the mantle, which is true only in some cases (some Arionidae) but not in others, for example in *Testacella* (Testacellidae) and *Daudebardia* (Zonitidae). Solem (1978) and Tillier's (1983, 1984, 1989) argumentation that all the characters mentioned above represent compensations for space alterations in the process of slug evolution must be reconsidered with a historical perspective. Within the Xanthonychidae, the semislug stage has occured only once, and thus the monophyly of the semislug genera is supported in this study.

The second monophyletic group delimited in the two analyses and also well supported by the statistical test of homoplasy (Fig. 1) is composed of *Helminthoglypta* + *Charodotes* + *Epiphragmophora* + *Cepolis* + *Dialeuca* and *Polymita.* This group occurs in all most parsimonious cladograms and thus is "unequivocally supported" by the data. The monophyly of this group is supported by "vas deferens looped around the dart sac," a character that later changes to "looped around the penial retractor muscle" in the clade *Cepolis* + *Polymita* + *Dialeuca.* The muscular basal genital sheath is the synapomorphy supporting the relationship between the Cepoliinae and *Epiphragmophora.* Baker (1943) had pointed out that *Helminthoglypta* and *Cepolis,* placed by Pilsbry (1939) in different subfamilies, could be related based on the common presence of the "dart sheath" (considered here as the basal genital sheath, character 27). Later, Baker (1961) proposed that the Cepolinae (*Cepolis, Polymita,* and *Dialeuca*) should be included with *Helminthoglypta* and *Micrarionta* in the Helminthoglyptinae as defined by Pilsbry (1939). However, both Nordsieck (1987) and Roth (1996) denied any relation between the two genera because of the "completely differently constructed glands" that they possess. In this study, the clade composed by *Helminthoglypta* + *Charodotes* + *Epiphragmophora* + *Cepolis* + *Polymita* and *Dialeuca* appeared as a monophyletic group with the addition of *Monadenia* in the second analysis (cladogram #2). The genus *Bradybaena* (representing the Bradybaenidae) is the sister group of this clade in both analyses, although its position is not strongly supported by any synapomorphy.

The third monophyletic group is composed of *Eremarionta* + *Micrarionta* + *Plesarionta* and *Xerarionta.* The monophyly of this group is sustained by character 23 (expansioned and spread of distal portion of mucous glands). *Eremarionta, Plesarionta* and *Xerarionta* were originally proposed as "sections" or subgenera of *Micrarionta.* Later, they were elevated to genera based on "major differences" that were unfortunately not well detailed (Bequaert & Miller, 1973; Miller, 1981). My results are consistent with Pearce's (1990) hypothesis of the relationships of these genera (Fig. 1a).

Because the type and shape of the mucous glands has been traditionally important in the various definitions of Xanthonychidae-Helminthoglyptidae-Helicidae, and often used as the only justification for splitting the families (Pilsbry, 1939; Miller & Naranjo-Garcia, 1991), the second analysis was performed for comparative purposes. In this analysis, there are three synapomorphies supporting the ingroup: bursa copulatrix duct not swollen at base, copulation reciprocal, and fertilization pouch-spermathecal complex buried in the albumen gland. *Helix* is internested in the ingroup. The hypothesis proposed in Figure 2, treating the mucous glands as homologous structures, suggests that the dart sac and mucous glands were originally absent in the

basal clades *Tryonigens* and *Sonorella,* and secondarily acquired (Figs. 2, 3) in the rest of Xanthonychidae. The hypothesis proposed in Figure 1, 1a, treating the mucous glands as non-homologous structures, suggests, however, the opposite situation: the dart sac and mucous glands inserted in the vagina were originally present and secondarily lost in separate groups. Comparisons among the cladograms presented (Figs. 1a, 2) are difficult to make because a different matrix was used for the analysis. Even if we compare the behavior of characters 19 and 20 of the first analysis and 35 and 36 of the second, which refer to the same structure coded differently, the mucous glands are highly homoplastic and therefore appear to be unreliable as characters for phylogenetic reconstruction. In any case, the mucous glands and dart sac have been overvalued in past studies, and many other nongenital characters have not been considered in previous classifications of the traditional Xanthonychidae.

The question of whether the genera that lack the dart complex form a monophyletic unit or whether the absence of these structures are products of parallel evolution has been discussed several times (Nordsieck, 1987; Schileyko, 1991). Based on the cladograms obtained in this study, neither analysis supports the idea of monophylly of taxa that lack dart complex, represented here by *Tryonigens* and *Sonorella.* The taxonomic position of *Monadenia,* originally placed in the Helminthoglyptinae by Pilsbry (1939) but later moved to Bradybaenidae (Miller & Naranjo-Garcia, 1991), remains controversial. In this study, the "swollen" in the terminal genitalia present in *Monadenia* was interpreted as an atrial sac. When the shape of the mucous glands are taken into consideration and the mucous glands are considered homologous structures (Fig. 2), the position of *Monadenia* is similar to the one traditionally accepted and proposed by Pilsbry (1939). However, it could be concluded that the relationships of *Monadenia* will remain obscure until a phylogeny of the Bradybaenidae-Xanthonychidae-Helicidae is proposed.

(a) The family Xanthonychidae (= Helminthoglyptidae) as defined and used by Pilsbry (1939) and Zilch (1959–1960) is paraphyletic.
(b) Xanthonychidae-Bradybaenidae-Helicidae conform a monophyletic group.
(c) The preferred hypothesis (Fig. 1a) supports the delimitation of three monophyletic groups within the traditionally named Xanthonychidae.
(d) Some characters used in previous studies, such as the shape and number of mucous glands, are of poor value for the reconstruction of the phylogeny of the Xanthonychidae.

Considering the monophyly of the Bradybaenidae-Helicidae-Xanthonychidae well established, further cladistic studies are needed as a basis for a revised, testable and informative classification of its components.

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APPENDIX I

Taxa studied:

Bradybaena similaris (Férussac):

ANSP 434, A8774: West Java, Bogor, Java. Feb. 1973.*Bunnya bernardinae* Baker

ANSP A16728: Ruins of Monastery 20 km southwest of Mexico City, El Desierto de Los Leones to La Venta, Distrito Federal, Mexico. July 1926. H.B. Baker!

Cepolis cepa (Müller):

UF 46191: Dept. du Sud, SE slope of Morne Formon, Haiti. 1500 m. Thompson! Jan. 1984.

UF 235002: Dept. Quest, 3 km S. Kenscoff, Haiti. 1470 m. Thompson & Auffenberg!

UF 48281: Dept. du Sud, Boia Dirant, SW of Morne Formon, 1250 m. June 1984, K.A. & R.W.P!

Charodotes traski (Newcomb):

ANSP A14976: 12 miles E. of Las Cruces, Baja California, Mexico. 1918. H. N. Lowe!

Cryptostrakon gabbi Binney:

ANSP 246310, A9639: Costa Rica. Lectotypes selected by Baker (1963).

Cryptostrakon corcovadensis Cuezzo (in press):

INBIO 468080: P. N. Corcovado, Estacion Sirena, Sendero a Río Los Patos, 10 mt., Madrigal, Puntarenas Province, Costa Rica. 18 Aug. 1994. Marianella Segura.

INBIO 468087: Madrigal, Puntarenas Province, P. N. Corcovado, Estacion Sirena, Sendero a Los Patos, Costa Rica, 10 m. 14 Aug. 1994. Marianella Segura!

INBIO 408059: Madrigal, Puntarenas Province, P. N. Corcovado, Estación Sirena, Sendero Los Espaveles, 20 m. 12 Aug. 1994. Marianella Segura!

Dialeuca nemoraloides (Adams):

ANSP A12685: Mandeville, Manchester Parish, Jamaica. June 1933. H. Baker!

Epiphragmophora hieronymi Doering:

FML A100: Quebrada del Tala, Catamarca, Argentina. Jan. 1993. Domínguez!

Eremarionta rowelli (Newcomb):
ANSP A11327B: Needles Peaks, Topock, Mohave County, Arizona, USA. *Ex* Ferriss.
ANSP 164956, A11327G: Rocky Hills, Punta Libertad. Feb. 1935. H. N. Lowe!

Helminthoglypta arrosa (Gould, in Binney):
ANSP A11345: 12 miles from end of Point Reyes, Marin County, California, USA. H. N. Lowe!

Helminthoglypta tudiculata (Binney):
ANSP 94237 A11344F: Oceanside, San Diego County, California, USA. Sept. 1907. *Ex* H. G. Eaton.

Humboldtiana humboldtiana (Valeciennes, in Pfeiffer):
ANSP A13281: El Desierto de Los Leones, Mexico.

Leptarionta quillarmodi (Shuttleworth):
ANSP A16727: 4 km North and slightly east of Cordova, foothills east and north of Toxpam (Hacienda de San Francisco), Cerro de Las Palmas, Vera Cruz, Mexico. June 1926. H. B. Baker!

Lysinoe ghiesbreghti (Nyst):
FMNH 206294/1: 2400 m, on trail, 1 km SW of Esquipulas Palo Gordo, San Marcos, Guatemala. 28 July 1980. Ken Young!
UF 190195: Alta Verapaz Prov., Guatemala. 10.5 Km. SE of Tactic. Feb. 1991. F. T. & S. P. Christman!

Metostracon mima Pilsbry:
ANSP 77245, A9636: Morelia, Michoacan, Mexico. S. N. Rhoads! Holotype.
ANSP A9635F: Uruapam del Progresso, Michoacan, Mexico. S. N. Rhoads! Paratype.

Metostracon mima Pilsbry:
ANSP A9410D: Near Alvarez at km 53, San Luis de Potosi, Mexico. July 1934. H. A. Pilsbry!
ANSP A9411A: Km 42, Potosi and Rio Verde Railroad, San Luis de Potosi, Mexico. Aug. 1934. H. A. Pilsbry!

Micrarionta facta (Newcomb):
ANSP 10789, A11342: Santa Barbara Island, Santa Barbara County, California, USA. Newcomb!

Micrarionta sp.:
ANSP 130897, A113321: 5 mi W of Leach Spring, Granite Mountains, California, USA. 1922. Ferris!

Monadenia fidelis (Gray):
ANSP 158283, A16079: Riverdale, Multnomah County, Oregon, USA. August 1929. H. B. Baker!
ANSP 158278 A16078: About 13 mi N of Klamath Falls, E side of upper Klamath Lake, Ouxy siding, Klamath County, Oregon, USA. July 1929. H. B. Baker!

Plesarionta stearsiana (Gabb):
ANSP 66091 A11336: Coronado Island, San Diego County, California, USA. 1895. A. W. Anthony!
ANSP 146098 A11332E: Near San Vicente Mission, Baja California Norte, Mexico. Dec. 1927. L. G. Ingles!

Polymita picta Born:
ANSP A13209: Cuba,
ANSP 154067, A9341: Mandinga de Yumuri, Oriente Province, Cuba. Welch!

Sonorella hachitana (Dall)
ANSP A10367: Florida Mountains, Luna County, New Mexico. 1906. H. A. Pilsbry!

Trichodiscina cordovana (Pfeiffer):
ANSP A16732: Steep Valley down from saddle, Twin Peak Valley, Estado Puebla, Mexico. July 1926. H. B. Baker!
ANSP A16734: Steep valley down from saddle, Twin Peak Valley, Estado Puebla, Mexico. July 1926. H. B. Baker!

Tryonigens remondi (Tryon):
ANSP 166233, A9415A: Hills around Panuco, Sinaloa, Mexico. Aug. 1935. H. A. Pilsbry!

Xanthonyx sp.:
ANSP A16735: 14 km from Cordoba towards Orizaba on Mexican railroad, hills southeast of town, on opposite side of canyon Sumidero, Vera Cruz State, Mexico. June 1926. H. B. Baker!

Xerarionta kelletti (Forbes):
ANSP 138972, A11332A: West of North Bay isthmus, hillside west of Isthmus Cove, Santa Catalina Island, Los Angeles County, California, USA. 1925. H. A. Pilsbry! ANSP A138973, A11332B: Avalon, Santa Catalina Island, Los Angeles County, California, USA. 1925. H. A. Pilsbry!

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DIVERGENCE AMONG MOBILE BASIN POPULATIONS OF THE PLEUROCERID SNAIL GENUS, *LEPTOXIS,* ESTIMATED BY ALLOZYME ELECTROPHORESIS

Robert T. Dillon, Jr.[1] and Charles Lydeard[2]

ABSTRACT

Although the Mobile River Basin of Alabama was historically a center of great pleurocerid diversity, populations today are small and scattered. We obtained samples of all four nominal species of Mobile Basin *Leptoxis* currently extant: *L. ampla* (3 populations), *L. picta* (1 population), *L. plicata* (2 populations), and *L. taeniata* (2 populations). Gene frequencies at nine variable enzyme loci were determined for about 30 individuals from each population using horizontal starch gel electrophoresis. Samples of about 30 individuals from three populations of the widespread *Leptoxis praerosa* were analyzed as controls. Within populations, 18 of 99 loci were polymorphic, none showing genotype frequencies significantly different from Hardy-Weinberg expectation. Between populations within species, statistically significant divergence was apparent at most loci. Comparisons among the nominal species showed *L. praerosa* and *L. plicata* to be quite distinct from each other, and from all other populations. Much lower levels of divergence among populations nominally *L. picta, L. ampla,* and *L. taeniata* seem more consistent with a hypothesis of geographic isolation than reproductive isolation. We refer to these three taxa as the "*Leptoxis picta* group." Our results compare favorably in most respects with previously published data on mitochondrial 16S rRNA gene sequence divergence among these taxa, especially in the genetic distinction of *L. plicata.* The relationships within the *L. picta* group warrant further study.

Key words: genetics, isozymes, speciation, freshwater, gastropods, Alabama, endangered species.

INTRODUCTION

The rivers and streams of Alabama's Mobile River Basin have recently attracted attention as a center of endemism for a variety of aquatic life, including turtles, fish, bivalves and prosobranch snails (Lydeard & Mayden, 1995). Based primarily on the revisions of Goodrich (1922, 1924, 1936, 1941), Burch (1989) recognized 77 species of pleurocerid snails from the region, 95% of which were unknown outside the Mobile Basin. Burch's list included 6 species of *Gyrotoma,* 5 species of *Pleurocera,* 52 species of *Elimia* (synonymizing *Goniobasis* as used by Goodrich), and 14 *Leptoxis* (lowering *Anculosa,* as used by Goodrich, to subgeneric level). During the present century, however, most of the larger rivers of the Mobile Basin have been impounded for hydroelectric power, channelized, or otherwise modified for navigation. The Mobile Basin pleurocerid fauna has also been adversely impacted by changing patterns of land use, first from siltation due to intensive agriculture, and more recently from pollution. Lydeard & Mayden (1995) presumed extinct 29 species of Mobile Basin pleurocerids, including all six species of the endemic genus *Gyrotoma.*

The *Leptoxis* species of the basin have been the object of special concern. Of the 11 species of *Leptoxis* known historically from the Coosa River, Bogan & Pierson (1993a) found only *L. taeniata* (Conrad, 1834), apparently restricted now to but a few small tributaries. Of the four *Leptoxis* species documented from the Cahaba River, only *L. ampla* (Anthony, 1855) apparently survives, inhabiting a 30 km reach of the main river and several smaller Cahaba tributaries (Bogan & Pierson, 1993b). The only *Leptoxis* population remaining in the Black Warrior drainage is *L. plicata* (Conrad, 1834), restricted to a short reach of Locust Fork. Based on these data, as well as extensive U. S. Fish & Wildlife Service field records, Hartfield (1997) identified *L. taeniata, L. ampla,* and *L. plicata* as candidates for addition to the U. S. list of endangered and threatened wildlife and plants. The status of the only other nominal *Leptoxis* species

[1]Department of Biology, College of Charleston, Charleston, South Carolina, 29424, U.S.A.
[2]Aquatic Biology Program, Department of Biological Sciences, University of Alabama, P.O. Box 870344, Tuscaloosa, Alabama 35487, U.S.A.

known with certainty to have survived in the Mobile Basin, *L. picta* (Conrad, 1834) of the main Alabama River, continues to be monitored.

But Hartfield noted that the genetic relationships among these four nominal species of *Leptoxis* are poorly understood. They are distinguished primarily by minor attributes of shell shape and size, traits long known for clinal variability (Goodrich, 1934, 1935). The non-genetic component of some aspects of pleurocerid shell morphology is well-documented (Chambers, 1982; Dillon, 1984a).

In light of these concerns, Lydeard et al. (1997) surveyed 15 pleurocerid populations from the Mobile Basin: seven *Elimia* and four *Pleurocera,* in addition to the four nominal *Leptoxis* species. A molecular phylogeny constructed from mitochondrial 16S rRNA gene sequences suggested that Alabama *Elimia* and *Pleurocera* are sister taxa. The four *Leptoxis* species were quite different from the *Elimia/Pleurocera* group, and depicted as paraphyletic. Levels of sequence divergence were low between *L. taeniata* and *L. ampla,* with *L. picta* and *L. plicata* appearing more distinct.

Allozyme electrophoresis is an older and more established technique for evaluating the specific status of pleurocerid populations, especially the large genus *Goniobasis.* Extensive surveys of variation at allozyme-encoding loci, involving at least 11 species and 58 populations, have established that *Goniobasis* shows unusually low levels of heterozygosity, high levels of divergence between populations within species, and very few shared alleles at any locus when compared among species (Chambers, 1978, 1980; Dillon, 1984b, 1988a; Dillon & Davis, 1980; Bianchi et al., 1994; Stiven & Kreiser, 1994). Recent evidence suggests similar trends in *Leptoxis,* although intrapopulation variation may be somewhat greater, and interpopulation variation less (Dillon & Ahlstedt, 1997).

The purposes of the present work are twofold. We survey the allozyme divergence displayed by populations representing the four nominal species of Mobile River basin *Leptoxis* to gather further evidence regarding their genetic distinction. We also compare the levels of allozyme divergence estimated here to the DNA sequence divergence estimates of Lydeard et al. (1997), as a possible guide to the future application of the newer technology.

METHODS

We analyzed eight populations of Alabama *Leptoxis* assigned to four species (Appendix). Our *L. taeniata* populations were sampled from Buxahatchee and Choccolocco creeks, two tributaries of the Coosa River. As no *Leptoxis* inhabit the 50 km reach of the Coosa River separating these two creeks, gene flow between the populations we designated Taebux and Taechc, respectively, would seem to be negligible at present. We obtained samples of *L. ampla* from three shoals of the Cahaba River separated about 20 river km from each other, labeled Ampcah1, Ampcah2, and Ampcah3 from upstream down. Our two samples of *L. plicata* are from Locust Fork, Pliloc1 about 15 river km upstream from Pliloc2. Our single sample of *L. picta* (Picala) was collected by boat from limestone walls and outcrops in the lower Alabama River.

We selected three populations of the well-characterized *Leptoxis praerosa* (Say, 1821) to provide calibration for our analysis. Populations of this species are common and widespread throughout the Ohio, Cumberland, and Tennessee river drainages. Our *L. praerosa* came from three tributaries of the Tennessee River, the Elk River (Praelk), the Duck River (Praduk), and the Sequatchie River (Praseq). *Leptoxis* from the Sequatchie and Duck rivers have been previously analyzed by Dillon & Ahlstedt (1997). Analyzing all 11 populations together, we were able to evaluate observed levels of genetic divergence among nominal Alabama species by comparison to divergence among *Leptoxis* populations known to be conspecific, isolated at approximately equivalent distances.

The geographic relationships among the 11 populations analyzed in this work are mapped in Figure 1, and locality data and sample sizes are given in the Appendix. Although our sample sizes were in most cases greater than 30, only 21 individual *L. picta* were available. The Appendix also provides catalog numbers for voucher specimens deposited in the Academy of Natural Sciences of Philadelphia.

Our equipment and techniques for horizontal starch gel electrophoresis of whole animal homogenates have been previously described (Dillon, 1985, 1992). Samples were initially run on gels of five different buffer systems and stained to visualize 13 different enzymes. We simultaneously screened these gels and stains by requiring that clearly inter-

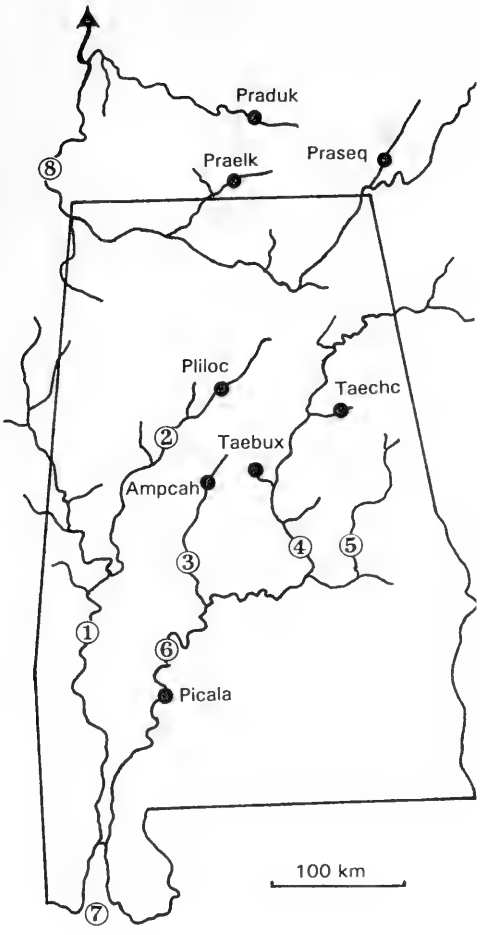


FIG. 1. The state of Alabama, showing major drainages and sample sites. (1) Tombigbee River, (2) Black Warrior River, (3) Cahaba River, (4) Coosa River, (5) Tallapoosa River, (6) Alabama River, (7) Mobile Bay, (8) Tennessee River.

pretable polymorphism be present in an initial comparison of Praduk and Ampcah1, selected as the most different pair of populations in our study. Ultimately we identified the products of nine putative gene loci for detailed analysis over all 11 populations.

The Poulik buffer (Poulik, 1957) was used to resolve glucose phosphate isomerase (GPI, EC 5.3.1.9) and octopine dehydrogenase (ODH, EC 1.5.1.11). The AP6 buffer (Clayton & Tretiak, 1972) was used to resolve mannose phosphate isomerase (MPI, EC 5.3.1.8), 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44), and isocitrate dehydrogenase (IDH, EC 1.1.1.42). The products of two putative loci were apparent on the IDH gel, one migrating cathodally ("IDHF") and the other anodally ("IDHS"). The TEB8 buffer (buffer III of Shaw & Prasad, 1970) was also employed for IDHF, xanthine dehydrogenase (XDH, EC 1.2.1.37), and esterase (EST1, EC 3.1.1.2). Superoxide dismutase (SOD, EC 1.15.1.1) activity was visualized as light bands on TEB8 gels darkly stained for XDH or IDH.

Allozyme phenotype has been shown to result from simple Mendelian inheritance of codominant alleles at the 6PGD locus by Chambers (1980), working with *Goniobasis floridana*. Dillon (1986) reported similar findings for GPI, ODH, and EST1, based on a mother-offspring analysis in *Goniobasis proxima*. Although the esterase stain employed here (α-napthyl acetate as substrate) yields a complex, multi-banded phenotype for each individual, only the slowly-migrating, strongly staining products of the single locus designated EST1 by Dillon (1986) were accorded a genetic interpretation in the present work.

Population Praduk served as the standard for allelic designations. We adopted here the same designations used for this population by Dillon & Ahlstedt (1997) for the four shared loci (EST1, GPI, MPI, and ODH), and labeled all new alleles accordingly. For the five loci not reported by Dillon & Ahlstedt (IDHS, IDHF, XDH, 6PGD, and SOD), the most common allele in Praduk was considered to migrate 100 mm and all other alleles labeled by their relative electrophoretic mobilities in millimeters faster or slower.

Gene frequencies, tests to Hardy-Weinberg expectation (by chi-square, with pooling for rare genotypes), and Nei's (1978) unbiased genetic identities and distances were calculated using BIOSYS version 1.7 (Swofford & Selander, 1981). Tests for homogeneity between populations within nominal species were by Fisher's exact method in 2×2 cases, otherwise by chi-square contingency tests, pooling the rarest rows or columns as necessary. We analyzed the matrix of genetic distances using the multidimensional scaling module of STATISTICA (Release 5.0, StatSoft, Inc.), with a standard Guttman-Lingoes (principal component) starting configuration. The distances between any pair of populations sharing no alleles at any locus (i.e., similarity = 0.0) were set to 5.0, a figure greater than any value actually observed.

RESULTS

Example shells from each of the four nominal Alabama *Leptoxis* species are shown in Figure 2. Their differences were not striking. The shells of *L. picta* tended to be heavier, with a higher spire, while those of *L. ampla* were lower and more rounded, and *L. taeniata* intermediate. Apical erosion made spire height difficult to evaluate, however, especially in the *L. ampla* population. The shells of *L. plicata* were less eroded, with more shouldered whorls. They were characterized by low folding (or plication) on the whorl periphery, barely visible in Figure 2. Although such plications have been reported to occur in *L. ampla,* we saw no evidence of them in our samples.

Gene frequencies are given in Table 1. Levels of intrapopulation variation were low, although perhaps not quite as low as in the better-studied *Goniobasis.* Over all $9 \times 11 = 99$ loci, we found 18 polymorphic as judged by the 95% criterion. Genotype frequencies at none of these 18 loci differed significantly from Hardy-Weinberg expectation.

All nominal species for which more than one population was sampled are listed in Table 2, along with the loci at which any intraspecific polymorphism was observed. Every nominal species showed significant interpopulation allelic frequency difference in at least one locus. This was especially striking at the ODH locus in *L. ampla* , and at both the ODH and EST1 loci in *L. plicata,* where the most common allele changed over distances as short as 15 river km. Not only did the three *L. praerosa* populations differ significantly from each other, the present Praseq and Praduk populations differed from the Sequatchie and Duck samples of Dillon & Ahlstedt (1997) located 20–30 km downstream.

Figure 3 shows Nei's unbiased genetic identities among all pairs of *Leptoxis* populations. The three *L. praerosa* populations were strikingly different from all others, as were the two *L. plicata* populations. The levels of genetic identity among *L. ampla, L. picta,* and *L. taeniata* populations were much higher. Figure 3 also depicts the Nei's genetic distances in two dimensions, from multidimensional scaling. After 100 iterations, the stress for this solution was 0.0015. The six populations comprising *L. ampla, L. picta,* and *L. taeniata* occupy one end of the long axis of the scale, the three *L. praerosa* the other end, and *L. plicata* appears intermediate.

DISCUSSION

The species concept under which the pleurocerid fauna of the Mobile Basin has been described and revised differs substantially from the biological concept in currency today. In his (1922) monograph on the "Anculosae" (*Leptoxis*) of Alabama, Goodrich wrote, "That collection of individuals in the Pleuroceridae may be called a species whose predominant characters are not the predominant characters of another collection of individuals. If we see only a few specimens of a single species its own peculiar characters may often seem to be submerged by characters linking it with another species. But in a long series the individual characters stand out, and we are compelled then to recognize the existence of definable differences and to proceed to describe them and provide the label of a name."

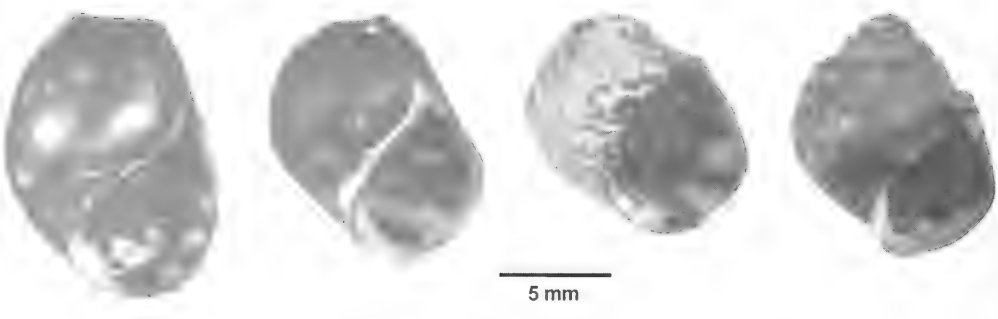


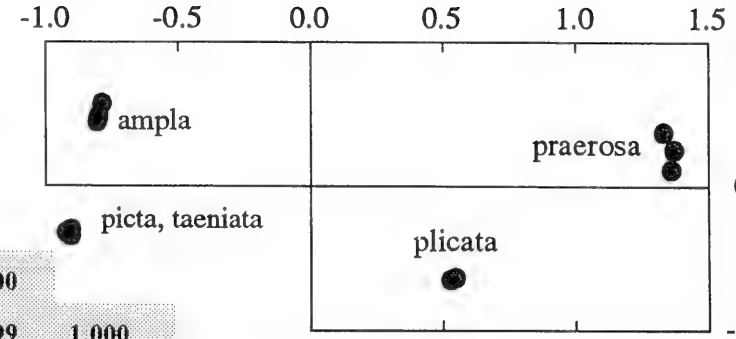
FIG. 2. Example shells of four Mobile Basin *Leptoxis* species. From left, *L. picta* (Picala), *L. taeniata* (Taechc), *L. ampla* (Ampcah1), and *L. plicata* (Pliloc2).

TABLE 1. Gene frequencies at nine allozyme loci for 11 populations of *Leptoxis*

Locus	Allele	Amp-cah1	Amp-cah2	Amp-cah3	Picala	Taechc	Taebux	Pliloc1	Pliloc2	Praduk	Praelk	Praseq
GPI	108				0.024			0.726	0.726			
	104	1.000	0.855	0.625	0.976	1.000	1.000					
	97							0.274	0.274	1.000	1.000	0.838
	94		0.145	0.375								
	90											0.162
MPI	100							1.000	1.000		0.016	
	98				0.929	1.000	1.000					
	95	1.000	1.000	1.000	0.071					1.000	0.984	1.000
EST1	106							0.613	0.387			
	105									1.000	1.000	1.000
	104	1.000	1.000	1.000	1.000	1.000	1.000					
	99							0.387	0.613			
6PGD	106	1.000	1.000	1.000	1.000	1.000	1.000	0.767	0.900			
	100							0.233	0.100	1.000	1.000	0.952
	94											0.048
ODH	121							0.355	0.242			
	118							0.065	0.048			
	115							0.323	0.532	0.855	0.177	0.054
	113							0.258	0.177	0.145	0.823	0.203
	110		0.057	0.682								
	107	1.000	0.943	0.318	1.000	1.000	1.000					0.743
IDHF	103							1.000	1.000			
	100									1.000	1.000	1.000
	98	1.000	1.000	1.000	1.000	0.875	1.000					
	95					0.125						
IDHS	103	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000			
	102											0.177
	100									1.000	1.000	0.823
XDH	103	1.000	1.000	1.000	1.000	1.000	1.000					
	100							1.000	1.000	1.000	1.000	1.000
SOD	110	1.000	1.000	1.000	1.000	1.000	1.000					
	100							1.000	1.000	1.000	1.000	1.000

Goodrich often reported that Mobile Basin species overlapped not just in character, but in geographic range as well. Populations identified by Goodrich (1922) as *L. picta* historically inhabited the lower Coosa River, the lower Cahaba River, and the Alabama River downstream to Claiborne, Monroe County. Goodrich reported the range of *L. taeniata* as substantially identical to that of *L. picta,* except that *L. taeniata* extended further up the Coosa River and its tributaries. Goodrich listed *L. ampla* from both the Coosa and Cahaba rivers and their tributaries, although not from the main stem of the Alabama River. Goodrich did not consider that the geographic range of *L. plicata* overlapped with those of *L. picta, L. taeniata,* or *L. ampla.* He recorded *L. plicata* as occurring in the Black Warrior River, the Tombigbee River, and their tributaries only.

The concept of the species differs today, as does the distribution of *Leptoxis* in the Mobile Basin. Under the biological species concept, local variation in gene frequencies (and by extension, external appearances) is a not-unexpected consequence of limited gene flow in



	Ampcah1	Ampcah2	Ampcah3	Picala	Taechc	Taebux	Pliloc1	Pliloc2	Praduk	Praelk	Praseq
Ampcah1	**1.000**										
Ampcah2	**0.998**	**1.000**									
Ampcah3	**0.932**	**0.949**	**1.000**								
Picala	0.903	0.899	0.829	**1.000**							
Taechc	0.886	0.881	0.810	0.998	**1.000**						
Taebux	0.889	0.884	0.814	1.000	**0.999**	**1.000**					
Pliloc1	0.222	0.227	0.234	0.226	0.225	0.222	**1.000**				
Pliloc2	0.234	0.239	0.248	0.239	0.237	0.234	**0.990**	**1.000**			
Praduk	0.113	0.115	0.119	0.008	0.000	0.000	0.359	0.357	**1.000**		
Praelk	0.111	0.114	0.118	0.008	0.000	0.000	0.358	0.331	**0.948**	**1.000**	
Praseq	0.206	0.206	0.155	0.097	0.089	0.088	0.338	0.314	**0.923**	**0.938**	**1.000**

FIG. 3. Below the diagonal are Nei (1978) unbiased genetic identities among *Leptoxis* populations. The identities of nominally conspecific populations are shaded darkly. Identities among the members of the *L. picta* group are lightly shaded. Above the diagonal is the result of a multidimensional scaling based on Nei (1978) genetic distances.

TABLE 2. The probability of homogeneity among nominally conspecific populations of *Leptoxis* (p_x from χ^2 tests, p_f from Fisher's exact tests). A subscript "c" indicates that rows or columns were combined for the test. The table is blank for loci where conspecific populations were not polymorphic.

	L. ampla	*L. taeniata*	*L. plicata*	*L. praerosa*
GPI	$p_{xc} < 0.001$		$p_f = 1.0$	$p_{fc} < 0.001$
EST1			$p_f = 0.019$	
6PGD			$p_f = 0.085$	
ODH	$p_x < 0.001$		$p_{xc} = 0.062$	$p_x < 0.001$
IDHF		$p_f = 0.006$		
IDHS				$p_{fc} < 0.001$

populations of organisms with dispersal capabilities as low as freshwater snails. For example, a culvert placed in a small North Carolina stream in the 1950s caused significant divergence at the ODH locus between upstream and downstream populations of *G. proxima* over a distance of just 10 meters (Dillon, 1988b). That this did not comprise a speciation event became clear when the barrier was removed, and the genetic difference disappeared. Indeed, geographically isolated populations of *G. proxima* sharing no alleles at as many as six allozyme loci have nevertheless demonstrated no evidence of reproductive isolation when transplanted (Dillon, 1986, 1988a). Evidence of similar interpopulation divergence is clear in our three samples of *L. ampla,* our two samples of *L. taeniata,* and our two samples of *L. plicata.* Whether the significant differences highlighted in Table 2 are due to some unrecognized barriers to dispersal, or whether they may be due to isolation by distance alone, cannot be told at present. But it is clear that our three populations of *L. ampla,* for example, do not constitute different species. It is also clear that, extending the levels of divergence illustrated within *L. ampla* down the 120 km length of the Cahaba River as was the situation earlier in this century, the *Leptoxis* of the Alabama River would be expected to show striking genetic differences with the *Leptoxis* of the headwaters, through isolation by distance. There is little expectation, however, that reproductive isolation will evolve in such a circumstance, or that headwaters populations and populations from the main river will speciate parapatrically.

The divergence among *L. taeniata, L. picta,* and *L. ampla* is negligible, given their geographic distance. *Leptoxis picta* has uncommon alleles at the GPI and MPI loci not detected in *L. taeniata,* and one *L. taeniata* population has an allele at IDHF not seen in *L. picta.* The levels of divergence appeared somewhat greater between *L. ampla* and *L. picta/taeniata,* due to the results at the MPI locus. But although *L. ampla* is fixed for an allele not seen in *L. taeniata,* Table 1 shows that both MPI alleles are found in the *L. picta* population that may have connected them in the main Alabama River. Such small and clinal differences are not comparable to those normally displayed by species of pleurocerid snails presumed distinct, as illustrated by *L. praerosa* and *L. plicata.* We therefore refer to all three of these taxa, *L. picta, L. taeniata,* and *L. ampla,* as the "*Leptoxis picta* group."

In most respects, our findings coincide with those based on 16S rRNA gene sequence divergence. Lydeard et al. (1997) also found *L. plicata* to be quite distinct from all other Mobile Basin *Leptoxis,* unique at about 20% of its nucleotide bases. Lydeard's mtDNA phylogeny depicted the three members of the *L. picta* group as a single clade when transversions were weighted more than transitions. But while very little sequence divergence was apparent between *L. taeniata* and *L. ampla* (only about 2%), Lydeard reported about 20% sequence divergence between *L. taeniata/ampla* and *L. picta.* So our finding that *L. picta* and *L. taeniata* are indistinguishable in their allozyme frequencies at nine loci was quite unexpected.

A similar discrepancy between allozyme and mtDNA divergence in oysters was attributed to balancing selection at multiple enzyme loci by Karl & Avise (1992), although much more data would be required before such a suggestion could be made in our case. Lydeard et al. (1997) only analyzed a single individual for each nominal *Leptoxis* species. There is a clear need for additional surveys of

16S rRNA sequence divergence focused below the species level.

A complete understanding of the genetic relationships among *L. picta, L. taeniata,* and *L. ampla* would have required samples from populations inhabiting the lower regions of the Cahaba and Coosa rivers, where the three nominal species were once reported to co-occur. All such populations are long extinct. Regardless of their specific status, the levels of genetic diversity displayed by the small populations of the *L. picta* group that remain today, as evidenced by both mtDNA and allozyme studies, argue strongly for conservation measures. The *L. plicata* population restricted now to just 20 km of Locust Fork (Hartfield, 1997) is clearly a unique species by all measures, and deserves immediate protection.

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APPENDIX

Locality data, sample sizes, and (where applicable) catalog numbers for voucher specimens deposited in the Academy of Natural Sciences of Philadelphia

Ampcah1—Cahaba River at Co. 52, 1.5 km W of Helen, Shelby County, Alabama. Same station as CA-61 of Bogan & Pierson (1993b). N = 25 *Leptoxis ampla.*

Ampcah2—Cahaba River at River Road, 3 km S of intersection of Co. 1 and Co. 13. Shelby County, Alabama. N = 36 *Leptoxis ampla.*

Ampcah3—Cahaba River at Co. 24, Bibb County, Alabama. Just downstream from station CA-64 of Bogan & Pierson (1993b), and 10 km downstream from the *L. ampla* site of Lydeard et al. (1997). N = 24 *Leptoxis ampla.* ANSP 400111.

Picala—Alabama River about 2 km south of U.S. 84 crossing, Monroe County, Alabama. Same site as the *L. picta* site of Lydeard et al. (1997). N = 21 *Leptoxis picta.* ANSP 400112.

Pliloc1—Locust Fork, 0.4 km upstream from the Mount Olive Road boat ramp, Jefferson County, Alabama. About 20 km south of *L. plicata* site of Lydeard et al. (1997). N = 31 *Leptoxis plicata.* ANSP 400113.

Pliloc2—Locust Fork, at shoal 10 km downstream from the Mount Olive Road boat ramp, Jefferson County, Alabama. N = 31 *Leptoxis plicata.*

Taebux—Buxahatchee Creek, Shelby County, Alabama. N = 34 *Leptoxis taeniata.*

Taechc—Choccolocco Creek, Talladega County, Alabama. Same site as the *L. taeniata* site of Lydeard et al. (1997). N = 32 *Leptoxis taeniata.* ANSP 400115.

Praduk—Duck River at Shelbyville, Bedford County, Tennessee. About 30 km upstream from Duck River site of Dillon & Ahlstedt (1997). N = 31 *Leptoxis praerosa.*

Praelk—Elk River at Stump Shoals Public Access near US 64 bridge, 8 km E of Fayetteville, Lincoln County, Tennessee. N = 31 *Leptoxis praerosa.* ANSP 400114.

Praseq—Sequatchie River at Tn 28 bridge, Whitwell, Marion County, Tennessee. About 20 km upstream from Sequatchie site of Dillon & Ahlstedt (1997). N = 37 *Leptoxis praerosa.*

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LARVAL FUSION AND DEVELOPMENT OF CONJOINED TERATOIDS IN *BIOMPHALARIA GLABRATA*

Charles S. Richards, Carolyn Patterson, Fred A. Lewis & Matty Knight

Biomedical Research Institute, 12111 Parklawn Drive, Rockville, Maryland 20852

ABSTRACT

Snails of a genetically isolated laboratory stock of *Biomphalaria glabrata,* if mated to snails of certain other stocks, produce polyzygotic egg capsules. When two or more embryos occupy a single capsule, some embryos spontaneously fuse during the trochophore larval stage into teratoids of usually two, but up to seven, conjoined snails. We have observed more than 950 of these fused teratoids, with some dizygotic conjoined twins surviving to adulthood. Allophenic cell-to-cell adhesions lead to unusual patterns of development, apparently determined by the areas of the embryos that are fused.

Key words: *Biomphalaria,* Pulmonata, development.

INTRODUCTION

Observations of conjoined twinning in mollusks have been previously reported [reviewed by Bigus (1981) and Mason & Copeland (1988)], most often as rare anomalies in otherwise normal populations. The unexpected occurrence of large numbers of spontaneously conjoining embryos in one of our snail stocks and the consistency with which we obtain these teratoids have provided an abundance of material for study. Allophenic cell membrane junctions, the development of conjoined embryos, and the unusual morphological patterns that result are areas of interest that lead us to continue the culture of these teratoids.

The pulmonate gastropod *Biomphalaria glabrata* is a simultaneous hermaphrodite and is an important intermediate host of the parasitic trematode *Schistosoma mansoni.* We maintain several named stocks of this snail in genetic isolation to conserve phenotypic variations in pigment and in susceptibility to *S. mansoni* (Richards & Shade, 1987). Pigmentation markers (Newton, 1954; Paraense, 1955) are useful in elucidating inheritance patterns of the susceptibility-resistant phenotypes (Richards, 1985; Richards et al., 1992).

MATERIALS & METHODS

Snails of one genetic stock, M3 636, produced a large number of polyzygotic eggs after out-crossing with a different stock (10R2 was used most often). M3 636 stock snails were isolated as juveniles and reared individually in 400 ml beakers until the onset of egg production by self-fertilization. Each snail was then put in a new 400 ml beaker with a partner of a different snail stock. After 2–7 days, during which each snail fertilized the other, the snails were re-isolated. A piece of clear plastic sheeting was floated in the beaker of each isolated snail. Most snails preferentially deposit clutches onto this plastic, which can then be removed from the beaker for examination under a low-power dissecting microscope. Hybrid pigmentation of offspring from the clutches of both parents indicated the two snails had each functioned as a male in the cross.

Many (> 200) egg clutches that contained polyzygotic egg capsules were sketched at intervals of their development to record the number of zygotes originally deposited in each capsule and the timing and pattern of embryo fusion.

Some conjoined teratoids apparently had difficulty hatching on their own, in which cases we used dissecting needles to break the egg capsule. Survival of teratoids after hatching was very limited until we began feeding the neonate teratoids with cultured *Nostoc* sp. (Liang et al., 1987). This filamentous cyanobacterium, in addition to being nutritionally sufficient, reduced the need for active foraging and allowed simultaneous feeding by component snails fused in a configuration that made motility and feeding problematic.

RESULTS

Polyzygotic Egg Capsules

Because we had not previously observed significant polyzygocy in self-fertilizing snails or in isogenic interbreeding populations of laboratory snail stocks, the production of large numbers of polyzygotic egg capsules by an out-crossed snail suggested that crossing genetically distant snails may trigger polyzygocy.

Most M3 636 snails exhibit a degree of self-sterility (Paraense, 1993) and produce, by self-fertilization, very few clutches, each of which contains few or no viable eggs. After mating with a snail from a different stock, M3 636 snails would generally produce normal clutches for several days before producing polyzygotic egg capsules. Polyzygocy increased during the next 4–6 weeks and then declined until only a few small clutches were produced. A subsequent out-crossing would often induce another cycle of polyzygocy.

Egg capsules that contain multiple zygotes are not larger than monozygotic capsules. Multiple embryos share the single portion of nutritive capsular fluid and are smaller at hatching than snails from normal monozygotic egg capsules. In clutches that contain both single embryo and polyzygotic egg capsules, it is sometimes evident that the egg capsules deposited first are those that are monozygotic. The egg capsules deposited last in the clutch contain increasing numbers of zygotes, as if the snail were unable to provide enough capsular fluid or other material to accommodate available zygotes. Zygotes are often observed at one end of the clutch, not contained in egg capsules, but loose in the fluid that surrounds the egg capsules.

Fusion of Larval Snails

Early observations of conjoined twins indicated that it was unlikely that they resulted from incomplete division of a single embryo. Conjoined snails of different pigment phenotypes (Fig. 1) confirmed that fusion of individuals occurred. In our many observations of new clutches, which were sketched and recorded at intervals in their development, we saw that the polyzygotic egg capsules contained excess fertilized oocytes and that division of a single zygote into multiple germs did not occur.

For embryos to fuse they must make contact. When the clutch is first deposited, the zygotes are non-motile and are spaced throughout the viscous capsular fluid. Cleavage continues with little change in the size of the embryo through the blastula stage. During this interval, up to about 24 hrs after the clutch is deposited, the embryos become more dense, and gravity acts to bring the embryos, now blastulae, together at the lowest point in the spherical egg capsule. Though the embryos seem to be in close contact, we have not seen fusion at this stage. With gastrulation, the vitelline membrane that had surrounded and isolated the embryo is lost (Kawano et al., 1992; Arambasic et al., 1989), the embryo increases in size, and the prototroch forms. The first larval motility is seen in this trochophore larva. We see fused embryos only after the multiple embryos in a polyzygotic capsule begin to move by means of the ciliated prototroch cells (Fig. 2). However, there may be some asynchrony in the development of embryos within the same egg mass, and the growing embryos may crowd each other in a polyzygotic egg capsule to the extent that fusion or the absence of fusion is not discernable. Also, because of the large number of polyzygotic egg capsules produced in our laboratory, we do not follow every clutch throughout larval development. Some conjoined twins are first observed when the component snails are long past the early trochophore stage. Fusion of separate embryos may sometimes occur in late larval stages.

FIG. 1. Conjoined *Biomphalaria glabrata* twins with one albino component and one black-eye (arrow) component. (length about 1 mm)

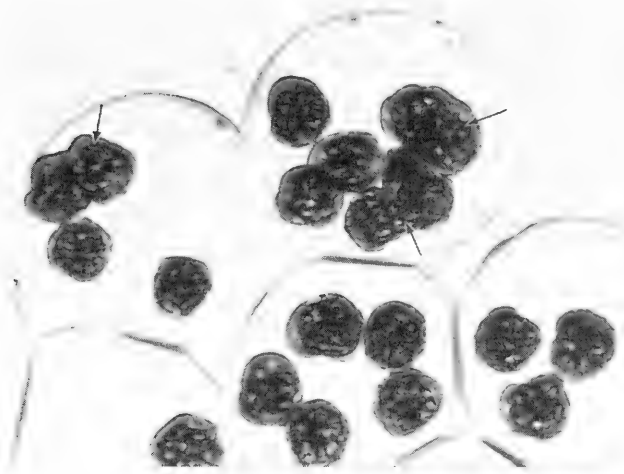

FIG. 2. Four egg capsules from a clutch laid by a M3 636 snail after crossing with a 10R2 snail: motile trochophore larvae of which three pairs (arrows) have fused.

Development of Conjoined Embryos

The differentiation and development of teratoids subsequent to larval fusion is, of course, affected by the regions of the embryos that are fused. Larval snails fuse in many configurations. Most teratoids with more than two components do not develop long past the trochophore stage (Michelson & Schork, 1958; Bigus, 1981). Therefore, the following generalizations will be discussed only in relation to the development of conjoined twins.

Fusion occurs between homologous structures. For example, two heads may be fused dorsally (Fig. 3) or laterally, occasionally two individuals may be joined at the edge of the foot, or the shell fields of two embryos may fuse and result in a two-headed individual under one shell (Fig. 4). Development of a twin that would have resulted from fusion of a pretrochal area of one component embryo with a posttrochal area of the other component embryo (Kawano et al., 1992) has not been seen.

Every conjoined twin that developed and survived to the veliger stage had two heads (Fig. 5). Mouth parts, including the radula and anterior esophagus, were not fused in surviving conjoined twins. Occasionally tentacles and eyes were partially fused, displaced, or distorted, but there was individual development of these structures in both component snails.

For twins that had fused in a configuration allowing normal development of two shells—for example, fusion of dorsal areas of the heads—each component developed a complete anatomy of basically normal morphology.

The fusion of the shell fields of two embryos often led to the formation of an aberrant shell shared by the two components. Often these teratoids would initially have two hearts, two kidneys, and other separate viscera. As development progressed with the single shell sheltering the two-headed teratoid, usually only one heart would persist and other internal organs would seemingly coalesce into an internal anatomy grossly similar to that of a normal individual. These individuals, however, did not produce eggs, although one lived several weeks (Fig.4).

Three sets of conjoined twins fused in configurations that allowed one component to develop fairly normally while the other component, after some early development, degenerated and became a vestigial tumor-like mass on the larger component. In these cases, head and mouth parts of the smaller component, although initially present, were lost. The larger component snails continued to develop and two produced offspring.

Conjoined snails, often with heads oriented

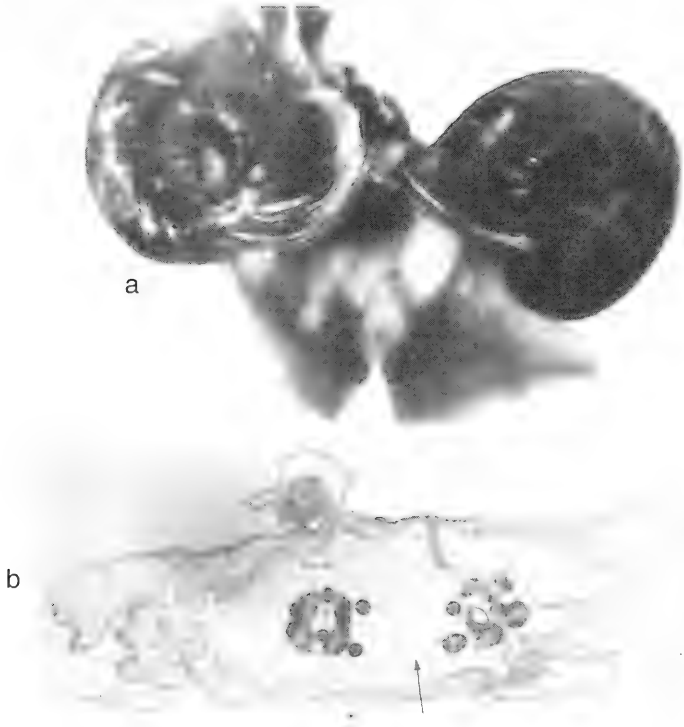

FIG. 3. (a) Teratoid that survived several weeks and produced offspring (each shell ~4 mm diameter) and (b) a stained section through the separate brains and shared cephalic sinus (arrow) of this teratoid.

FIG. 4. Conjoined pair sharing one aberrant shell (grid = 2 mm). This teratoid initially had two hearts, only one of which persisted.

FIG. 5. A teratoid with one component that initially had an everted mouth and was unable to feed. The other component fed normally, developed a complete anatomy, and produced offspring. The mouth of the non-feeding component reverted to the normal configuration concurrent with the eversion of its preputium (shell diameter approximately 5 mm).

in different planes or facing opposite directions, are sometimes unable to hatch without assistance and do not forage and feed efficiently. Nearly all teratoids are considerably smaller than individual snails of the same age, and most do not approach normal adult size.

DISCUSSION

Polyzygocy is a necessary condition for the formation of the conjoined teratoids described in this report. The highly determinate spiral cleavage in *B. glabrata* and other mollusks would all but preclude monozygotic twinning (Bigus, 1981; Crabb, 1931). One previous report (Eyster, 1995) of conjoined teratoids involves one of the many mollusks for which polyzygocy is normal. Mason & Copeland (1988) report some increase in the frequency of double embryo egg capsules in one generation of selected breeding of the normally mono-embryonic pulmonate slug *Lehmannia valentiana.* Studies of other conjoined mollusks for which polyzygocy is unusual have not demonstrated any heritability of the trait (Bigus, 1981; Crabb, 1931; Hall, 1925). In the past, we have seen occasional polyzygotic egg capsules in most of our laboratory stocks of *B. glabrata,* but attempts in these cases to increase its frequency by selection always failed. The fact that snails of one of our genetically isolated stocks of *B. glabrata,* when mated with snails of certain other stocks, consistently produce polyzygotic egg capsules indicates that there is indeed a genetic factor in this case of polyzygocy.

Bigus (1981) reports that polyzygocy in *Physa acuta* increases as reproductive senescence approaches, concurrent with a reduction in the thickness of the capsule and in the amount of capsular fluid. Although we have found no age dependence in our study, the very small clutch size of most self-fertilizing M3 636 snails suggests that polyzygocy may serve as a reproductive strategy that maximizes number of offspring when resources for egg capsule or clutch production are for some reason limited or declining while production of zygotes continues or increases.

Larval motility does not limit fusion and may in some instances be necessary to bring the trochophore larvae into contact with each other. The embryos of *B. glabrata* do not move until the vitelline membrane is lost. *Lehmannia* embryos lack a vitelline membrane and are reportedly motile as zygotes when the polar bodies are visible (Mason & Copeland, 1988). Although these authors observed early paired zygotes of *Lehmannia* in apparent contact, they do not state when in embryonic development fusion may have occurred, only that teratoids survived to hatch. Eyster (1995) increased the number of conjoined teratoids, first observed as veliger larvae, by subjecting the polyzygotic egg capsules of *Crepidula* to acidified seawater, possibly disrupting or destroying the vitelline membrane. We have not been able to demonstrably remove this membrane from *B. glabrata* embryos and have not seen larval motility, indicating loss of the membrane, before the early trochophore stage.

By the trochophore larva stage, the morphogenetic fields of *B. glabrata* have been established (Camey & Verdonk, 1970; Kawano et al., 1992), and cell membranes on the exterior of the embryo are evidently primed to form cell-to-cell junctions (Serras et al., 1990). Further study of live and fixed, whole-mount and sectioned, fused embryos will help determine which cells form these junctions, the nature of the junctions, whether non-homologous areas of separate embryos can fuse, and the effect on development of communication between allophenic cells.

The morphologies of conjoined teratoids in our laboratory are analogous to morphologies reported for teratoids of other molluscan species, and most closely resemble those reported by Bigus (1981) for *Physa acuta,* another pulmonate snail. The survival to maturity of bizarrely configured teratoids of *Lehmannia* (Mason & Copeland, 1988) may be due to the lack of shell to interfere with mobility or to limit the spatial arrangement of multiple organs.

From the morphologies we have observed, it is apparent that the development of head regions is not as greatly modified or suppressed as the development of other regions often is. We cannot readily determine the extent to which internal organs are mosaic in a developed teratoid, but the head and mouth parts are clearly distinct. When separate hearts are initially present, in some cases, one will persist as the other seems to be resorbed. Other organ systems, including the kidney, reproductive tract, and the digestive tract posterior to the mouth, develop later than the heart and are not as readily visible under the shell as is the beating heart. To what degree these and other systems are histologically a mosaic of genetically different cells or are the result of suppression or degeneration of one genetic

line of cells while the other cell line develops is not known.

ACKNOWLEDGEMENTS

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COMPARATIVE SPERM MORPHOLOGY AND PHYLOGENETIC CLASSIFICATION OF RECENT MYTILOIDEA (BIVALVIA)

Alexander I. Kafanov & Anatoly L. Drozdov

Institute of Marine Biology, Far East Branch, Russian Academy of Sciences, Vladivostok 690041, Russia

ABSTRACT

In sperm morphology, the genera of Recent Mytiloidea that have been studied thus far — *Adula, Arcuatula, Aulacomya, Brachidontes, Choromytilus, Crenomytilus, Modiolus, Musculista, Musculus, Mytilus, Perna, Semimytilus* and *Septifer* — may be classified into several groups based on size of the tip, mode of chromatin packing, number of mitochondria, and presence or absence of an axial rod in the acrosomes. Spermatozoa of *Modiolus* might be regarded as a basal or plesiomorphic type within the Mytiloidea. The available sperm morphology data, together with conchological characters, is adequate for suggesting a modified classification of Recent Mytiloidea, representing a sort of trade-off between the arrangements of Soot-Ryen (1969) and Scarlato & Starobogatov (1979, 1984). Only one family (Mytilidae) is conventionally acknowledged among Recent Mytiloidea. The family Septiferidae Scarlato & Starobogatov, 1979, has been ranked as a tribe in the subfamily Modiolinae. The subfamily Perninae Scarlato & Starobogatov, 1979, *non* Zittel, 1895, has been abolished.

Key works: Mytilidae, sperm morphology, classification.

INTRODUCTION

It is commonly accepted that, besides the Triassic Mysidiellidae Cox, 1964, the superfamily Mytiloidea includes the large family Mytilidae Rafinesque, 1815, incorporating at least 57 Recent and fossil taxa of the genus group. Numerous Mytilidae are of commercial importance in fisheries and aquaculture and are of biostratigraphical use. For these reasons, the systematics of the family is of long-standing interest to researchers.

After the revision by Soot-Ryen (1969) for the *Treatise on Invertebrate Paleontology,* it became the usual practice to subdivide Mytilidae into four or five separate subfamilies: Mytilinae, Crenellinae Gray, 1840, Musculinae Iredale, 1939, Lithophaginae H. Adams & A. Adams, 1857, and Modiolinae Keen, 1958 (Kafanov, 1987). Subsequently, these subfamilies were supplemented by the monotypic Dacrydiinae (Ockelmann, 1983: 112).

A very different scheme was suggested by Scarlato & Starobogatov (1979, 1984; Starobogatov, 1992), who subdivided the family Mytilidae into four separate families — Mytilidae, Septiferidae Scarlato & Starobogatov, 1979, Crenellidae, and Lithophagidae — and they established an additional 13 subfamilies. Unfortunately, their diagnoses of the taxa are extremely brief and they did not give arguments to support their classification. At the same time, a need for new morphological criteria for the classification of the Mytiloidea and the taxonomic importance of sperm morphology in other groups made us pay attention to specific features of spermatozoon morphology.

TAXONOMIC IMPORTANCE OF SPERMATOZOON MORPHOLOGY

In Recent years, gamete ultrastructure has been used extensively for solving various systematic and phylogenetic problems in the Metazoa (Afzelius, 1979; Jamieson & Rouse, 1989; Ferraguti & Gelder, 1991; Justine, 1991; Jamieson et al., 1995). Species specificity for spermatozoa ultrastructure was initially established for Mammalia (Bishop & Austin, 1957) and subsequently confirmed for other animal groups (Baccetti & Afzelius, 1976), including bivalve molluscs (Drozdov & Reunov, 1986b). Structural features of spermatozoa have been successfully used for identification of sibling species (Meier et al., 1972; Aksenova, 1978). Species specificity of spermatozoon morphology, acrosome structure in particular, is thought (Popham, 1979) to contribute to reproductive isolation of closely related species

on a cytological level. In the opinion of a number of researchers (Ockelman, 1964, 1965; Popham, 1979; Franzen, 1970, 1983; Drozdov & Kasyanov, 1985; Pashchenko & Drozdov, 1991), the spermatozoon structure of bivalve molluscs is dependent on the structure of egg membrane and specific insemination features.

A significant amount of data is presently available concerning gamete-specific morphological features for families of various animal groups: scleractinias (Steiner, 1991), archiannelids (Franzen, 1982; Franzen & Sensenbaugh, 1984), and chitons (Hodgson et al., 1988; Pashchenko & Drozdov, 1994, 1997). Such features also are known in the Bivalvia (Karpevich, 1961, 1964; Gharagozlou-Van Ginneken & Pochon-Masson, 1971; Thompson, 1973; Popham, 1974, 1979; Maxwell, 1983; Drozdov & Kasyanov, 1985; Eckelbarger et al., 1990; Hodgson et al., 1987, 1990; Healy, 1995, 1996). In particular, acrosomal morphology displays characteristic features that provide information on phylogenetic relationships (Baccetti, 1970).

Spermatozoon morphology of Mytiloidea has been dealt with in several papers (Table 1), and sufficient data now exists for a review of previous classifications.

GENERIC FEATURES OF SPERMATOZOON MORPHOLOGY IN MYTILOIDEA

All mytiloidean sperm show essentially the same organization of the midpiece, that is, spherical mitochondria are grouped in a ring around the proximal and distal centrioles (centrioles arranged at approximately 90° to each other), a small rootlet connects the proximal centriole to the nucleus, and a satellite fibre complex of nine terminally forked fibres anchors the distal centriole to the plasma membrane. Some generic differences in spermatozoon morphology are given below and in Table 2.

Modiolus Lamarck, 1799 (Fig. 1a). Large-sized spermatozoa with flask-shaped head and pointed acrosome. Barrel-shaped nucleus 2.0 μm in length and 2.7 μm in diameter.

TABLE 1. References to the morphology of spermatozoa in Recent Mytiloidea

Species	Authors
Adula falcatoides Habe, 1955	Reunov & Drozdov, 1986
Arcuatula capensis (Krauss, 1848)	Reunov & Hodgson, 1994
Aulacomya ater (Molina, 1782)	Hodgson & Bernard, 1986a; Garrido & Gallardo, 1996
Brachidontes semistriatus (Krauss, 1848)	Reunov & Hodgson, 1994
Choromytilus chorus (Molina, 1782)	Garrido & Gallardo, 1996
Choromytilus meridionalis (Krauss, 1848)	Hodgson & Bernard, 1986a
Crenomytilus grayanus (Dunker, 1853)	Drozdov, 1979, 1983; Drozdov & Mashansky, 1979; Drozdov et al., 1981; Drozdov & Kasyanov, 1985
Modiolus americanus (Leach, 1815)	Hylander & Summers, 1977
M. kurilensis Bernard, 1983	Drozdov & Kasyanov, 1985; Drozdov & Reunov, 1986a
M. modiolus (Linnaeus, 1758)	Franzen, 1955
Musculista senhousia (Benson, in Cantor, 1842)	Drozdov, 1992
Musculus discors (Linnaeus, 1767), including *M. laevigatus* (Gray, 1824)	Kaufman, 1977; Franzen, 1983; Drozdov & Kasyanov, 1985
Mytilus chilensis Hupé, 1854	Garrido & Gallardo, 1996
M. coruscus Gould, 1861	Reunov & Drozdov, 1987
Mytilus of the group of *M. edulis* [*M. edulis* Linnaeus, 1758 + *M. trossulus* Gould, 1850]	Niijima & Dan, 1965; Longo & Dornfeld, 1967; Endo, 1976; Drozdov & Reunov, 1986b; Hodgson & Bernard, 1986b
M. galloprovincialis Lamarck, 1819	Hodgson & Bernard, 1986b; Crespo et al., 1990; Drozdov, 1992
Perna perna (Linnaeus, 1758)	Boucart et al., 1965; Bernard & Hodgson, 1985; Hodgson & Bernard, 1986a
P. viridis (Linnaeus, 1758)	Drozdov, 1992
Semimytilus algosus (Gould, 1850)	Garrido & Gallardo, 1996
Septifer keenae Nomura, 1936	Reunov & Drozdov, 1986

TABLE 2. Morphology and sizes of Mytilidae spermatozoa

Genera	Nucleus length, µm	Acrosome length, µm	Shape of head	Quantity of DNA in nucleus, pkg*	DNA density, pkg/µm^3*	Axial rod presence/ absence	Number of mitochondria	Figure herein
Adula	2.9	1.4	flask (conical)	2.14	0.49	no	5	1c
Arcuatula	1.3	0.8	same	?	?	no	?	1g
Aulacomya	2.2	3.7	same	?	?	no	5–6	1b
Brachidontes	2.0	2.5	same	?	?	no	?	1d
Choromytilus	1.5–1.6	2.0–2.1	same	?	?	no	4–6	1e
Crenomytilus	2.0	2.0	same	2.39	0.52	yes	5	2c
Modiolus	2.0	2.5	same	3.99	0.42	no	12–14	1a
Musculista	1.4	1.1	bullet	?	?	no	5	1f
Musculus	7.2–7.7	0.8	extended conical	?	?	yes	4	3a,b
Mytilus	2.0–2.1	2.0–5.0	flask (conical)	1.47–2.24	0.45–0.46	yes	5	2a,b,d,e
Perna	1.6–1.7	2.0–5.3	same	?	?	yes	5	2f
Semimytilus	1.2	2.4	same	?	?	no	?	
Septifer	0.5	0.7	bullet	1.29	0.72	no	5	1h

*After Tuturova, 1989.

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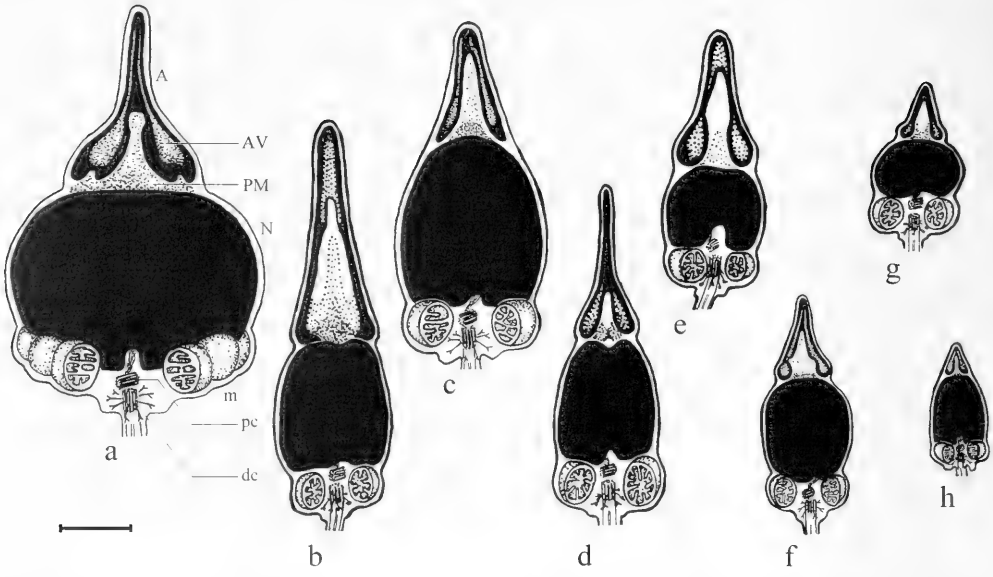

FIG. 1. Structural pattern of spermatozoa of the subfamily Modiolinae: a—*Modiolus kurilensis,* b—*Aulacomya ater,* c—*Adula falcatoides,* d—*Brachidontes semistriatus,* e—*Choromytilus meridionalis,* f—*Musculista senhousia,* g—*Arcuatula capensis,* h—*Septifer keenae.* A: acrosome; N: nucleus; AR: axial rod; m: mitochondria; dc: distal centriole; pc: proximal centriole; AV: acrosomal vesicle; PM: periacrosomal material. Bar = 1 μm.

No axial rod typically present. Acrosomal complex of two parts: cone-shaped acrosomal vesicle and periacrosomal granular material arranged between the vesicle and nucleus. On side of nucleus, acrosomal vesicle with a contraction filled by periacrosomal material. Midpiece of spermatozoon formed by two mutually perpendicular centrioles, surrounded by 13–14 mitochondria.

Aulacomya Mörch, 1853 (Fig. 2b). Sperms with flask-shaped head, containing a barrel-shaped nucleus of about 2.2 μm length and a large (3.7 μm long) cone-shaped acrosomal vesicle with a large cavity filled by granular periacrosomal material. The midpiece of spermatozoon with five, sometimes six, spherical mitochondria.

Adula H. & A. Adams, 1857 (Fig. 1c). Acrosomal complex (1.4 μm long) with a conical electron-dense acrosomal vesicle with a contraction in a distal acrosomal part, filled by electron-lucent periacrosomal material. Middle part of spermatozoon formed by five mitochondria encircling two centrioles.

Brachidontes Swainson, 1840 (Fig. 1d), and *Arcuatula* Lamy, 1919, *ex* Jousseaume, MS (Fig. 1g). Sperm head of *B. semistriatus* 4.5 μm long, containing a barrel-shaped nucleus (2.0 × 1.9 μm) with minute anterior hole. Nucleus adjoining flask-shaped acrosome of 2.5 μm length, with a long anterior projection.

Sperm of *A. capensis* with a relatively long head, a barrel-shaped nucleus (length, 1.2 μm, average diameter 1.5 μm) and a small conical acrosome, 0.8 μm long).

Choromytilus Soot-Ryen, 1952 (Fig. 1e). Sperms of *C. meridionalis* with a comparatively rounded nucleus of 1.6 μm diameter and a 2 μm-long acrosome crowning the head. Acrosome with a large-sized conic cavity filled by granular electronically compact material. The middle part of spermatozoon includes four (sometimes five) spheric mitochondriae of 6 μm diameter.

Musculista Yamamoto & Habe, 1958 (Fig. 1f). Unlike *Modiolus,* spermatozoa comparatively small-sized (bullet-shaped head of sperm about 2.5 μm, middle piece of spermatozoon 0.4 μm, top head width 1.2 μm). Flask-shaped acrosome with two components: acrosomal vesicle widened distally and narrowed towards the top, possessing a contraction in the distal part filled by globular periacrosomal material. Proximal and distal centrioles perpendicular.

Septifer (*Mytilisepta*) Habe, 1951 (Fig. 1h). Sperms with a bullet-shaped head of 1.2 length and 1.4 μm width. Conical acrosome 0.7 μm-long with cupola-shaped acrosomal vesicle filled with electron-lucent periacrosomal material. Two centrioles surrounded by five mitochondriae of 0.4 μm diameter.

Mytilus Linnaeus, 1758 (Figs. 2a, b, d, e), *Crenomytilus* Soot-Ryen, 1955 (Fig. 2c), and *Perna* Retzius, 1788 (Figs. 2f, g). Spermatozoa of several species of these genera with flask-shaped head between 3.6 μm (*Perna viridis*) and 7.0 μm (*Mytilus coruscus*) in length. Barrel-shaped nucleus with pointed acrosome anteriorly. Posteriorly, nucleus adjacent to middle part of spermatozoon containing five spheric mitochondria and two centrioles.

Musculus Röding, 1798 (Fig. 3a, b). Sperms with extended cone-shaped head of about 8.0 μm length and 0.8 μm base diameter. Acrosomal complex of two components: apical part with an acrosomal vesicle of approximately 0.8 μm diameter, surrounded by a membrane. Periacrosomal material branching off from vesicle and consisting of a bundle of actine threads entering nucleus channel and a small amount of globular material. In some specimens, the axial rod reaching midpart of spermatozoon (*M. discors* from North Pacific), whereas in other specimens only reaching middle of nucleus (same species from North Atlantic and Arctic Ocean). Midportion of sperm with four spheric mitochondriae of 0.4 μm diameter encircling two perpendicular centrioles.

TAXONOMIC ANALYSIS

Although a thorough taxonomic analysis would require the availability of sperm morphology data for a larger number of genera, the available data (Table 1) is adequate for some conclusions on alternatives for the systematics of the Mytiloidea (Table 3). The more so because the taxonomic value of a character depends on its adaptive significance

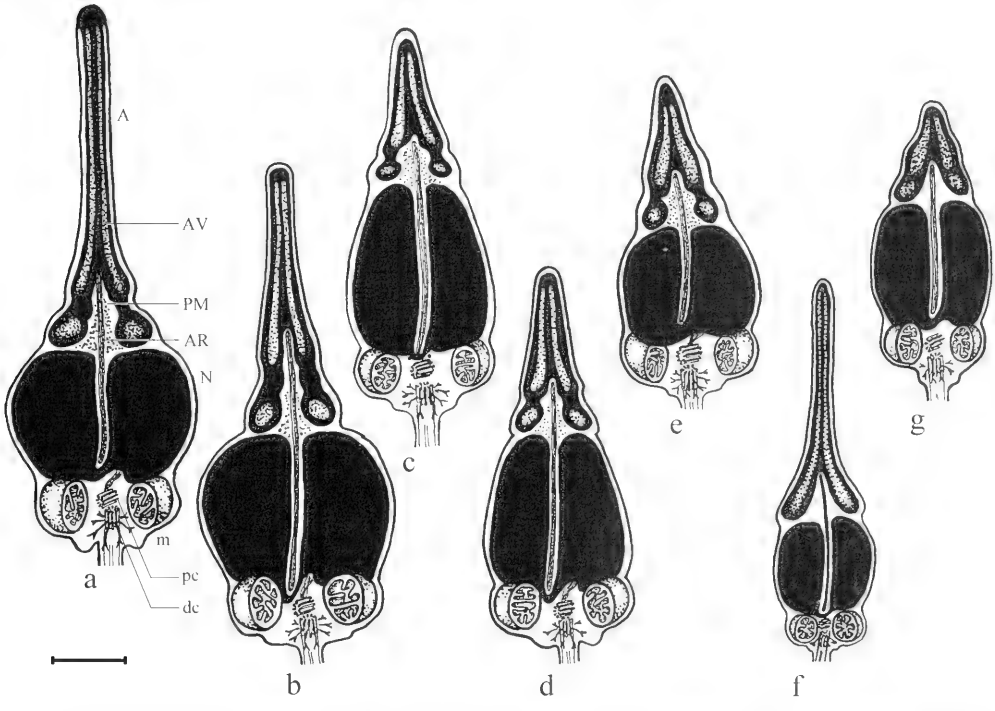

FIG. 2. Structural pattern of spermatozoa of the subfamily Mytilinae: a—*Mytilus coruscus*, b—*Mytilus trossulus*, c—*Crenomytilus grayanus*, d—*Mytilus galloprovincialis*, e—*Mytilus edulis*, f—*Perna perna*, g—*Perna viridis*. Bar = 1 μm.

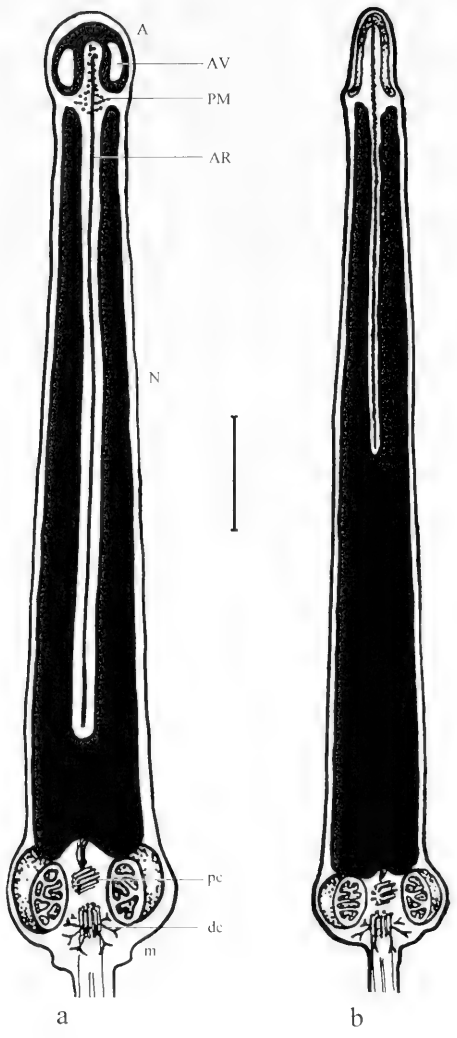

FIG. 3. Structural pattern of spermatozoa of the subfamily Musculinae: a, b—*Musculus discors.* Note that the length of the axial rod varies among specimens of one species. Bar = 1 μm.

(Mayr, 1969), there is no reason to treat spermatozoon morphology as less taxonomically important than the conventional conchological characters.

In terms of the presence or absence of an axial rod in acrosomes of intact spermatozoa, all the analysed representatives of Mytiloidea may be clearly classed into two major groups: those possessing no axial rod (*Modiolus, Musculista, Septifer, Adula, Choromytilus, Aulacomya, Brachidontes, Semimytilus,* and *Arcuatula*) and those possessing an axial rod (*Mytilus, Crenomytilus, Perna,* and *Musculus*). The axial rod of the acrosome consists of a bundle of actine filaments which, in the course of acrosomal reaction, form the basis for the formation of acrosomal filament (Drozdov & Mashansky, 1979; Drozdov et al., 1981; Drozdov & Podgornaja, 1982; Drozdov, 1992). Because the acrosomal reaction has a role in fertilization and the onset of ontogenesis, it should be assumed that the presence or absence of acrosomal the axial rod is of great taxonomic importance. The absence of an acrosomal axial rod provides evidence concerning the more primitive spermatozoon structure in animals with external fertilization (Popham, 1979).

Spermatozoons of *Modiolus* are distinguished by an unusually large head with a large amount (3.99 pkg) of loosely packed (0.42 pkg/μm^3) DNA (Tuturova, 1989), large, variable number (10–14) of mitochondria in the middle part of the spermatozoon and, as previously mentioned, the absence of an axial rod in the acrosome. These characters present strong evidence concerning the apomorphic structure of the spermatozoa of *Modiolus,* which is confirmed by its long (Devonian through Recent) geological history compared to the remaining Mytilidae (Soot-Ryen, 1969).

In terms of acrosomic rod presence/absence, Recent Mytiloidea form two major groups around *Modiolus* and *Mytilus.* The rank of these taxa is open to debate, because there are no objective criteria for establishment of taxa above the species rank (Mayr, 1969). Analoguous information about other superfamilies provides a perspective on how many families should comprise the superfamily Mytiloidea. In this connection, Cardioidea is notable. For instance, the spermatozoa morphology of *Keenocardium californiense* (Deshayes, 1839) (Clinocardiinae) and various representatives of Lymnocardiinae are very different from each other (Fig. 4). Nevertheless, in the most modern classification (Schneider, 1992, 1995), the Cardioidea includes only one family Cardiidae. Among Recent Mytiloidea, the degree of morphological variability of spermatozoa is much less.

Because all the spermatozoon types of Mytiloidea may be derived from that of *Modiolus,* as well as the fact that even the classification of Scarlato & Starobogatov (1979, 1984) imparts only subfamily status to Modio-

TABLE 3. Comparison of the classifications of Recent Mytiloidea by Soot-Ryen (1969), Scarlato & Starobogatov (1979, 1984) and proposed system

Soot-Ryen, 1969	Scarlato & Starobogatov, 1979, 1984	Proposed herein
fam. Mytilidae Rafinesque, 1815	fam. Mytilidae Rafinesque, 1815	fam. Mytilidae Rafinesque, 1815
subfam. Mytilinae Rafinesque, 1815	subfam. Mytilinae Rafinesque, 1815	subfam. Mytilinae Rafinesque, 1815
	subfam. Arcuatulinae Scarlato & Starobogatov, 1979[1]	
	subfam. Musculinae Iredale, 1939	subfam. Musculinae Iredale, 1939
subfam. Modiolinae Keen, 1958	subfam. Modiolinae Keen, 1958	subfam. Modiolinae Keen, 1958
		tribe Modiolini Keen, 1958
	subfam. Brachidontinae Scarlato & Starobogatov, 1979[2]	tribe Brachidontini Scarlato & Starobogatov, 1979
	subfam. Perninae Scarlato & Starobogatov, 1979[3]	
	subfam. Trichomyinae Scarlato & Starobogatov, 1979	
	fam. Septiferidae Scarlato & Starobogatov, 1979	tribe Septiferini Scarlato & Starobogatov, 1979
	subfam. Septiferinae Scarlato & Starobogatov, 1979	
	subfam. Limnoperninae Scarlato & Starobogatov, 1979	
subfam. Crenellinae Gray, 1840	fam. Crenellidae Gray, 1840	subfam. Crenellinae Gray, 1840
	subfam. Crenellinae Gray, 1840	
	subfam. Botulinae Scarlato & Starobogatov, 1979[4]	
subfam. Lithophaginae H. Adams & A. Adams, 1857	fam. Lithophagidae H. Adams & A. Adams, 1857	subfam. Lithophaginae H. Adams & A. Adams, 1857
	subfam. Lithophaginae H. Adams & A. Adams, 1857	
	subfam. Adulinae Scarlato & Starobogatov, 1979	

Notes. [1]Type-genus not given in the original publication. Established (according to Sysoev & Kantor, 1992), by name formation, on *Arcuatula* Lamy, 1919, *ex* Jousseaume MS, *non* Gugenberger, 1934, nec Soot-Ryen, 1955. [2]*Nom. correct.* (Kafanov, 1987) *pro* Brachiodontinae Scarlato & Starobogatov, 1979. [3]Invalid because preoccupied repeatedly as family-group name based on *Perna* Bruguière, 1789, *non* Retzius, 1788 [Pernaridia Rafinesque, 1815; Pernadae Fleming, 1828, also Guilding, 1828; Pernidae Zittel, 1895]. [4]Dacrydiinae Ockelmann, 1983, may be a junior synonym.

linae, there is no sound basis for identifying any other separate families among Recent Mytiloidea.

The genera grouped around *Modiolus* differ in levels of synplesio- and synapomorphy (in the sense of Hennig, 1950, 1966). Morphological evolutionary transformations of spermatozoons of the *Modiolus*-type were then accompanied by compactification of DNA, diminution of spermatozoon head size, and by the development of an acrosomal axial rod.

The most advanced spermatozoons appear to be those of *Septifer,* possessing a small-sized bullet-shaped head with a negligible (1.29 pkg) but very tightly packed (0.72 pkg/μ^3) amount of DNA (Tuturova, 1989) and a minute acrosome. Spermatozoons of other genera characterized by the absence of an axial rod in the acrosome (*Musculista, Adula, Choromytilus, Semimytilus, Arcuatula*), being variable in details, occupy an intermediary position between *Modiolus* and *Septifer.* In this

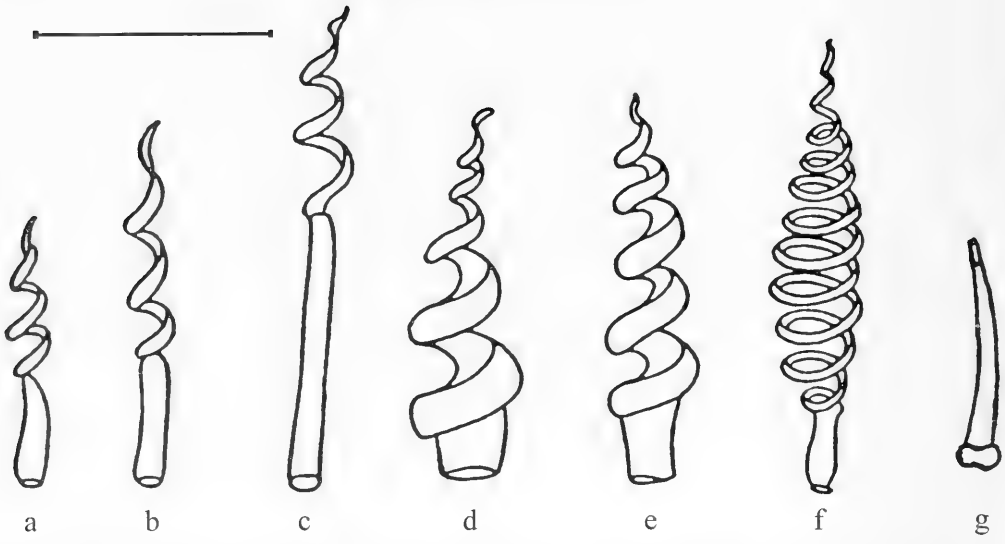

FIG. 4. Structural pattern (light microscope) of spermatozoa in cardiid subfamilies Lymnocardiinae Stoliczka, 1870 (a–f), and Clinocardiinae Kafanov, 1975 (g). Species: a—*Hypanis (Monodacna) colorata* (Eichwald, 1829), b—*Hypanis (Monodacna)* sp. 1, c—*Hypanis (Monodacna)* sp. 2, d—*Hypanis (Adacna) laeviuscula* (Eichwald, 1829), e—*Hypanis (Adacna) vitrea* (Eichwald, 1829), f—*Hypanis (Adacna) minima* (Ostroumoff, 1907) (Figs. a-f from: Karpevich, 1964), g—*Keenocardium californiense* (Deshayes, 1839) (from Drozdov, 1992). Bar = 8 μm.

case, the spermatozoons of *Modiolus* and *Musculista* are most similar, closely corresponding to their allocation to the same subfamily Modiolinae by Scarlato & Starobogatov (1979, 1984) and Scarlato (1981).

Among spermatozoa of *Mytilus*-type, possessing an axial rod, the most primitive are those of *Musculus,* which possess a long (approximately 8 μm), narrow head crowned with a minute acrosomal vesicle, from which an axial rod almost reaches the middle part of spermatozoon and which also possess five mitochondriae. These features may indicate some specialization, because of the large (about 600 μm) eggs of *Musculus* compared to those of *Mytilus* (about 70 μm) and to features of their insemination (Drozdov & Kasyanov, 1985).

In spermatozoon structure, as well as conchological features (Siddall, 1980), the subfamily Perninae Scarlato & Starobogatov, 1979, appears to be paraphyletic. Although a close taxonomic relation among *Perna, Mytilus, Choromytilus,* and *Aulacomya,* was proposed by Soot-Ryen (1952), spermatozoa of *Perna* show no morphological differences from those of *Mytilus* and *Crenomytilus,* whereas spermatozoa of *Chromytilus* and *Aulacomya* prove to be similar to the spermatozoa of *Modiolus.* This makes it impossible to consider *Choromytilus* Soot-Ryen, 1952, to be a subgenus of *Perna* Retzius, 1788, as was suggested by Soot-Ryen (1969). In any case, however, Perninae Scarlato & Starobogatov, 1979, is invalid for nomenclatural reasons (Table 2).

The available spermatozoan data, together with conchological characters, is adequate for suggesting an slightly modified systematics of Recent Mytiloidea, representing a trade-off between the classifications of Soot-Ryen (1969) and Scarlato & Starobogatov (1979, 1984) (Table 3). The subfamily status of Lithophaginae in this system is the result of its boring habitat, generally uncommon for mytilids, resulting in major transformations in shell morphology. In any case, the Lithophaginae is more closely related to Modiolinae than to the Mytilinae.

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INFLUENCE OF WATER TEMPERATURE ON THE ACTIVITY OF *PLANORBARIUS CORNEUS* (L.) (PULMONATA, PLANORBIDAE)

Katherine Costil[1] & Stuart E. R. Bailey[2]

ABSTRACT

This study complements our previous study of the influence of temperature on the life-history traits of the freshwater snail *Planorbarius corneus* by examining the effect of temperature on activity. The activity of 24 groups of three individuals was recorded over 24 h in a 12 h light:12 h dark photoperiod with six groups at each of four temperatures — 10, 15, 20 and 25°C.

Despite considerable variations between individuals, activity increased significantly with temperature, principally due to an increase in time spent feeding. Time spent on non-displacement movements declined above 10°C, but time spent on locomotor activity did not alter with temperature. However, the speed and total distance moved increased significantly with temperature, as did the number of contacts made with the water surface and with other individuals. Locomotion, feeding and non-displacement movements showed no diurnal rhythms at any temperature. The present study shows that a behavioural component contributes to the influence of temperature on life-history traits.

Key words: freshwater snail, behavioural ecology, temperature, light, locomotor and feeding activities.

INTRODUCTION

Planorbarius corneus is a large basommatophoran snail, common in eutrophic ponds of northwest France (Costil & Clément, 1996). It is often collected in association with *Lymnaea stagnalis,* and both species contribute to communities showing high species richness and diversity (Costil, 1994b). *Planorbarius corneus* plays an important part in the invertebrate communities of eutrophic ponds in this region, where it is eaten by both fish and birds.

Natural populations of freshwater snails have to adapt to various abiotic constraints, for example, climate, desiccation and water chemistry. Temperature is particularly important for freshwater pulmonates, because they inhabit shallower waters than most prosobranch molluscs, and thus experience a seasonal range of temperatures almost as great as that on land (MacMahon, 1983). We have previously studied the life-history traits of *P. corneus* and demonstrated the importance of water temperature in the control of these snail populations (Costil, 1994a; Costil & Daguzan, 1995a, b). To complete this study, we have now examined the impact of temperature on the activity of *P. corneus.*

Activity level is an important component of adaptive strategy. For example, locomotion in most animal species is an essential component of fitness, being necessary for both reproduction (finding mates and oviposition sites) and survival (finding food and avoiding predators) (Calow, 1974). Most studies of molluscan activity have concerned land pulmonates, and concentrated on their diurnal activity rhythms (e.g., Rollo, 1982; Dainton & Wright, 1985; Ford & Cook, 1987; and Wareing & Bailey, 1985, on slugs; and Cameron, 1970a, b; Bailey & Lazaridou-Dimitriadou, 1986; and Lorvelec, 1988, on helicid snails). The fewer studies on the activity of freshwater snails have focused on locomotion of tropical species (Beeston & Morgan, 1977; Chaudry & Morgan, 1983; Pimentel-Souza et al., 1984), the North American planorbid *Helisoma trivolvis* (Kavaliers, 1981) and the European species *Lymnaea stagnalis* (MacDonald, 1973), *Ancylus fluviatilis* and *Planorbis contortus* (Calow, 1974). In *P. corneus,* locomotion involves mucus, muscles and cilia covering the sole of the foot. Deliagina & Orlovsky (1990) established the locomotory repertoire of this species and the nervous control of locomotion.

Little attention has been paid to the time budget of different activities, and the effect of

[1]Laboratoire de Zoologie et d'Ecophysiologie, Campus de Beaulieu, Université de Rennes I, 35042 Rennes Cedex, France.
[2]School of Biological Sciences, 3.614 Stopford Building, Oxford Road, Manchester M13 9PT, United Kingdom

temperature on this budget, or on the consequences of increased speed of movement induced by elevated temperatures on distances travelled and encounters made, for example, with other individuals and the water surface. This study addresses those aspects.

METHODS

The snails were collected from a pond near Rennes, northwest France, where the life cycle of the population had been studied (Costil & Daguzan, 1995b). The snails were immature when collected and belonged to the same cohort. Brought into the laboratory at 18°C, they grew to maturity and after nine months were grouped into four experimental groups of 18 snails, each group having a similar mean diameter (close to 18.8 mm).

The water temperature of each group was gradually adjusted to one of the four experimental temperatures of 10, 15, 20 or 25°C. The experimental temperatures are within the range generally recorded in the region. After an acclimatization period of at least three weeks at the target temperature, the activity of the snails was studied by filming them with a video-camera for 24 h. The advantages and disadvantages of this technique were emphasised by Bailey (1994). The recording apparatus consisted of a black-and-white camera sensitive to dim red light (6300 to 7500 A), a monitor, and a time-lapse video-recorder. Recordings were made at one frame per sec, so that 24 h of recording could be played back in one hour at normal speed to analyse activity.

A total of 72 snails were tested, 18 at each temperature. The snails were filmed in six groups of three. Individuals were identified by painting spots on one snail, stripes on a second, and leaving the third snail unpainted. The snails were fed with rectangular pieces of lettuce (5 × 7 cm) which were weighted down to the bottom of the tank. Each tank (23 × 17 × 9 cm) was filled with 1800 ml of pond water. To aid the recording of distances moved by the snails, a grid of lines was drawn on the bottom and sides of the tank. There was no artificial aeration of the water in the tanks. Recordings were made in a photoperiod of 12 h light (from 08h00 to 20h00) and 12 h dark. As in Lorvelec's study (1988), a red light was used for filming during the dark phase, since pulmonates respond only weakly to long wavelengths (Kerkut & Walker, 1975). When the lights went on or off, the snails either did not react, or immediately stopped crawling for a short period, usually a few seconds.

Activity was analysed for each individual snail, recording its behaviour in each of the 288 5-min units over the 24 h. Snail activity was classified into five categories:

—inactive (I);
—locomotion (L); a displacement of 2 cm or more in the 5 min;
—moving without displacement (M), often consisting of rocking movements of the shell;
—feeding (F), with obvious scraping movements with the head on the lettuce;
—copulating (C).

If a snail showed two activity categories (e.g., locomotion and feeding) within a five-min time unit, both categories were scored as a half unit. More than two categories never occurred within one time unit.

The following additional variables were also calculated for each individual:

—longest period of inactivity in 24 h (LPI), in min;
—total distance covered in 24 h (TD), in m;
—mean speed (MNS), the total distance moved divided by five times the number of five-min units spent locomoting, in cm min^{-1};
—maximum speed (MXS), recorded over any 5-min period;
—number of contacts with the water surface in 24 h (CWS), including contacts with head or foot of a snail near the walls of the tank as well as contacts when the snail crawls with foot extended on the surface;
—number of contacts with fellow snails in 24 h (CFS).

The data on inactivity (I and LPI) were subjected to non-parametric tests (Mann Whitney test and Kruskal Wallis test), because the conditions of normality and equivariance required for an analysis of variance were not met, even after various transformations.

The data on activity were subjected to multivariate analyses of variance (MANOVA), preceeded by univariate analyses and Newman Keuls tests (Morrison, 1967; Dagnelie, 1977). The effects of temperature and light on the different types of activity were tested using a two-way MANOVA with two factors (temperature and light), 18 replicates, and three variables (times spent locomoting, feeding, and in non-displacement movements). The effect of temperature on activity was tested with a one-way

MANOVA applied to eight variables (L, F, M, MNS, MXS, TD, CWS and CFS); M and TD were transformed to Log (x + 1) to meet the requirements of the analysis of variance. After MANOVA, we used multiple comparisons which were in agreement, and thus the results of WILKS test and ROY test alone are presented here. Calculations were performed with STATVIEW (1988) and STAT-ITCF (1988) programs.

RESULTS

Egg masses were laid at 20 and 25°C, but the egg masses of *Planorbarius corneus* are almost colourless, and not sufficiently visible to enable us to reliably distinguish ovipositing during recording. Mating activity was observed only at 20°C. Two matings occurred: one began at 18h30 and lasted for 5.33 h, the other began at 5h40 and lasted 6.50 h. To compare the results at 20°C with the others, we have excluded those 5.6% of time units which were occupied by copulatory activity, and brought the percentage occupied by other activities back to 100.

As the high standard deviations given in Table 1 indicate, there are considerable differences between individuals, and at all temperatures, some snails were continuously active. For example, at 10°C, three snails were continuously active while four other snails were inactive for three quarters of the time. At 15°C, the percentage of time that individuals spent feeding varied from 1 to 75%. At 20°C, the distance covered by individual snails varied from less than three to more than 32 m. At 25°C, some snails made ten times more contacts with the surface than other snails.

Temperature significantly affects both the total time spent inactive (H = 28.01, p = 0.0001, Kruskal Wallis test), and the longest period of inactivity (H = 26.89, p = 0.0001, Kruskal Wallis test). The time spent inactive decreases at higher temperatures, and this decrease was most noticeable between 15 and 20°C (Fig. 1). The mean length of the longest period of inactivity (LPI) was negatively related to temperature (Fig. 2); LPI at 25°C was 35 min, but at 10°C it was > 21 h.

The proportion of time spent feeding increases with temperature, from 17.1% at 10°C to 56.2% at 25°C. The increase in time devoted to feeding accounts for most of the decline in inactivity seen with increasing temperature. Non-displacement movements occupy 10.2% of the time at 10°C, but only 3.3% to 5.4% of the time at higher temperatures. Time spent in locomotion was independent of temperature. Figure 3 shows no consistent or

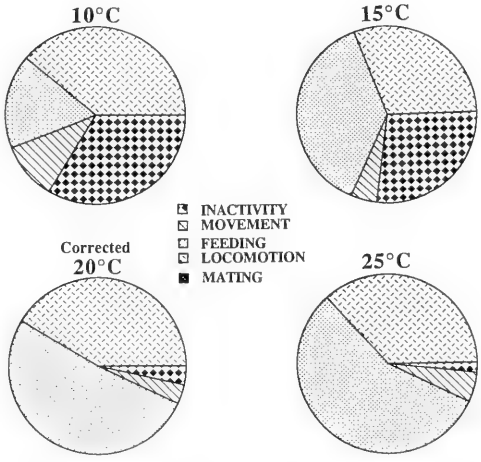

FIG. 1. Mean percentages of inactivity and different types of activity in *Planorbarius corneus* individuals at four temperatures (10, 15, 20 and 25°C) over 24 h corresponding to 288 activity units of 5 min. The results obtained at 20°C were corrected by excluding the activity units corresponding to mating.

TABLE 1. Mean numbers (± S.D.) of activity units spent by the individuals of *Planorbarius corneus* in inactivity and different types of activity (locomotion, feeding, movement without displacement and mating) at 10, 15, 20 and 25°C (n = 18). The results obtained at 20°C were corrected by excluding the activity units corresponding to mating, thus bringing back the total number of activity units to 288.

TEMPERATURE	INACTIVITY	LOCOMOTION	FEEDING	MOVEMENT	MATING
10°C	97.28 ± 103.89	112.00 ± 71.90	49.28 ± 45.96	29.4 ± 22.11	0
15°C	79.06 ± 65.63	87.61 ± 57.32	107.72 ± 64.58	13.61 ± 9.78	0
20°C	9.39 ± 10.36	114.08 ± 43.08	138.81 ± 34.11	9.61 ± 7.27	16.11 ± 31.29
Corrected 20°C	10.00 ± 12.02	118.92 ± 41.29	148.86 ± 43.27	10.22 ± 12.02	0
25°C	5.28 ± 4.87	105.08 ± 48.68	161.97 ± 47.30	15.67 ± 10.13	0

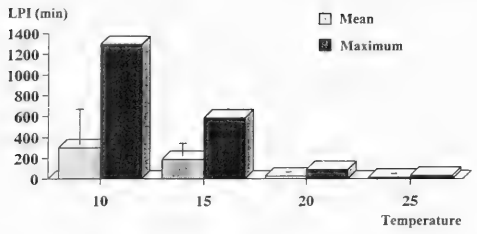

FIG. 2. Mean (± S.D.) and maximum values of the longest period of inactivity (LPI) in *Planobarius corneus* put at 10, 15, 20 and 25°C.

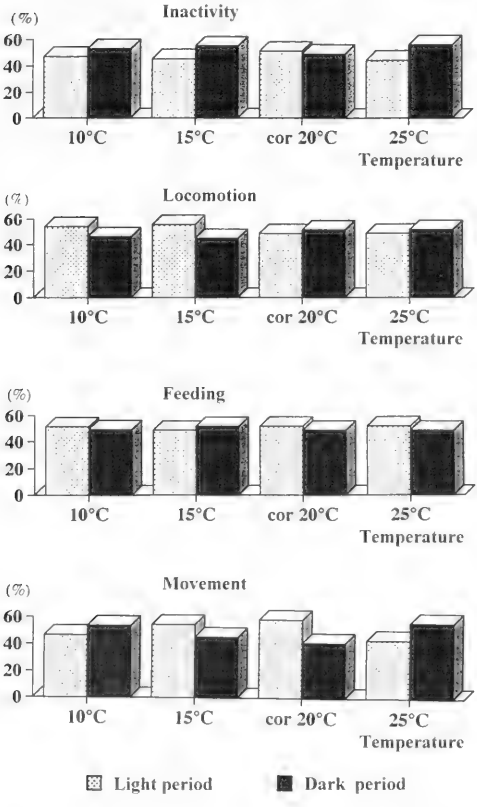

FIG. 3. Mean percentages of inactivity and different types of activity occurring during a 12h light and 12h dark period in *Planorbarius corneus* at 10, 15, 20 and 25°C.

sizeable differences between the light and dark phases in the proportion of time spent in any activity category, and applying a two-way MANOVA using both Wilks test and Roy test, no significant differences were found for either light or the light × temperature interaction (Table 2). Temperature emerges as the only factor influencing activity. As Table 3 shows, the time spent feeding changes significantly throughout the temperature range, while non-displacement movements only alter significantly over the lower part of the range, and there are no significant differences in the time spent in locomotor activity over the range of temperatures employed.

In general, the higher the temperature, the faster the snails moved (Fig. 4). At 25°C, the mean speed was on average 3.5 cm min^{-1}, with a maximum speed of 7.5 cm min^{-1}. The mean distances covered at 25°C were twice and four times those recorded at 15 and 10°C respectively, and one snail at 25°C covered 42 m in 24 h. The number of contacts between snails increased with temperature to reach a mean of 45.3 encounters in 24 h at 25°C, while at 10°C four snails never encountered a fellow snail. When two *P. corneus* snails met, one snail generally pushed the other and continued on its way. At the three highest temperatures, phoretic behaviour (carrying other individuals) was often observed. Individuals at 25°C has significantly more contacts with fellows than individuals at 10°C, probably because, although they do not move for any longer period of time, they move faster, and cover more distance.

The number of contacts with the water surface in general increased with temperature; the mean values are lowest at 15°C and highest at 25°C. However, the differences were not significant Although these contacts with surface included breathing visits, it is very difficult to be see breathing from a videorecording, and only in a few instances were lung ventilations with the pneumostome obviously open observed. The results of a one-way MANOVA applied to eight activity variables (L, F, M, TD, MNS, MXS, CWS and CFS) show a significant effect of temperature (Wilks Test, $F = 9.327$, $p < 0.0001$; ROY Test, eigen value = 5.179, critical value = 0.414, $p < 0.001$). Among these variables, temperature affects time spent feeding, total distance, mean and maximum speeds, and number of encounters with fellow snails (Table 4).

DISCUSSION

The large differences in the level of activity of individual snails under identical conditions that we observed were also noted by

TABLE 2. Results of a two-way multivariate analysis of variance using two different tests (Wilks test and Roy test) and applied to three variables (locomotion, feeding, movement without displacement) of *Planorbarius corneus* activity.

	WILKS TEST		ROY TEST		
FACTORS	F	p	Eigen Value	Critical Value	p
TEMPERATURE	10.807	0.0000	0.723	0.102	0.0000
LIGHT	0.330	0.8062	0.007	0.060	0.8065
INTERACTION Temp. × Light	0.451	0.9062	0.021	0.102	0.9202

TABLE 3. For each activity, temperatures for which some significant differences were calculated with Roy multiple comparison method used after the two-way multivariate analysis of variance.

	LOCOMOTION	FEEDING	MOVEMENT
Temperatures (°C) for which some differences were calculated	NO	10–15 10–20 20–25 15–25	10–20

MacDonald (1973), who found that the sequences and rates of activity patterns of individual *L. stagnalis* were so different as to preclude summing results. Such heterogeneity necessitates the use of sufficient numbers of individuals, and cautious interpretation of results. Video recordings often fail to show whether snails and slugs feed all the time that they are in contact with food (Bailey, 1994), but the use of visible scraping movements proves to be an efficient definition of feeding, and allows a firm relationship to be established between increasing temperature and increase in time spent feeding.

Despite the inter-individual differences, this study has demonstrated that *P. corneus* spends more time active, or, conversely, less time inactive, at higher temperatures. This may seem an unremarkable conclusion, but it does, in fact, contrast with Cameron's (1970b) observations on helicid snails, and Dainton & Wright's (1985) data on a terrestrial slug. Ford & Cook (1987) also showed that *Limax pseudoflavus* is initially stimulated when the temperature is lowered from 17°C to 4°C. Furthermore, the present study shows that the component activities (locomotion, non-displacement movement, feeding, mating) show different and non-linear relationships to temperature. The time spent in locomotion is not affected by temperature. The principal component of activity which increases with temperature is the time spent feeding. This activity levels off between 20 and 25°C, and it is possible that at higher temperatures, feeding would be depressed, as shown in *L. stagnalis* at 30°C (MacDonald, 1973). Rollo (1982) also found a curvilinear relationship between temperature and activity in the slug *Limax maximus,* and no activity was found above 19.5°C. Non-displacement movement of *P. corneus* is not linearly related to temperature, the mean value being minimum at 20°C and maximum at 10°C (the sole significant difference): moreover, this activity showed no significant relationship to temperature when the MANOVA was applied to a higher number of variables.

The observations of egg-laying and mating at higher temperatures are consistent with our previous demonstration that the population of *P. corneus* from which these animals were taken reproduces at and above 15°C and is most fecund at 20°C, all reproduction parameters being affected by temperature (Costil & Daguzan, 1995a). No complex courtship was seen, the first contact between partners occurring about 10 min before the start of copulation. Durations of mating varying from 3 to 5 h are generally reported for planorbid species (Madsen et al., 1983), slightly shorter than the times noted here. However, the low number of observed matings do not allow us to draw any firm conclusions.

A snail's activity pattern is an energetic compromise between different activities, and pedal mucus production accounts for 13–32% of the energy assimilated (Calow, 1974). According to Denny (1980), "it seems likely that the high cost of movement has affected the lifestyle of these animals, for example by limiting the dis-

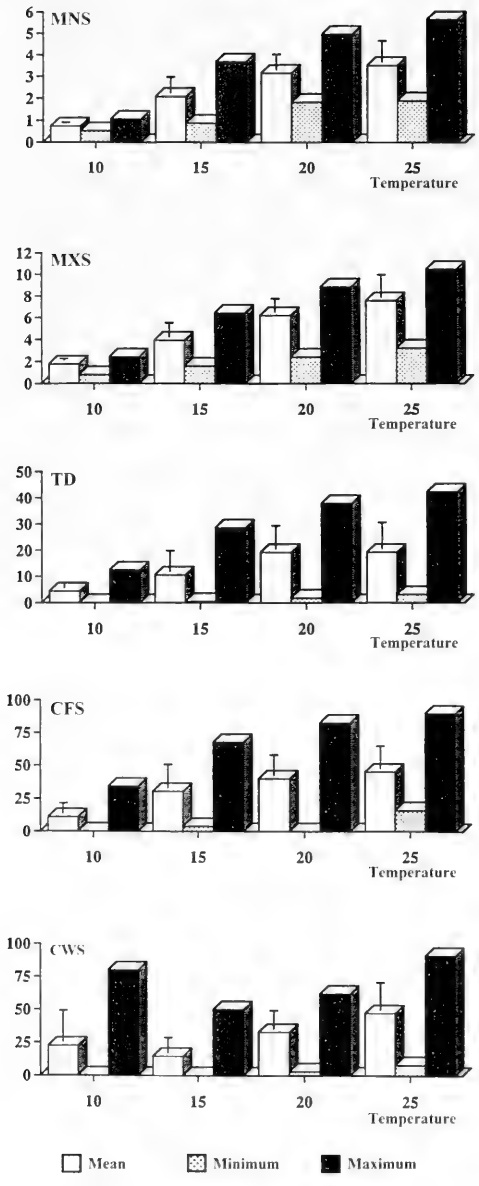

FIG. 4. Mean (± S.D.), minimum and maximum values of activity variables calculated in *Planorbarius corneus* put at 10, 15, 20 and 25°C. MNS = mean speed for 24 h filmed; MXS = maximum speed; TD = total distance covered by snails; CFS = number of meetings with the fellow snails; CWS = number of contacts with the water surface.

tance over which it is profitable to crawl in search of mates or food." Mouritsen & Jensen (1994) found that infection by larval trematodes reduced locomotor activity of *Hydrobia ulvae,* and suggested that the energy for locomotion is probably re-allocated to parasite metabolism and excessive growth of the host.

The persistence of locomotion when temperature decreases is important for species that make regular seasonal migrations or burrow into the substratum in winter. Cheatum (1934) reported that during autumn, when temperatures are declining, some species migrate from shallow littoral waters to overwinter in deeper waters. Several authors have confirmed such migrations, but there is no definite information on *P. corneus.* Caution is required when attempting to extend laboratory observations on the effect of temperature on speed of movement to field conditions: movement over a substratum of mud, plants and stones could be expected to be slower than on the glass of an aquarium tank. Furthermore, Calow (1974) showed that the speed of *Planorbis contortus* is affected by starvation, food availability and water movement, whereas Dimock (1985) found that after three weeks in an aquarium, there was a 50% reduction in speed and a significant decrease in overall activity in the marine mud snail *Ilyanassa obsoleta.* Nonetheless, DeWit (1955) reported a maximum speed of 7 cm min^{-1} in *P. corneus,* close to our highest recorded speed, and speeds of 8.5 cm min^{-1} for the slightly larger *L. stagnalis* and 6.5 cm min^{-1} for the smaller *Physa fontinalis.* In our experiments, the mean distance travelled in 24 h ranged from 4.38 m at 10°C to 19.29 m at 25°C, with a maximum value of 42 m. Boss et al. (1984) recorded much smaller displacements in 24 h by marked individuals of three aquatic species of snail — less than 40% moved one metre or more from the release point, and the maximum distance moved in the speediest species, *Physa integra,* was less than seven m. However, these authors observed snail positions only at intervals and did not follow the complete tracks of the snails. The direction of movement is also important, and Deliagina & Orlovsky (1990) reported that when searching for food, *P. corneus* exhibited very sinuous tracks, turning at irregular intervals. In random movement, the mean displacement is close to the square root of tracklength (Bailey, 1989).

Freshwater pulmonates renew the air in the mantle cavity lung by periodic visits to the sur-

TABLE 4. Temperatures for which some significant differences were calculated with Roy multiple comparison method used after the one-way multivariate analysis of variance. L = locomotion, F = feeding, M = movement, TD = total distance covered by the snails, MNS = mean speed, MXS = maximum speed, CWS = number of contacts with water surface, CFS = number of meetings with fellow snails.

	L	F	M	TD	MNS	MXS	CWS	CFS
Temperatures for which some differences were calculated	NO	10–20 10–25	NO	10–20 10–25	10–20 20–25	10–20 20–25 15–25	10–25	NO

face. The interval between lung ventilations varies considerably between species: in nine species studied by Cheatum (1934) the range was from 52 min in *Lymnaea palustris* to 1493 min in *Physa sayii crassa.* The pseudobranch of *P. corneus* enables this species to obtain over 50% of its total oxygen uptake by cutaneous exchange at much lower levels than the similarly sized *L. stagnalis,* and the high affinity of planorbid haemoglobin makes better use of pulmonary oxygen, allowing it to remain submerged for extended periods, even burrowing into substrate (Jones, 1961, 1964). Water contains less dissolved oxygen at higher temperatures, and at 11°C, the average interval between breathing periods for Cheatum's nine species was nearly 22 times that in water at 21°C. In *L. stagnalis,* the percentage of time breathing increased with temperature (MacDonald, 1973). Although snails would be expected to visit the surface more frequently merely because of the increased distances they moved at higher temperatures, surface visits increase in response to lowered oxygen tensions even at the same temperature (Jones, 1961).

Our conclusion that snail activity was unaffected by the presence or absence of light, might appear surprising, given the well-known nocturnality of terrestrial gastropods (e.g., *Limax pseudoflavus,* Ford & Cook, 1987; *Helix aspersa,* Lorvelec, 1988; and *Helix lucorum,* Bailey & Lazaridou-Dimitriadou, 1986). Unlike terrestrial snails and slugs, however, freshwater gastropods are not subjected to the low humidity that reduces the activity of helicids (Cameron, 1970a). Thus, they are not constrained to place most of their activities during the night. Several studies on the influence of light, reviewed by MacMahon (1983), suggest that the activity of freshwater gastropods increases during darkness, or at dusk and dawn. In *Bulinus tropicus,* locomotion, feeding, excretion, ovipositing and hatching all follow a diurnal pattern (Chaudry & Morgan, 1983). Endogenous dawn and dusk activity peaks are reported in the prosobranch *Melanoides tuberculata* by Beeston & Morgan (1979). Infected individuals of *L. stagnalis* are most active in the first few hours of illumination (Anderson et al., 1976). In the absence of food, *P. corneus* exhibited maximum locomotor activity during the day (Deliagina & Orlovsky, 1990). Truscott et al. (1995) found maximum activity in *Lymnaea stagnalis* in the morning, and minimum activity around midnight, but the differences were small. Intertidal gastropods could constitute an intermediate case: the pulmonate limpets *Siphonaria capensis* are active at low tide both by day and night in intertidal pools, whereas limpets exposed to air at low tide are active only during nocturnal or late evening low tides (Branch & Cherry, 1985). Nevertheless, the siphonarian species *S. sirius,* inhabiting the low shore, is active only during daytime and when awash and submerged both at ebb and flood tides (Iwasaki, 1995). Barnes (1986) suggested that the activity variation in *Hydrobia ulvae* was most likely a direct response to changes in light intensity and water cover.

The tropical planorbid *Biomphalaria glabrata* exemplifies the great variation in activity that can be encountered in a single species under the influence of several factors, including parasitization. Moreover, activity, in common with many life-history traits, probably differs between different populations of a species. Pimentel-Souza et al. (1984) reported a dusk and a dawn peak of activity in *B. glabrata.* The levels of locomotion are similar in constant darkness and constant light, although significantly lower than levels shown under a natural cycle of illumination. *Biomphalaria glabrata* shows more locomotor activity by day than by night, with a distinct maximum in the second hour of light, in contrast to feeding and egg-laying activities (Hien & Disko, 1981). Gerard (1996) also found that uninfected *B. glabrata* move less during the

night than during the day, but the locomotor activity of infected individuals is not influenced by the time of day or night.

The importance of light in directly controlling activity or synchronising the endogenous rhythm of activity of terrestrial gastropods has diverted attention away from the subtle effects of temperature in terrestrial species. However, Cameron (1970b) found significant effects of temperature and time on the activity of three species of land snails. All species had their maximum daytime activity at 8°C but became increasingly nocturnal as temperature increased. *Arianta arbustorum,* the least nocturnal species, reached maximum activity at 8°C, but *Cepaea nemoralis* and *C. hortensis* were most active at 22°C. In aquatic snails, also, temperature interacts with endogenous activity cycles in subtle ways: Kavaliers (1981) showed that the planorbid *Helisoma trivolvis* has a circadian rhythm of behavioural thermoregulation, selecting maximum temperatures of 21–22°C in a thermal gradient during the dark phase, and minimum temperatures (17–18°C) during the light phase (Kavaliers, 1981). This rhythm had an endogenous basis, and temperature selection continued in constant darkness.

Temperature and light influence molluscan behaviour in different ways. From the present results and those already discussed, we may conclude that temperature, light and endogenous circadian rhythm act together to control the behaviour of terrestrial gastropods, whereas temperature is the most important factor controlling the behaviour of freshwater species. Interspecific differences probably reflect different environmental constraints to which different species have adapted. Basommatophoran snails, including the planorbids, are pulmonates that have secondarily readapted to aquatic life, and different species show varying degrees of re-adaptation (Russell-Hunter, 1978). It would be of greatest interest to compare the influence of temperature and light on the behaviour of a less aquatic species, such as *L. truncatula,* with that of the more aquatic *P. corneus.*

In northwest France, *P. corneus* has a spring generation each year, and sometimes an autumn one as well (Costil & Daguzan, 1995b). Compared to *Planorbis planorbis, P. corneus* appears to be more influenced by climate, and there are strong differences in the growth patterns of the spring and autumn generations. Shell growth was fastest in Spring, and very slow or nil in Winter. In the laboratory, growth increases with temperature (Costil, 1994a): this is connected to the enhancement of metabolic processes with a temperature coefficient of about two (Ricklefs, 1990). However, the present study demonstrates an additional behavioural explanation for enhanced growth. The increased feeding activity at higher temperatures is important in meeting the increased requirements for growth and reproduction.

This study also provides a behavioural explanation for the precocious senility at higher temperatures. At 10°C, growth is slow, and there are many mortalities (Costil, 1994a); this is accompanied by inactivity, slow locomotion, and little feeding activity. However, the snails reared at the two highest temperatures expended energy on fast somatic growth and then became senile earlier. Life expectancy from hatching falls from 2.64 years at 15°C to 1.96 y at 20°C and 1.26 y at 25°C (Costil, 1994a). Snails at the two highest temperatures were rarely inactive and moved quickly.

Our wider study demonstrates the importance of temperature on the life history of *P. corneus,* and the present study emphasises the induction of life history traits by behavioural components, as well as physiological ones.

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SHELL REPAIR FREQUENCIES IN WHELKS AND MOON SNAILS FROM DELAWARE AND SOUTHERN NEW JERSEY

Gregory P. Dietl[1] & Richard R. Alexander[2]

ABSTRACT

More than 1,500 specimens combined from the extant moon snails *Euspira heros* (Say) and *Neverita duplicata* (Say), and the whelks *Busycon carica* (Gmelin) and *Busycotypus canaliculatus* (Linnaeus) from southern New Jersey tidal flats were examined for breakage-induced shell repair. Additionally, 500 specimens of *E. heros* and *N. duplicata* and *Busycon scalarispira* (Conrad) from the Miocene Kirkwood Formation collected from northern Delaware were examined. On each specimen, body whorl diameter (WD) and apertural lip thickness (ALT) at three positions, namely, anterior-most, mid-length, and posterior-most location on the lip, were measured and number of scars per final whorl counted. Although mean number of repairs per specimen differed among the five species, averages were comparable for Recent moon snails (1.1 for *N. duplicata* and 1.0 for *E. heros*) and whelks (5.3 for *B. carica* and 5.2 for *B. canaliculatus*). Mean repairs/specimen were appreciably lower for Miocene naticids (0.4) and the melongenid (0.7). Repair frequencies/shell ranged from zero to 12 for Recent whelk species and Recent *N. duplicata,* zero to seven for the Recent *E. heros,* and zero to four for Miocene moon snails and whelks. Only four and three percent of shells of *B. canaliculatus* and *B. carica* lack repairs, whereas 48% and 57% of Recent *E. heros* and *N. duplicata,* respectively, lack repairs. The majority of shells in every size class of Miocene whelks and moon snails lacked repairs, save for the largest size class of *B. scalarispira.* Repair frequencies accumulated at a greater rate (regression line slope) and are more strongly correlated (higher r value) with WD and ALT for thicker lipped Recent whelks than either thinner lipped moon snails or Miocene whelks. Presence of an umbilical plug in *N. duplicata* renders posteriorly located breaks on the apertural lip repairable, unlike the plug-lacking *E. heros.* Greater rate of scar accumulation with increasing shell size among Recent naticids reflects increase in breakage-localizing shell thickness since the Miocene. Greater mean repair frequencies in Recent whelks relative to Recent moon snails is attributed to the additive effect of sublethal predation on whelks plus prey-induced apertural lip fracture during valve-wedging by feeding whelks. The substantially lower frequency of repair/shell in *B. scalarispira* versus *B. carica* and *B. canaliculatus* suggests that the habit of shell-wedging of prey had not yet evolved, or was ineffectually practiced by Miocene whelks.

Key words: shell repair, moon snails, whelks, durophages, shell-wedging.

INTRODUCTION

Shell breakage by durophagous predators is potentially a more important agent of antipredatory adaptive selection with respect to gastropod armor today than in pre-Cretaceous time. The most effective shell-breaking predators in Recent gastropod communities, brachyuran crabs, elasmobranchs and teleost fishes, were rare, absent or had not yet achieved durophagy in the Paleozoic and early Mesozoic eras. Vermeij et al. (1981) postulated that "if all breakage were lethal, there would be no selection between weak and strong shell variants, and no shells would show the scars that record nonlethal injury." Therefore, breakage-induced shell repair, although not a measure of the magnitude of selection, is a conservative estimate for the importance of selection in favoring the evolution of durophage-resistant gastropod armor. Low frequencies of repair implies either that shell-breaking predators were rare or that most shells were successfully lethally broken, regardless of predator abundance. High frequencies of breakage-induced shell repair (among other defenses) reveals the effectiveness of shell characteristics in protecting the snail against contemporaneous durophages. Repair frequencies are expected to vary within and between snail species both spatially and temporally.

[1]Department of Ecology and Evolution, State University of New York, Stony Brook, New York 11794-5245, U.S.A.
[2]Department of Geological and Marine Sciences, Rider University, Lawrenceville, New Jersey 08648-3099, U.S.A.

Several factors influence differences in accumulation of repairs of sublethal shell breakage on different gastropod species with unornamented, oval to tear dropped-shaped apertures, among taxonomically closely and distantly related species. Shell size, thickness, strength, and shape of the gastropod prey is of primary importance (Vermeij, 1976, 1977; Curry & Kohn, 1976; Palmer, 1979; Vermeij & Curry, 1980; Kitchell et al., 1966; Bertness & Cunningham, 1981), but other factors, including differences in diversity and abundance of shell-breaking predators (Vermeij, 1987), size refugia from predators, within-habitat refugia (e.g., vegetated vs. barren substrata or duration of intertidal exposure) from predators, age-structure of different prey species populations, difference in locomotion or escape habit of prey species, tissue toxicity and palatability, as well as differences in mode of subjugation and invasion of prey of compared predatory snail species influence repair frequencies. In order to determine the importance of any one, or set of factors, it is desirable to control or eliminate many other variables. Studies of co-habitating gastropod species afford the opportunity to eliminate differences in frequencies of shell repair due to disparities in predator abundances and within-habitat refugia, and focus on the architecture and size of the prey species and differences in their escape and/or feeding habits. Accordingly, the low intertidal to shallow subtidal, vegetation-barren, shifting, rippled, fine sandy bottomed community of the New Jersey coast offers an opportunity to study repair frequencies on shells of four large, burrowing, predatory gastropods that are prone to durophagous predation.

Durophagous predators on snails crush or peel their prey. Crushing involves compression between two rigid surfaces, such as the claws of many crabs (Vermeij, 1976, 1977, 1978; Zipser & Vermeij, 1978; Brown et al., 1979). Peeling involves breakage of the outer shell lip in a spiral direction (Muntz et al., 1965; Shoup, 1968; Vermeij, 1978; Vermeij et al., 1980). Shell characteristics that deter durophagy include a low spire, small aperture, thick outer lip, tight coiling, external sculpture, and retractility (Vermeij, 1982). In the case of moon snails and whelks, deeply embayed fractures (Fig. 2-1, 2-8) and scalloped fractures (Figs. 2-3, 5) in the body whorl suggest that peeling and crushing are employed by crabs preying on whelks and moon snails. The four large gastropods common to the southern New Jersey coast are the globose naticids *Euspira heros* (Say), *Neverita duplicata* (Say) and the subpyriform melongenids *Busycon carica* (Gmelin) and *Busycotypus canaliculatus* (Linnaeus) (Figs. 1, 2). Their common shell-crushing and shell-peeling predator is the blue crab *Callinectes sapidus.* Sampled gastropod species from New Jersey are compared to the fossil gastropod assemblage from the Miocene Kirkwood Formation of Delaware (Ward, 1992), which includes both *E. heros* and *N. duplicata,* plus the whelk *Busycon scalarispira* (Conrad).

Objectives of this investigation were determination of significant differences, if any, in: (1) size-dependent accumulation of shell repair among the co-existing species; and (2), relation between apertural lip thickness at selected anteroposterior positions and number of repairs per shell among the co-existing modern and fossil species. Furthermore, this study utilizes several perspectives. The evolutionary perspective deals with whether or not repair frequencies for the temperate *E. heros* and *N. duplicata* are similar to congeneric and conspecific fossil populations from the Late Cretaceous to Miocene. The latitudinal perspective bears on the issue of different abundance and diversity of predators on whelks and moon snails from different communities. Are repair frequencies in these temperate latitude gastropods higher or lower than those reported on tropical congeneric Recent assemblages (Vermeij, 1982)? The architectural-size perspective focuses on whether or not co-habitating, confamilial and congeneric species with differing shell thicknesses differ in their frequency of breakage-induced repair (i.e., *E. heros* vs. *N. duplicata* and *B. carica* vs. *B. canaliculatus*). The trophic habit perspective focuses on possible differences in repair frequencies among co-habitating melongenids versus naticids given that whelks risk, and moon snails do not risk, fracture of their apertural lip during invasion of their molluscan prey.

MATERIALS AND METHODS

Empty and hermit crab-occupied shells of the naticid snails *N. duplicata* and *E. heros* and the melongenid whelks *B. carica* and *B. canaliculatus* were collected from Hereford Inlet (HI), Cape May County, and Longsport Beach in Great Egg Harbor (GEH), Atlantic County, New Jersey. Collections of *E. heros, N. duplicata,* and *B. scalarispira* from the Miocene Kirkwood Formation of Delaware were loaned by Lauck Ward, Virginia Museum

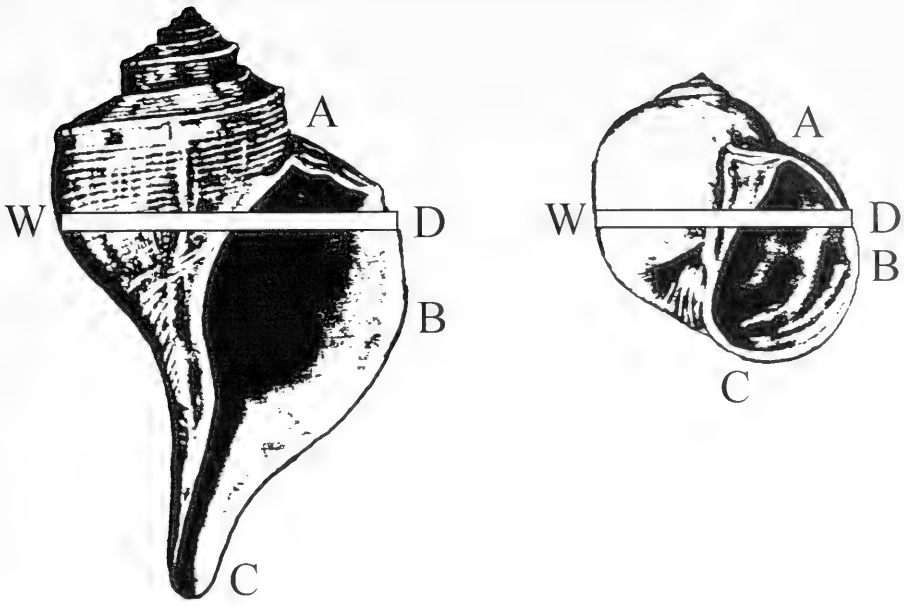

FIG. 1. Apertural view of *Busycotypus canaliculatus* and *Euspira heros* showing position of maximum whorl diameter (WD) measured for body whorl and apertural lip thickness (ALT) measured (A) anteriorly near junction with penultimate whorl, (B) at lip mid-length, and (C) posteriorly at siphonal canal in whelks and nearest umbilicus in moon snails.

of Natural History. The fauna of the Kirkwood Formation correlates with the Calvert Formation of Maryland, which is the lowest of the Chesapeake Group (Richards & Harbison, 1942). Whorl diameter (WD) and apertural lip thickness (ALT) at three positions, namely, the anterior-most, mid-length, and posterior-most location on the lip (Fig. 1) were measured with Vernier calipers to the nearest 0.05 mm on more than 1,500 specimens combined from the extant moon snails *N. duplicata* and *E. heros,* and the whelks *B. carica* and *B. canaliculatus.* These same measurements were made on more than 500 fossil specimens of *E. heros, N. duplicata* and *B. scalariispira.* Traces of sublethal breakage may be seen as scars that cut across the growth lines or axial sculpture of the shell (Fig. 2). Number of sublethal scars per final whorl were counted for each specimen and the repair frequency determined. Repair frequency was calculated as the number of repairs per shell divided by the total sample size, following the definition by Vermeij et al. (1982), Vermeij & Dudley (1982), and Schindel et al. (1982).

Accordingly, the number of repair scars per shell, an index of repair frequency, was regressed on WD, the index of size, for each species in the Recent and Miocene samples to determine if repairs were correlated with size (age) for either moon snail and/or whelk species. Both linear and second-order polynomial regressions were executed for number of repairs regressed on WD. The greater correlation coefficient generated by these two methods was used in statistical comparisons (Tukey-HSD test) of r values within and between species. Slope of the regression equations, beta, were also statistically compared (F-test) between and within species in order to evaluate if the rate of accumulation of repairs with increasing size differ within and between species.

Shells of all species were sorted according to WD into six size-class divisions, namely, class 1, < 19 mm; class 2, 20–39 mm; class 3, 40–59 mm; class 4, 60–79 mm; class 5, 80–99 mm; and class 6, 100–119 mm. Repair frequencies were calculated for specimens in each size class by dividing the total number of repairs in the sample by the number of shells examined in the size class (Table 1). The proportion of shells with a given number of scars per final whorl, which ranged from zero to 12,

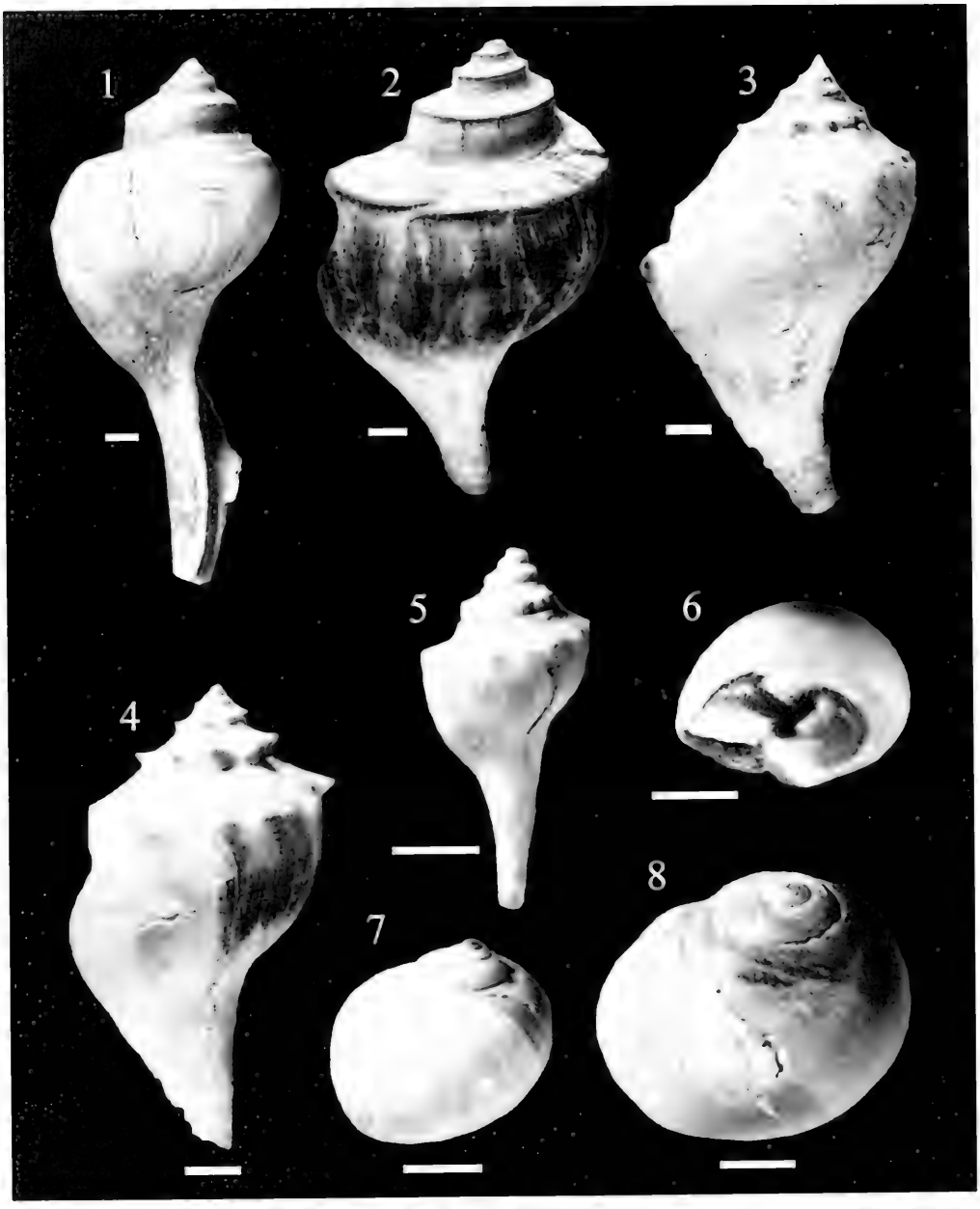

FIG. 2. Specimens of *Busycotypus canaliculatus* (1–2), *Busycon carica* (3–5), *Neverita duplicata* (6, 8), and *Euspira heros* (7) with shell repairs. Multiple repairs in body whorl illustrated in 3, 7. Anteriorly concentrated repair in apertural lip shown in 2, mid-length lip repair in 4, 8, and posteriorly concentrated repair near umbilicus of moon snail shown in 6. Repair that extends anteriorly across whorl shoulder to penultimate whorl of juvenile whelk shown in 5. Width of bar equals one cm.

TABLE 1. Frequency of shell repair in relation to size class (based on whorl diameter). N = number of specimens; f = shell repair frequency. Repair frequencies are calculated as total repairs in a sample divided by number of shells examined in that sample.

Taxon	Location	Size Class (mm)	N	f
E. heros	GEH	<19	0	0.00
Recent		20–39	23	0.69
		40–59	29	1.00
		60–79	10	2.10
	HI	<19	12	0.58
		20–39	79	0.72
		40–59	86	0.96
		60–79	33	1.51
		80–99	13	1.54
E. heros	Kirkwood	<19	71	0.48
Miocene	Fm; Delaware	20–39	28	0.18
		40–59	7	0.57
N. duplicata	GEH	<19	0	0.00
Recent		20–39	64	0.61
		40–59	324	1.05
		60–79	53	2.13
	HI	<19	17	0.35
		20–39	193	0.73
		40–59	207	1.37
		60–79	132	2.04
N. duplicata	Kirkwood	<19	69	0.26
Miocene	Fm; Delaware	20–39	82	0.73
		40–59	5	0.80
B. carica	HI	<19	12	1.67
Recent		20–39	49	3.80
		40–59	20	4.55
		60–79	28	6.10
		80–99	40	7.00
B. canaliculatus	HI	<19	3	0.33
Recent		20–39	41	3.00
		40–59	21	3.42
		60–79	28	5.03
		80–99	42	7.78
		100–119	10	7.90
B. scalarispira	Kirkwood	<19	24	0.13
	Fm; Delaware	20–39	28	0.43
		40–59	58	0.59
		60–79	66	0.83
		80–99	25	1.32
		100–119	3	1.67

Locations: GEH = Great Egg Harbor, Atlantic County, NJ; HI = Hereford Inlet, Cape May County, New Jersey. Kirkwood Fm = Pollac site, Kent County, Delaware.

was determined for each size class in each species. Hereafter this calculation is referred to as "proportion of repair" (Table 2). One-way ANOVA was used to determine if mean number of repairs per specimen was significantly different between the five species. A Kolmogorov-Smirnov test was used to determine if there were differences in the percent-frequency of shells with increasing number of repair scars per shell between contemporaneous and noncontemporaneous whelk and moon snail species (Table 3).

TABLE 2. Proportion of shells of *Euspira heros, Neverita duplicata, Busycon carica, B. scalarispira,* and *Busycotypus canaliculatus,* with increasing number of repairs per shell (0–12) in relation to size class defined by whorl diameter (mm).

Taxon & Geol. Age	LOC	Size Class (mm)	Number of repairs per shell												
			0	1	2	3	4	5	6	7	8	9	10	11	12
Eh	GEH	<19													
Recent		20–39	0.57	0.30	0.09			0.04							
		40–59	0.44	0.30	0.13	0.13									
		60–79	0.20	0.20	0.30	0.10	0.10		0.10						
		80–99	1.0												
	HI	<19	0.70	0.10	0.10	0.10									
		20–39	0.43	0.24	0.20	0.07	0.05	0.01							
		40–59	0.53	0.29	0.11	0.06	0.01								
		60–79	0.30	0.21	0.30		0.09	0.03	0.03						
		80–99	0.31	0.23	0.31	0.08			0.08						
Nd	GEH	<19													
Recent		20–39	0.53	0.38	0.08		0.02								
		40–59	0.44	0.33	0.14	0.04	0.02	0.01	0.01	0.01		0.05			0.05
		60–79	0.21	0.34	0.17	0.09	0.06	0.02	0.08		0.02				0.02
	HI	<19	0.61	0.39											
		20–39	0.51	0.38	0.11	0.04	0.01								
		40–59	0.33	0.34	0.17	0.06	0.06	0.02	0.02	0.03					
		60–79	0.16	0.12	0.16	0.23	0.08	0.12	0.04	0.04	0.08				
Bcn	HI	<19	0.67	0.33											
Recent		20–39		0.18	0.25	0.25	0.10	0.13	0.03	0.03			0.03		0.03
		40–59	0.11	0.16	0.16	0.21	0.11	0.21		0.05					
		60–79	0.04	0.04	.04	0.14	0.25	0.14	0.11	0.11	0.04	0.04	0.04		0.04
		80–99				0.02	0.07	0.12	0.07	0.17	0.14	0.17	0.12	0.07	0.05
		100–119				0.20			0.10	0.20			0.40	0.10	
Bcr	HI	<19	0.17	0.08	0.50	0.25									
Recent		20–39	0.08	0.06	0.12	0.20	0.16	0.10	0.14	0.06	0.04		0.02		
		40–59	0.11			0.16	0.11	0.37	0.11	0.05		0.11			
		60–79		0.07	0.07		0.17	0.10	0.10	0.17	0.14	0.10	0.07		
		80–99	0.03		0.03	0.05	0.05	0.10	0.18	0.10	0.10	0.13	0.13	0.05	0.08
		100–119				0.04	0.08	0.25	0.17	0.08	0.17	0.13	0.04	0.04	
Nd		<19	0.76	0.21	0.03										
Miocene		20–39	0.70	0.21	0.06	0.02									
Kirkwood		40–59	0.50	0.50											
Eh		<19	0.66	0.24	0.06	0.04									
Miocene		20–39	0.82	0.18											
Kirkwood		40–59	0.86				0.14								
Bs		<19	0.88	0.12											
Miocene		20–39	0.71	0.18	0.07	0.04									
Kirkwood		40–59	0.65	0.21	0.09	0.02	0.04								
		60–79	0.50	0.30	0.12	0.02	0.06								
		80–99	0.24	0.32	0.36	0.04	0.04								
		100–119	0.33		0.33	0.33									

Species: Eh - *Euspira heros;* Nd - *Neverita duplicata;* Bcn - *Busycotypus canaliculatus;* Bcr - *Busycon carica.;* Bs - *Busycon scalarispira;* LOC = Locations; GEH - Great Egg Harbor, Atlantic County, New Jersey; HI - Hereford Inlet, Cape May County, New Jersey; Miocene Kirkwood Fm - Pollac site, Kent County, Delaware.

Vermeij (1982) reported that species with thickened lips have significantly lower frequencies of repair than do thin-lipped species, because thick-lipped species experience many unsuccessful attacks that leave the lip unscathed and, therefore, unrepaired. Accordingly, a two-way ANOVA was used for both Recent and Miocene moon snails and

TABLE 3. Multiple comparison Fisher PLSD test for equality among mean number of repairs and Kolmogorov-Smirnov test for differences in percent-frequency of shells with increasing number of repair scars per shell.

Comparison	Fisher PLSD	K-S Chi-square
Within naticids		
Neverita duplicata (Recent) vs. *Euspira heros* (Recent)	0.225ns	1.5ns
vs. *E. heros* (Miocene)	0.334*	35.5**
vs. *N. duplicata* (Miocene)	0.249*	67.4***
E. heros (Recent) vs. *E. heros* (Miocene)	0.379*	21.8*
vs. *N. duplicata* (Miocene)	0.296*	35.8**
E. heros (Miocene) vs. *N. duplicata* (Miocene)	0.394ns	0.173ns
Within melongenids		
Busycotypus canaliculatus (Recent) vs. *Busycon carica* (Recent)	0.378ns	2.1ns
vs. *B. scalarispira* (Miocene)	0.365*	173.1***
B. carica (Recent) vs. *B. scalarispira* (Miocene)	0.346*	220.7***

P-values: ns = nonsignificant
* = < 0.05
** = < 0.01
*** = < 0.001

whelks treated separately to determine if mean thickness of the apertural lip for each species varies significantly anteriorly to posteriorly, that is, at lip positions A, B, or C (Fig. 1). Because repairs are often localized along a portion of the apertural margin (Figs. 2–4), the number of repair scars per shell was regressed on thickness of the apertural lip separately at positions A, B, or C for each species to determine the susceptibility of differing parts of apertural lip to sustaining repeated, repairable, breakage. As with regressions involving size (WD), both linear and second order polynomial regressions of the number of repairs versus apertural lip thickness were generated for each sample of each species. The greater r value generated by either method was used in statistical comparisons of correlation coefficients (Tukey-HSD test). Similarly, the slopes of the regression lines, beta, were statistically compared (F-test) to evaluate possible significant differences in the rate of accumulation of repairs with increasing lip thickness between and within species.

RESULTS

Frequency of shell repair is greater in all size classes of Recent whelks relative to the same size class among Recent moon snails (Table 1). However, frequencies of shell repair are similar among same size classes of compared Miocene whelks versus Miocene moon snails (Table 1). Number of repair scars per specimen ranged form zero to 12 for both Recent whelk species, zero to 12 for Recent *N. duplicata,* and zero to seven for Recent *E. heros* (Table 2). Only four and three percent of *B. carica* and *B. canaliculatus,* respectively, lack repairs, whereas 48% and 57% of Recent *E. heros* and *N. duplicata,* respectively, lack repairs. Recent whelks display significantly higher proportions of shells with more than six repairs relative to moon snails (Table 2). There is a significant difference ($p < 0.001$) between noncontemporaneous congeneric melongenids in percent-frequency distributions of shells with increasing number of repair scars per shell, as well as noncontemporaneous confamilial and conspecific Miocene and Recent naticids (Table 3). Furthermore, the thinner Recent moon snail *N. duplicata* and whelk *B. canaliculatus* (Table 4) do not have percent-frequency distributions of shells with increasing number of repair scars per shell different from their thicker-lipped Recent relatives *E. heros* and *B. carica,* respectively (Table 3). Among Miocene naticid shells, all size classes are dominated by shells without repairs. The maximum number of repairs per shell for any size class is four (Table 2). Among Miocene whelks, no shell had more than four repairs, and the majority of the specimens (72%) in the 19–79 mm size classes lacked repairs (Table 2).

The apertural lip of *E. heros* is thicker on average at all three positions (Fig. 1) relative to *N. duplicata* (Table 4), although the shell of the latter species is more massive due to an umbilical plug. Average thickness of the apertural lip increases by a factor of three at positions A

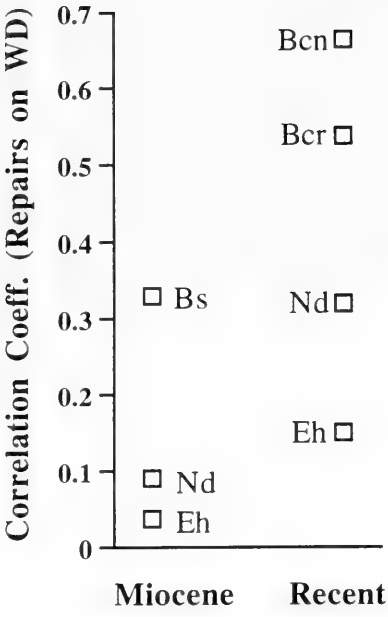

FIG. 3. Distribution of correlation coefficient, r, for number of repairs on shell regressed on size (indexed by whorl diameter, WD) for Miocene versus Recent samples of *Euspira heros* (Eh), *Neverita duplicata* (Nd), *Busycon carica* (Bcr), *Busycotypus canaliculatus* (Bcn), and *Busycon scalarispira* (Bs). Multiple comparison Tukey-HSD test reveal correlation coefficients are significantly different ($p < 0.01$) among naticids only in comparisons between Nd (Recent vs. Miocene). Among melongenids, r values are significantly different in comparisons of Bs vs. Bcr and Bs vs. Bcn ($p < 0.05$). Greater r value between linear or second order polynomial regression for a sample used in all comparisons.

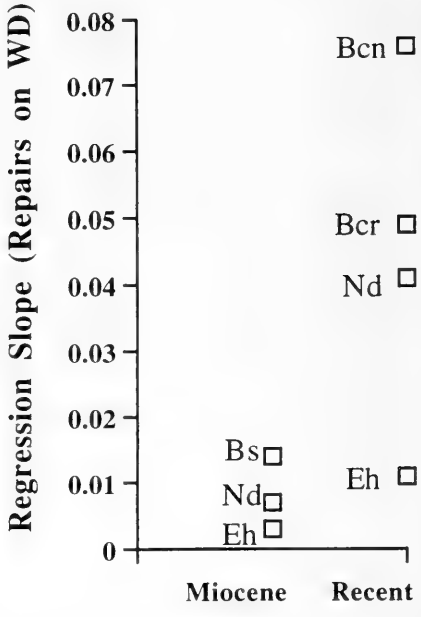

FIG. 4. Distribution of slope of regression lines, beta, for number of repairs on shell regressed on size (indexed by whorl diameter, WD) for Miocene versus Recent samples of *Euspira heros* (Eh), *Neverita duplicata* (Nd), *Busycon carica* (Bcr), *Busycotypus canaliculatus* (Bcn), and *Busycon scalarispira* (Bs). Multiple comparison F-tests reveal slopes of regression lines are significantly different ($p < 0.001$) in comparisons between Nd (Recent) and all other naticid samples. Among melongenids, regressions lines for Recent whelks (Bcn and Bcr) have significantly greater slopes ($p < 0.001$) than that for the Miocene whelk (Bs). Slope of regression line of Bcn is significantly greater ($p < 0.001$) than that for Bcr. Slopes for regression line of Recent whelks are significantly greater ($p < 0.001$) than all naticids.

and B, and a factor of four at position C (Fig. 1) from Miocene to Recent samples of *E. heros* (Table 4). Average thickness values at the same three positions on the apertural lip more than doubled from Miocene to Recent samples of *N. duplicata* (Table 4). Among whelks, shells of *B. carica* are on average twice as thick as those of *B. canaliculatus* at position A, but is no thicker at position C than *B. canaliculatus* or *B. scalarispira* (Table 4).

Mean number of repairs per specimen was different among the five species (ANOVA, $p = 0.0001$), although the average was comparable for the two moon snails, namely 1.1 and 1.0 for Recent confamilial *N. duplicata* and *E. heros,* respectively (Table 3). Miocene naticids *N. duplicata* (0.36) and *E. heros* (0.41), as well as the Recent whelks *B. carica* (5.3) and *B. canaliculatus* (5.2), also show comparable averages (Table 3). Mean number of repairs for Miocene *B. scalarispira* (0.7) and Miocene naticids are significantly different ($p < 0.05$) than Recent congeneric whelks and Recent naticids (Table 3). Among naticids, mean values are substantially greater for Recent temperate latitude *N. duplicata* and *E. heros,* versus Recent tropical naticids and Upper Triassic to Miocene naticids (Table 5). Among the melongenids, Recent species have a seven times greater repair frequency than the Miocene species (Table 6).

Repair frequency is more positively correlated with size and apertural lip thickness for all

TABLE 4. Two-way ANOVA for moon snails indicates that species ($p = 0.0001$) and position ($p = 0.0001$) are significant factors in mean differences in apertural lip thickness (ALT). See Fig. 1 for location of position A, B, and C on apertural lip. Factor interaction (species in combination with ALT position) is also significant ($p = 0.0001$). Two-way ANOVA for whelks indicates that species ($p = 0.0001$) and position ($p = 0.0001$) are significant factors in mean differences in ALT. Factor interaction (species in combination with position) is also significant ($p = 0.0001$); n = sample size.

Taxon	Mean ALT (mm) at position A	Mean ALT (mm) at position B	Mean ALT (mm) at position C
E. heros Recent	0.89 n = 241	0.76 n = 241	2.08 n = 240
E. heros Miocene	0.29 n = 81	0.22 n = 80	0.50 n = 80
N. duplicata Recent	0.75 n = 779	0.68 n = 780	1.62 n = 780
N. duplicata Miocene	0.37 n = 216	0.26 n = 222	0.66 n = 198
B. canaliculatus Recent	1.26 n = 143	1.36 n = 143	1.30 n = 143
B. carica Recent	2.11 n = 173	1.60 n = 173	1.28 n = 173
B. scalarispira Miocene	1.99 n = 194	1.73 n = 195	1.29 n = 195

TABLE 5. Comparison of repair frequencies, f (the number of scars per shell), in umbilicate species from the present study with extant and fossil populations.

Taxon	Geological Age/Formation	Location	f	Reference
Euspira heros	Recent	Southern New Jersey	1.0	Present study
Neverita duplicata	Recent	Southern New Jersey	1.1	Present study
Euspira sp.	Recent	Tropical	0–0.05	Vermeij, 1982
Polinices tumidus	Recent	Aru Islands	0.10	Vermeij, 1982
P. uber	Recent	Panama	0.13	Vermeij, 1982
Natica chemnitzii	Recent	Panama	0.50	Vermeij, 1982
E. heros	Miocene (Kirkwood Fm.)	Delaware	0.41	Present study
N. duplicata	Miocene (Kirkwood Fm.)	Delaware	0.36	Present study
E. rectilabrum	Late Cretaceous (Ripley Formation)	Mississippi	0.71	Vermeij & Dudley, 1982
Euspira sp.	Late Cretaceous (Ripley Formation)	Alabama	0.64	Vermeij & Dudley, 1982
Amauropsis paludinaris	Upper Triassic St. Cassian Gr.	Costalaresc, Italy	0.051	Vermeij et al., 1982

three positions (Fig. 1) for Recent melongenids versus Recent naticids (Figs. 3, 5, 7, 9). Furthermore, rate of accumulation of repairs is significantly greater for Recent whelks versus moon snails based on slopes of the regression lines for repairs regressed on WD and ALT (Figs. 4, 6, 8, 10). Repair frequency is more positively correlated with size and apertural lip thickness in Recent whelks relative to Miocene whelks (Figs. 3, 5, 7, 9). In contrast, the only significant difference occurs in the r values of repair frequency regressed on size for Miocene versus Recent samples of *N. duplicata* (Fig. 3). No significant differences occur in

TABLE 6. Comparison of repair frequencies, f (the number of scars per shell), in extant and fossil whelk populations.

Taxon	Geological Age/Formation	Location	f	Reference
Busycon carica	Recent	Southern New Jersey	5.3	Present study
Busycotypus canaliculatus	Recent	Southern New Jersey	5.2	Present study
Busycon scalarispira	Miocene (Kirkwood Fm.)	Delaware	0.7	Present study

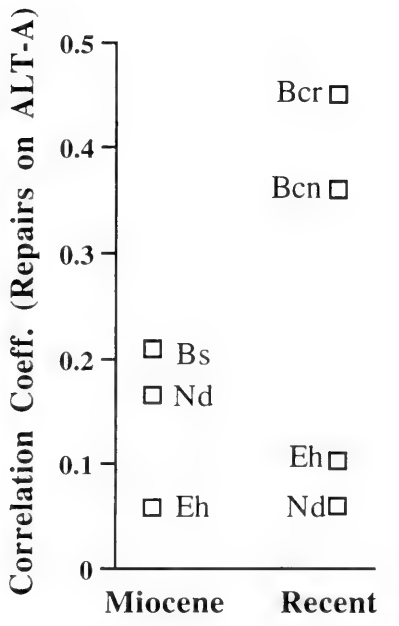

FIG. 5. Distribution of correlation coefficient, r, for number of repairs on shell regressed on apertural lip thickness at position A (see Fig. 1) for Miocene versus Recent samples of *Euspira heros* (Eh), *Neverita duplicata* (Nd), *Busycon carica* (Bcr), *Busycotypus canaliculatus* (Bcn), and *Busycon scalarispira* (Bs). Multiple comparison Tukey-HSD test reveal correlation coefficients are significantly different ($p < 0.05$) only in comparison between *B. carica* and *B. scalarispira*. Greater r value between linear or second order polynomial regression for a sample used in all comparisons of correlation coefficients.

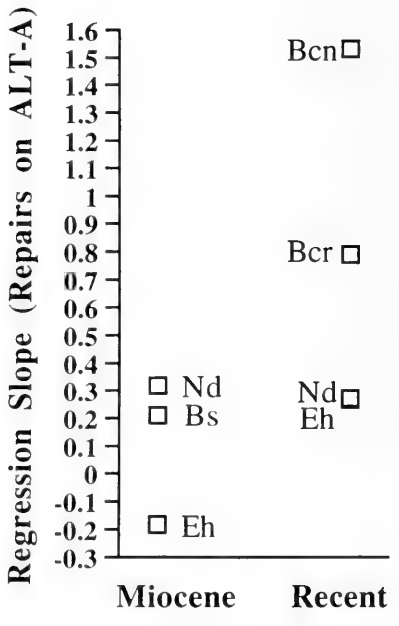

FIG. 6. Distribution of slope of regression lines, beta, for number of repairs on shell regressed on apertural lip thickness at position A (see Fig. 1) for Miocene versus Recent samples of *Euspira heros* (Eh), *Neverita duplicata* (Nd), *Busycon carica* (Bcr), *Busycotypus canaliculatus* (Bcn), and *Busycon scalarispira* (Bs). Multiple comparison F-tests reveal slopes of regression lines are significantly different only in comparisons involving melongenids. Regression lines for Recent whelks (Bcn and Bcr) have significantly greater slopes ($p < 0.001$) than that for the Miocene whelk (Bs) and Recent naticids (Eh and Nd). Slope of regression line of Bcn significantly greater ($p < 0.001$) than that for Bcr.

comparison of r values involving shell thickness (Figs. 5–10). Furthermore, no significant difference occurs between compared correlation coefficients or slopes of regression lines for Miocene and Recent samples of *E. heros* for either size or lip thickness (Figs. 3–10).

DISCUSSION

The significant correlation ($p < 0.05$) between the number of repairs per shell and size (WD) for Recent whelk and moon snail species (Fig. 3) indicates that age-dependent accumu-

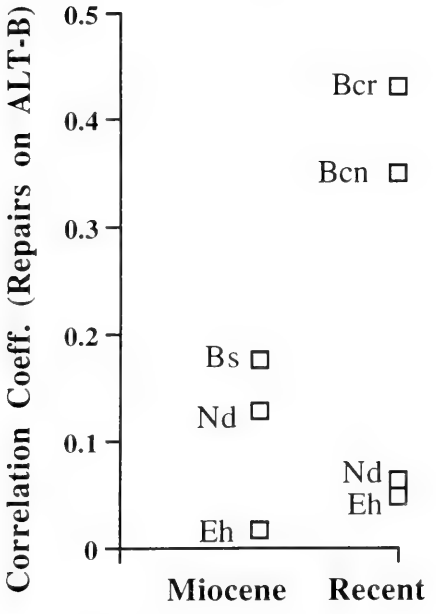

FIG. 7. Distribution of correlation coefficient, r, for number of repairs on shell regressed on apertural lip thickness at position B (see Fig. 1) for Miocene versus Recent samples of *Euspira heros* (Eh), *Neverita duplicata* (Nd), *Busycon carica* (Bcr), *Busycotypus canaliculatus* (Bcn), and *Busycon scalarispira* (Bs). Multiple comparison Tukey-HSD test reveal correlation coefficients are significantly different ($P < 0.05$) only in comparison between *B. carica* and *B. scalarispira*. Greater r value between linear or second order polynomial regression for a sample used in all comparisons of correlation coefficients.

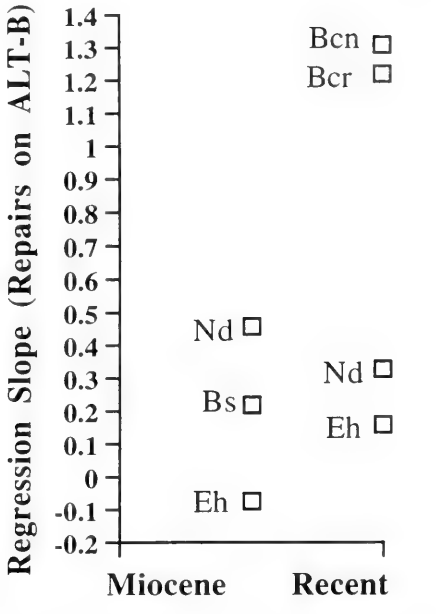

FIG. 8. Distribution of slope of regression lines, beta, for number of repairs on shell regressed on apertural lip thickness at position B (see Fig. 1) for Miocene versus Recent samples of *Euspira heros* (Eh), *Neverita duplicata* (Nd), *Busycon carica* (Bcr), *Busycotypus canaliculatus* (Bcn), and *Busycon scalarispira* (Bs). Multiple comparison F-tests reveal slopes of regression lines are significantly different only in comparisons involving melongenids. Regression lines for Recent whelks (Bcn and Bcr) have significantly greater slopes ($p < 0.001$) than that for the Miocene whelk (Bs) and Recent naticids (Eh and Nd).

lation of scars increased as exposure time to potential predators increased over the life span of the prey (Fig. 3). In most Recent gastropods, incidence of sublethal shell breakage increases as shell length increases (Vermeij et al., 1980; Zipser & Vermeij, 1980; Vermeij, 1982; Vermeij & Dudley, 1982; Vermeij et al., 1982; Raffaelli, 1978; Dudley, 1980). Similarly, Alexander (1989) showed that the frequency of repaired valves correlates significantly with valve surface area (index of size) for Late Ordovician brachiopods. Large size also decreases the probability that a shell-breaking predator will lethally fracture the shell (Hughes & Elner, 1979; Seed, 1978; Elner & Raffaelli, 1980; Preston et al., 1996), which may also explain why successively larger size classes have greater frequencies of shell repair for all five gastropod species in this investigation (Table 1). However, repaired fractures do not continue to accumulate on shells of *E. heros* (maximum = 6) from the penultimate to the largest size classes (60–79 vs. 80–99) as they do between the two largest size classes of *N. duplicata* (Table 2), suggesting a possible size refugia from predation for *E. heros*. In contrast, the largest individuals of *N. duplicata* had a WD in the 60–79 mm size class (Table 2) and had scars near the periphery of the apertural lip.

Failure to continue to accumulate repairs on shells of *E. heros* with increasing size beyond the 80 mm WD threshold in the population at Hereford Inlet (Table 2) may account for the lower correlation coefficient and slope value between repair frequency and WD for *E. heros* relative to the slightly smaller *N. duplicata* (Figs. 3–4). Preston et al. (1996) commented

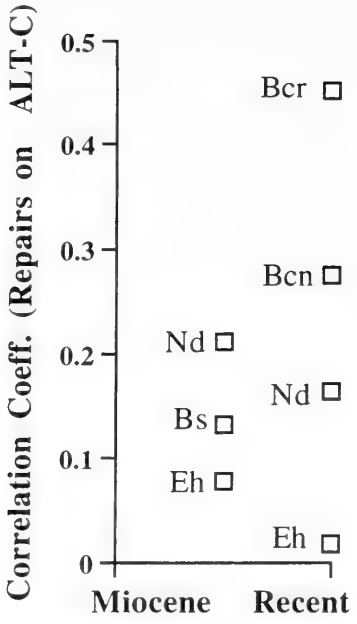

FIG. 9. Distribution of correlation coefficient, r, for number of repairs on shell regressed on apertural lip thickness at position C (see Fig. 1) for Miocene versus Recent samples of *Euspira heros* (Eh), *Neverita duplicata* (Nd), *Busycon carica* (Bcr), *Busycotypus canaliculatus* (Bcn), and *Busycon scalarispira* (Bs). Multiple comparison Tukey-HSD test reveal correlation coefficients are significantly different ($p < 0.01$) only in comparison of *B. carica* vs. *B. scalarispira*. Greater r value between linear or second order polynomial regression for a sample used in all comparisons.

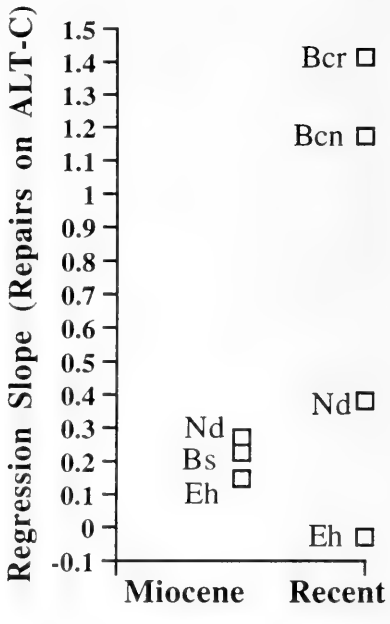

FIG. 10. Distribution of slope of regression lines, beta, for number of repairs on shell regressed on apertural lip thickness at position C (see Fig. 1) for Miocene versus Recent samples of *Euspira heros* (Eh), *Neverita duplicata* (Nd), *Busycon carica* (Bcr), *Busycotypus canaliculatus* (Bcn), and *Busycon scalarispira* (Bs). Multiple comparison F-tests reveal slopes of regression lines are significantly different ($p < 0.001$) among naticids only in comparisons between Recent *N. duplicata* and Recent *E. heros*. Regression lines for Recent whelks (Bcn and Bcr) have significantly greater slopes ($p < 0.001$) than that for the Miocene whelk (Bs) and Recent naticids (Eh and Nd).

that with increasing shell size, more attacks from predators will fail to break the thicker lip. Rate of accumulation of repairs is thus expected to decline when the predator can no longer readily inflict damage to the thicker shelled gastropods, a contrast to durophagy on skeletonized invertebrates with delicate appendages that can be sheared off at any body size, such as with crinoid arms (Oji, 1996).

The significantly greater correlation coefficient ($p < 0.05$) between repair frequency and lip thickness at position C ($r = 0.16$) vs. position A ($r = 0.06$) or B ($r = 0.06$) (Fig. 9 vs. Figs. 5 and 7) for both Recent and Miocene *N. duplicata* may indicate where the crushing elements of the durophage more often exerted their peeling force, namely posteriorly near the umbilicus (Fig. 1, position C). Peeled shells of *N. duplicata* more often have shell material of the body whorl closest to the umbilical plug removed, but retain a wrapped around "awning" of shell material anteriorly, nearest the suture with the penultimate whorl. The aperture is widest posteriorly at position C, in *N. duplicata,* allowing the claw of a crab to secure the deepest purchase onto the lip and into the body whorl. Hence most sublethal shell breakage originates posteriorly on the lip. Thus, the thickness of the lip posteriorly (position C), where peeling forces are exerted on the shell, may be more important than the thickness of the lip anteriorly (position A) in the determination if a fracture initiated by a durophage will be localized by the prey. The significantly greater correlation of repair frequency with lip thickness at position C rather than position A is harmonious with that reasoning.

Among repaired shells the umbilical plug

functioned to stop propagation of fractures through this posterior region into the basal cavity and thereby render repairable any posteriorly localized breakage. Vermeij (1987) stated that the structural weakness of the umbilicus can be strengthened by the formation of the umbilical plug closing the basal cavity. Conversely, lack of correlation between ALT at any position and number of shell repairs for the other Recent moon snail, *E. heros* (Figs. 5, 7, 9) may indicate an architectural weakness of this unplugged umbilicate design to localized fractures initiated in the apertural lip. As the probability of lethal fractures increased in this unplugged moon snail, frequency of sublethal fractures decrease. The statistical consequence is diminished correlation between ALT and repair frequency in shells of *E. heros* (Vermeij, 1982).

The better correlations between ALT at any lip position for whelks versus moon snails (Figs. 5–10) may reflect the fact the whelks have a higher probability of accumulating sublethal fractures on their shell as a consequence of their feeding habit. They wedge apart their bivalve prey (Colton, 1908; Warren, 1916; Carriker, 1951), an activity that is not likely to induce an unrepairable fracture. Indeed, most repairs are scallop-like, shallow indentations from the normal axial growth line (Figs. 2, 3), a pattern suggestive of breakage during valve-wedging of bivalve prey. Only deep, irregular embayments from the normal axial growth line (Fig. 2-1) indicate repairs of shell-breakage more likely to have been inflicted by blue crabs. Thus, the feeding habit of the whelk is probably more important than its victimization by crabs in accumulation of shell repairs with increasing size and apertural thickness (Tables 1, 2).

Vermeij (1997) speculated that predator-prey escalation between armored gastropods and their shell-breaking enemies can be used as a prediction that shell-breakage became increasingly important as a selective factor for gastropods through Mesozoic and Cenozoic time. Interpretations of repair frequencies cannot estimate either predator intensity or predator efficiency but can reflect the effectiveness of the predator's crushing ability and the prey's resistance to crushing (Schindel & Vermeij, 1982). Based on these assumptions, Vermeij, after several investigations, concluded that repair frequencies remained constant from the Late Carboniferous to the Late Triassic (Schindel et al., 1982; Vermeij et al., 1982), increased from the Late Triassic to the Late Cretaceous (Vermeij & Dudley, 1982), and then again became relatively constant approaching Recent levels (Vermeij, 1982). These trends, coupled with an increase in power and diversification of shell-breaking predators from the Mesozoic to Cenozoic, formed the basis of Vermeij's conclusions.

Vermeij & Dudley (1982) reported that *Euspira* sp. from the Ripley Formation (Late Cretaceous) had higher frequencies of repair (0.71, Table 5) than do Recent tropical populations of *Euspira* sp. (0-0.5; Vermeij, 1982). Data in this investigation show a frequency of repair which is even higher (1.0) than those of Late Cretaceous members (0.71), suggesting higher incidence and stronger expression of breakage-resistant armor in temperate members of this family. However, repair frequency in the Miocene *E. heros* is lower (0.4) than that reported for the Late Cretaceous (Table 5), evidence in support of Vermeij's contention that repair frequencies did not continue to increase after the Cretaceous. Disparity in repair frequencies between Recent tropical (0.05) and temperate *Euspira* sp. (1.0) may reflect differences in both the strength and the abundance of predators relative to the strength and abundance of the prey. Similarly, repair frequencies for *N. duplicata* (1.1) are higher than ecologically similar Recent tropical populations of *Polinices tumidus* (0.1) and *Polinices uber* (0.13)(Vermeij, 1982) (Table 5). Vermeij (1983) reported that an increase in relative abundance of the predator would increase the frequency of repair.

Size of individual shells is important in comparison of repair frequencies. Vermeij (1982) sorted 21 specimens of *Natica chemnitzii* among four size classes, that is, 5–9 mm; 10–19 mm; 20–29 mm; and 30–39 mm. The repair frequency of 0.67 in the 20–29 mm size class (n = 9; Vermeij, 1982), is comparable to the repair frequency values in the 20–39 mm size class in this study, namely, 0.61 (GEH) and 0.73 (HI) for *N. duplicata* and 0.69 (GEH) and 0.72 (HI) for *E. heros* (Table 1). However, for all size classes, repair frequency for shells of *N. chemnitzii* was 0.50 compared with 1.1 and 1.0 for *N. duplicata* and *E. heros,* respectively (Table 5). This difference may be the result of the inclusion of large numbers of shells in the 40–99 mm size classes in this study.

The higher frequency of repair for both *B. canaliculatus* (5.2) and *B. carica* (5.3) is attributed to a combination of shell breakage from crabs preying on whelks combined with apertural lip fracture (Fig. 2) during attempts by whelks to employ the outer shell lip to wedge open the valves of tightly closed bivalve prey.

The rate of accumulation of repairs, reflected in the slopes of repair frequencies regressed on size and lip thickness (Figs. 4, 6, 8, 10), is greater for whelks in comparison to moon snails. Because the shell lip of predatory whelks is often slightly damaged during attacks on prey, valve-wedging species frequently have a high incidence of repaired breaks than non-valve-wedging species. The frequencies of repair for Recent whelk species in this study are higher than that reported for any other gastropod in the literature. The frequency is more than seven times greater than that for *B. scalarispira* from the Miocene. Vermeij (1987: 182) suggested that the lip-wedging technique may not have evolved until the Middle Pliocene on the Atlantic Coastal Plain of North America, although no evidence was presented. The majority of repaired fractures in *B. scalarispira* extend across the varices onto the abaxial edge of the sutural shelf of the final whorl (Fig. 2–5). In contrast, most repairs in Recent whelks were concentrated at the lip mid-length (Fig. 2–4, 2–8). Thus, varices appear to be effective in limiting the extent of unrepairable damage from lip-wedging but not the direct attention to the lip by the durophage. This discrepancy suggests that Miocene melongenids had not evolved, or perfected, valve-wedging, and as a consequence the frequency of repairs probably reflects the contribution of predators sublethally peeling the whelks.

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WITHIN-CLUTCH EGG CANNIBALISM VARIABILITY IN HATCHLINGS OF THE LAND SNAIL *HELIX ASPERSA* (PULMONATA: STYLOMMATOPHORA): INFLUENCE OF TWO PROXIMATE FACTORS

Christophe Desbuquois & Luc Madec

U.M.R. CNRS 6553, Laboratoire de Zoologie et d'Ecophysiologie (U.A. INRA), Faculté des Sciences, Université de Rennes 1, Avenue du Général Leclerc, 35042 Rennes CEDEX, France

ABSTRACT

This study investigated, under laboratory conditions, the influence of two proximate factors, temperature and humidity, on the rate of egg cannibalism in hatchlings of *Helix aspersa*. Nine combinations were tested involving three different temperatures (15, 20, 25°C) and relative humidities (40, 70, 100% R.H.). Each new-born snail was maintained with a conspecific egg of its laying, previously incubated at 15°C instead of 20°C for the tested snails, so that its incubation duration was enhanced. Three measurements were carried out, namely the percentage of cannibalism, the snail mortality and the egg desiccation. Temperature, humidity and their interaction had a significant influence on egg cannibalism. For every combination of these two factors, the percentage of hatchlings having consumed the available egg increased with time. Those snails not fed could survive at least four days at high humidity. However, four days after hatching, non-cannibalistic hatchlings exhibited a higher mortality than cannibalistic ones, essentially due to humidity constraint. Hence, egg cannibalism may represent an adaptive mechanism to resist to adverse climatic conditions, such as high temperature and low humidity. In natural conditions, a higher rate of egg cannibalism might be expected at low temperature and low humidity in comparison with the one observed in this experiment. Egg cannibalism by juveniles might improve survival during adverse conditions (via water ingestion) and would be able to affect other life history traits, such as the subsequent growth rate of juveniles having consumed one egg immediately after the hatching. It might represent a mean to avoid juvenile size-dependent mortality due to the seasonal variations of abiotic conditions.

Key words: nutrition, intraspecific oophagy, egg cannibalism, proximate factors, *Helix aspersa*.

INTRODUCTION

Egg cannibalism in hatchlings of different land snail species has been reviewed by Baur (1992). In pulmonates, two species have been especially investigated, namely *Helix pomatia* Linnaeus (Baur, 1988a,d) and *Arianta arbustorum* Linnaeus (Baur 1988a-c, 1993; Baur & Baur, 1986). However, few studies deal with this aspect of juvenile nutrition in *Helix aspersa* Müller, although Elmslie (1988) demonstrated its existence in hatchlings of this species and Fearnley (1993) gave hypotheses on its consequences in life history. As in *H. pomatia* (Baur, 1988d), oophagy is restricted to new-born snails during the hatching period.

Different life history traits may be affected by oophagy; for example, subsequent survivorship and growth enhancements were observed in the snail *H. aspersa* having consumed one egg immediately after hatching (Desbuquois, 1997). Adaptation for terrestrial life involves strategies of survival and development under various proximate factors. Special forms of behaviour, namely aestivation and hibernation, may have evolved in land snails to offer some resistance to adverse abiotic conditions (Riddle, 1983). Such dormancies have a diapause value in *H. aspersa* (Bailey, 1981, 1983; Lorvelec 1988) and, in the context of life history theory, are often related with an adversity selection (Greenslade, 1983). In the same way, egg cannibalism might be one of these behavioural tactics with important repercussions on survival during winter via the size reached by juvenile snails before diapause (Biannic, 1995).

Within-clutch egg cannibalism variation can be related to microhabitat differences, especially in temperature and moisture (Baur, 1994; Baur & Baur, 1986), which were also subject, in Western Europe, to great variation during daily and annual cycles. Thus, the aim of our study is to test the influence of this vari-

ation during hatching, on the extent of egg cannibalism in hatchlings of *H. aspersa*.

MATERIAL AND METHODS

Relevant Natural History

The hermaphroditic land snail *H. aspersa* lays its eggs in batches in a nest excavated in the soil. One to three clutches are deposited per breeding season, with a mean clutch size of around 100 eggs. Newly hatched snails might remain in their nest from 3 to 16 days after hatching (6 days on average) before emergence (Herzberg & Herzberg, 1962); this may allow first born hatchlings to feed on unhatched eggs. Juvenile and adult snails are herbivorous.

In the present experiment, adults of *H. aspersa* were collected in August 1994 in a population living in the salt-pans at Guerande (South Brittany, France). They were maintained in hibernation in a room at 4°C for three months before breeding.

Breeding

The study was carried out on egg-layings obtained from 20 January to 23 March 1995 from adults reared in polythene boxes under constant artificial conditions (temperature: 20 ± 1°C, relative humidity: 80 ± 5%, 18/6 light/dark cycle) promoting reproduction (Daguzan, 1981). They were fed with a cereal composed snail food (produced by the company Arrivé) supplied *ad libitum* and renewed twice a week. Four laying jars containing moist soil were placed in each cage allowing snails to deposit their clutches.

Experimental Conditions

After each clutch was washed, it was divided into two equal groups of eggs, which were incubated in small Petri dishes (diameter: 55 mm) lined with moistened filter paper to obtain air moisture saturation. Half of the eggs were placed at 15°C to slow down the embryonic development, the other at 20°C to obtain the snails that will be fed with the 15°C-incubated eggs. This method, used by Baur (1988a, 1993) and Baur & Baur (1986), allowed to reproduce the natural hatching asynchronism, that is, a delay between the first and the last hatching. According to Le Calvé (1987), these conditions lead to a hatching delay of about nine days for the 15°C-incubated eggs.

Each new-born snail (one day old) was placed on a moistened filter paper disc in a small aluminium container (diameter: 20 mm; height: 6 mm), open at the top, and received a conspecific egg incubated at 15°C.

Animals tested were maintained in the shade at different temperatures in hermetically closed plexiglass boxes (24 × 18 × 10 cm). Constant humidities were obtained within the boxes by means of NaOH/water mixtures (Madge, 1961), which were introduced more than three days before starting the experiment. Three temperatures (15, 20, 25°C) and relative humidities (40, 70, 100% R.H.) and their eventual interactions were tested. The ranges of air temperature and relative humidity chosen were commonly encountered in South Brittany during the breeding season of this species. Containers were maintained above the NaOH solution with a wired prop placed 2 cm above the bottom of the boxes and were covered with a polythene net. The boxes were opened daily in order to allow observations and air renewal; at the same time, a drop of water was deposited on each disc of paper.

Measurements Used

Egg predation was observed under 12.5× magnification; the rate of egg cannibalism (percent) was defined as the ratio of hatchlings having consumed the egg divided by the number of snails tested per batch. Snail mortality was equal to the number of hatchlings dead divided by the number of snails tested (individuals of all clutches were considered together). When an egg was dehydrated, his weight became very low and his colour white and opaque. Egg desiccation was the delay (in days) before each egg not consumed in a batch was dehydrated. Three or four clutches were used for each combination of temperature and humidity. A total of 1,555 hatchlings was tested for egg cannibalism in the different thermohygrometric associations (i.e., from 130 to 260 hatchlings per combination).

Statistical Analyses

First, we calculated regressions of the percentage of egg cannibalism on the age in the different thermohygrometric conditions. Then, the effects of temperature, relative humidity and their eventual interactions on egg canni-

balism were tested using ANOVA on residuals of regressions, that is, log(age) was introduced as a covariable in the analysis. SNK multiple comparison tests were carried out when means were heterogeneous. The percentages of mortality were compared using $\chi 2$ tests of association. The assumption of normality of the residuals was checked using Lilliefors test on BIOMECO (1988). Other analyses were run with MINITAB (1991).

RESULTS

Egg Cannibalism

For every combination of temperature and humidity, the percentage of hatchlings having consumed the available egg increased with the logarithm of time (Fig. 1). Temperature significantly affected egg cannibalism by hatchlings (Table 1; ANOVA, $P < 0.001$): oophagy was highest at 20°C and 25°C and significantly lower at 15°C (SNK tests, $P < 0.001$). The percentage of egg cannibalism was also different according to relative humidity (Table 1; $P < 0.001$) and was higher at 100% R.H. than at the two other humidities (SNK tests, $P < 0.005$). Moreover, a significant interaction between the two factors studied was found (Table 1; $P < 0.001$) especially involving the 20°C-100% R.H. and 25°C-100% R.H. modalities (SNK tests, $P < 0.05$).

Other Mortality Factors

Snail survival and egg desiccation were highly affected by humidity and, to a lesser extent, by temperature.

Thus, hatchling mortality was higher and earlier at 40% R.H., much lower and later at 100% R.H. After 4 days, mortality was significantly higher in non-cannibalistic hatchlings, that is, snails having not consumed the available egg, except for 15°C-100% and 20°C-100% R.H. ($\chi 2$ test, $P < 0.001$), where no mortality occurred (Table 2A). In low relative humidity (40% R.H.) or high temperature (25°C) conditions, lethal water loss was especially important in non-cannibalistic hatchlings. After 6 days, results were not different but several combinations could not be tested because of egg desiccation which prevented snails from cannibalism (Table 2B). Obviously, humidity drastically affected the time of dehydration of the eggs (Table 3). At 100% relative humidity, eggs did not suffer from desiccation with time so they could be consumed more than ten days after the beginning of the experiment. At 40% R.H., all eggs were dehydrated after 2 to 4 days. At 70% R.H., egg desiccation increased with temperature. Hence, egg availability duration for potentially cannibalistic hatchlings was different according to thermohygrometric conditions.

DISCUSSION AND CONCLUSIONS

The present experiment shows that egg cannibalism is influenced by the two environmental variables studied which might operate at two levels: (i) hatchling survival, and probably activity and time spent by hatchlings in the nest, that is, egg cannibalism opportunity, and (ii) egg desiccation and hatching asynchronism of the clutch, that is, egg availability. Obviously, the longer a hatchling is in presence of non-dehydrated eggs, the more likely it is to eat them.

As was recorded in other mollusc species (Machin, 1975), juveniles of *H. aspersa* are particularly sensitive to dehydration: at 15°C and 20°C, water loss occurs under 90% relative humidity; at a temperature of 25°C, three-month-old snails lose water even if they are maintained at 100% relative humidity (Charrier, 1980). Klein-Rollais (1993) showed that the rate of water ingestion of juvenile snails during the first weeks of life was highest above 20°C, and decreased when the relative humidity increased from 60% to 100%. Under mild climatic conditions, individuals may survive more than four days without feeding, whereas under harsh conditions of temperature and relative humidity, mortality is very high in non-cannibalistic hatchlings. Thus, snail mortality is greatly influenced by temperature-humidity interaction through dehydration and impossibility of water intake. Therefore, egg cannibalism, through water intake, might be considered as an adaptive mechanism which allow some resistance to conditions which promote water loss.

In *H. aspersa,* locomotor activity is influenced by snail water content (Charrier, 1980; Biannic, 1995), which is itself dependent on temperature and humidity (Klein-Rollais, 1993). Thus, as in other slug and snail species (Prior, 1985), activity of *H. aspersa* is also closely related to these two factors (Herzberg & Herzberg, 1962; Dan, 1978). Moreover, juveniles are active in a higher range of temperatures and humidities than adults (Biannic,

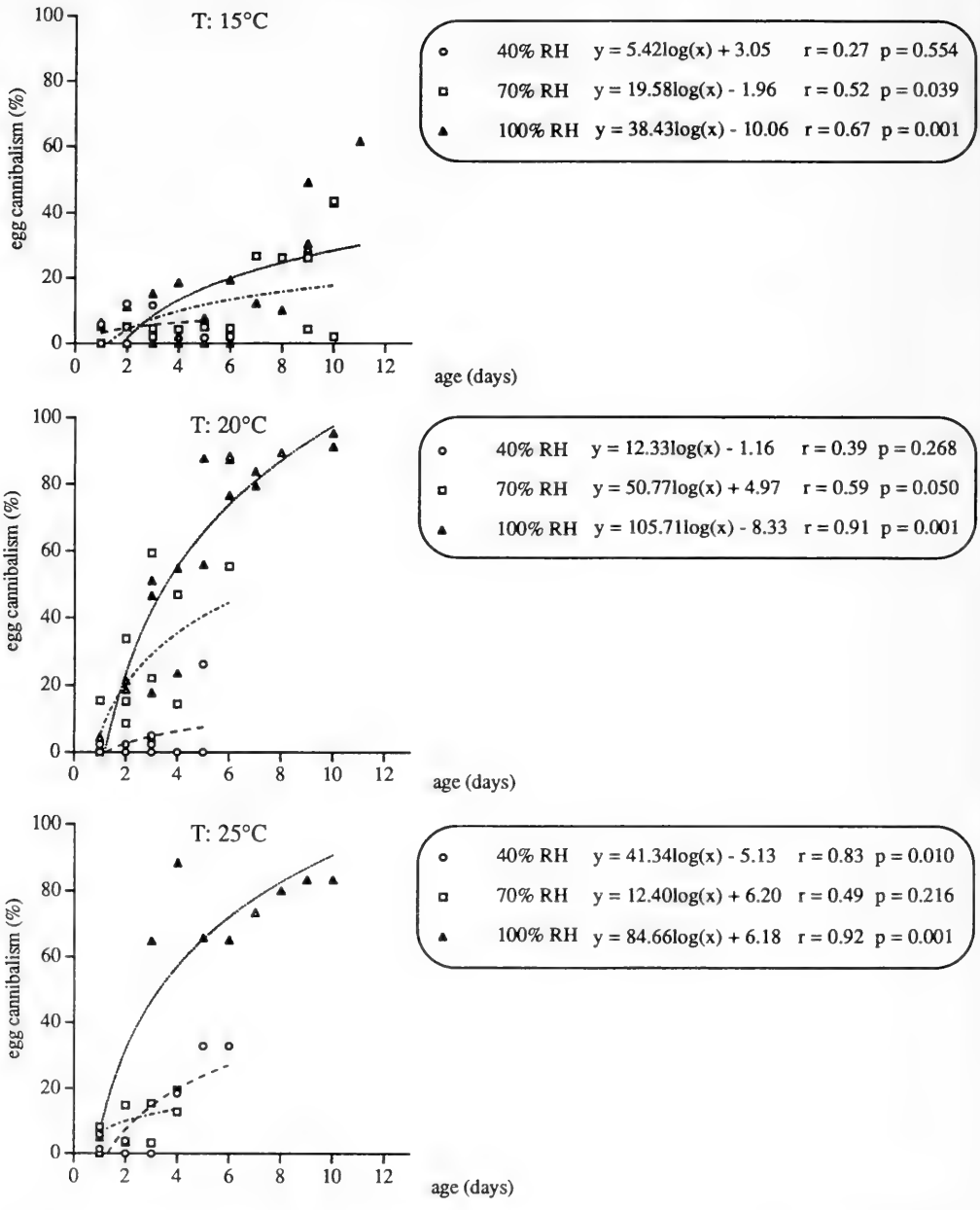

FIG. 1. Regressions between the rate of egg cannibalism and the age of hatchlings of *Helix aspersa* in relation to ambient temperature (T) and relative humidity (RH).

1995). Thus, the two factors tested are indirectly able to affect the extent of egg cannibalism.

In addition, egg cannibalism was also dependent on these two abiotic factors via egg desiccation which influences egg availability. Eggs are particularly sensitive to dehydration (Machin, 1975; Riddle, 1983). At 25°C, the rate of desiccation of isolated eggs of *H. aspersa* placed at 25% R.H. is 6.4 times higher than for

TABLE 1. Analysis of variance for egg cannibalism by hatchlings of *Helix aspersa* according to ambient temperature and relative humidity

Source of variation	Degrees of freedom	Sum of squares	F-values	P
Temperature (T)	2	33.09	20.87	<0.001
Relative humidity (RH)	2	18.03	24.61	<0.001
T × RH	4	18.67	11.59	<0.001
Error	102	41.07		

TABLE 2. Mean values of hatchling mortality (%) of *Helix aspersa* according to ambient temperature and relative humidity (left value: cannibalistic hatchlings; right value: non-cannibalistic hatchlings) (number of clutches in parentheses)
A. After four days of life. B. After six days of life (When no value appears, the experiment was stopped before day six because all the eggs were dehydrated)

		Temperature (°C)		
A	Relative humidity (%)	15	20	25
	40	28.6/85.2 (3)	16.7/53.5 (3)	0.0/43.8 (3)
	70	0.0/6.9 (3)	0.0/3.3 (3)	0.0/19.4 (3)
	100	0.0/0.0 (4)	0.0/0.0 (3)	0.0/4.8 (3)
		Temperature (°C)		
B	Relative humidity (%)	15	20	25
	40	—	—	—
	70	0.0/8.3	0.0/3.8	—
	100	0.0/0.0	0.0/20.0	0.0/4.8

eggs maintained at 90% R.H. (Bayne, 1968). Thus, egg cannibalism decreased when temperature increased and/or humidity decreased because eggs lost water and became uneatable.

There is also a negative correlation between the humidity and the durations of incubation and hatching in *H. aspersa* (Guéméné & Daguzan, 1983) and a positive one between temperature of incubation and hatching synchronism (Le Calvé & Daguzan, 1989). Thermohygrometric conditions can increase hatching asynchronism of clutches incubated in natural conditions and lead to egg cannibalism. The occurence of oophagy is also related to the time spent by hatchlings in the nest. Temperature enhances this period from four days at 25°C to 10 days at 15°C (Le Calvé, 1987). A low soil humidity may also increase this delay, because dry soil might prevent snails from emerging.

In natural clutches, as eggs are arranged in groups, egg dehydration is significantly lower and outer eggs may dry more rapidly (Bayne, 1969). Therefore, in a dry soil, outer eggs of the clutch dehydrate more rapidly and hatch later than the inner ones, so that they may be consumed by newly hatched snails from the internal eggs, as hypothetised by Baur & Baur (1986) for *A. arbustorum*. In *A. arbustorum*, Baur (1988b) noted a preference for wet oviposition sites and assumed that this choice induced a higher hatching success. Egg cannibalism might therefore be influenced by the choice of the oviposition site (parental manipulation), which produces an alteration of the hatching asynchronism (Baur, 1992).

In snail farms, the rates of hatching are often around 70% to 90% (Daguzan, 1981); although no value on egg cannibalism are available in the literature, it seems to be rare.

TABLE 3. Range of egg dehydration time (in days) of all eggs available of several batches of *Helix aspersa* according to ambient temperature and relative humidity (number of clutches in parentheses)

Relative humidity (%)	Temperature (°C)		
	15	20	25
40	2–4 (3)	2–4 (3)	2–4 (3)
70	>10 (3)	2–5 (3)	2–4 (3)
100	>10 (4)	>10 (3)	>10 (3)

In the wild, numerous causes of egg mortality exist in the clutches, but the extent of egg cannibalism is unknown.

High rates of egg cannibalism observed in this experiment compared with farm studies and probably wild conditions might be explained by two reasons: (i) the incubation time was artificially prolonged for half of the hatch; natural hatching asynchronism is probably lower in field conditions for the reason that the eggs of a clutch never undergoes such different environmental conditions, that is a range of 5°C in a egg laying site. In the case of high temperature and humidity in natural conditions or in snail farms where incubation conditions were nearly constant, the hatching synchronism of the eggs might have prevented egg cannibalism, (ii) in snail farms and when the conditions were favourable in the wild, the time spent by hatchlings in the nest is low. Thus, the length of time during which hatchlings may consume eggs is lower than in this experiment. On the other hand, egg cannibalism in natural conditions may increase at low temperature and low humidity in comparison with our results because hatchings were not synchronized and the length of time spent in the nest was high.

The low availability of an alternative food tends to favour cannibalism in different species (Elgar & Crespi, 1992). However, preliminary experiments demonstrated that newborn hatchlings of *H. aspersa* exhibited similar rate of cannibalism in presence and absence of humus (unpublished data). Thus, the lack of an alternative food can not explain the high rates of egg cannibalism observed in this experiment.

According to Baur & Baur (1986), egg cannibalism during dry weather might be a survival mechanism for the reason that nutritional and energetic benefits of egg consumption allow an increase in growth and survival of hatchlings. In addition, water intake by egg feeding leads, in those dry conditions, to a rehydration of hatchlings, which gives them the opportunity to wait for humidity and therefore increases their survival. Because of a juvenile size-dependent mortality based on harsh conditions encountered in winter for European populations of *Helix aspersa,* a high juvenile growth rate might represent an interesting element to avoid mortality due to low temperatures during winter, because juveniles with shell breadth below 19 mm are not able to really hibernate and thus, exhibit high mortality (Biannic, 1995). The plasticity of egg size and the correlation between egg size and hatchling size may lead to a seasonal enhancement of the juvenile growth rate, for autumnal clutches (Madec, 1989; Madec et al., in press). Egg cannibalism may be an alternative solution favouring higher growth rates. On that account, egg cannibalism and its plasticity, via their action on juvenile survival and on size at maturity (in preparation), may become an important component of the fitness in populations subject to periods of dryness.

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EXPRESSED SEQUENCE TAGS (ESTs) OF *BIOMPHALARIA GLABRATA,* AN INTERMEDIATE SNAIL HOST OF *SCHISTOSOMA MANSONI:* USE IN THE IDENTIFICATION OF RFLP MARKERS

Matty Knight[1], Andre N. Miller[1], Neil S. M. Geoghagen[2], Fred A. Lewis[1], & Anthony R. Kerlavage[2]

ABSTRACT

To identify some of the genes expressed in the snail *Biomphalaria glabrata,* a major intermediate host for the trematode parasite *Schistosoma mansoni,* we sequenced random cDNA clones from either a whole body or a cerebral ganglia cDNA library to generate 111 expressed sequence tags (ESTs). Searches of existing public databases showed that the majority of the snail sequences (54%) showed no significant homology to genes listed in either DNA or protein databases. Thirty one EST sequences showed significant matches with other genes in the databases. These included genes involved in gene expression, such as ribosomal proteins and translation factors, and those involved in cell communication, such as acetylcholine receptor and ATP-dependent transporter. Some ESTs used as probes demonstrated the occurrence of restriction fragment length polymorphisms (RFLPs) between parasite-resistant and parasite-susceptible snail stocks. Southern hybridization of parasite DNA with the snail EST encoding the acetylcholine receptor as probe showed the presence of a related sequence in the parasite genome, with the heterologous probe, indicating that this may be a useful method to identify closely related genes between the host and parasite.

Key words: *Biomphalaria glabrata,* cDNA libraries, Expressed Sequence Tags (ESTs), RFLP, *Schistosoma mansoni* resistant/susceptible.

INTRODUCTION

Considerable progress has been made in the analysis of genes of complex organisms by partial sequencing of random cDNAs. This method of generating expressed sequence tags (ESTs), first developed by Adams et al. (1991) for the human genome project, has rapidly expanded our knowledge of the identity and diversity of transcripts in organisms for which relatively little genetic information previously existed. For the parasitic helminths, *Schistosoma mansoni* and *Brugia malayi* for example, ESTs compiled in recent years have greatly expanded the number of cloned transcripts listed in DNA and protein databases from these organisms (Franco et al., 1995; Blaxter et al., 1996; Johnston, 1997). The identification of previously unknown genes from these invertebrates, and homologies with those from other organisms, may lead to a better understanding of their biology and to more effective treatment of the diseases they cause.

Much less is known about the genes of the vectors or intermediate hosts that transmit these parasites. For the mollusc *Biomphalaria glabrata,* an important intermediate host of *Schistosoma mansoni,* infection by the parasite is influenced by both snail and parasite genes (Richards, 1973, 1975). In adult snails, resistance to parasite infection is controlled by a single gene, which is inherited in Mendelian fashion, with resistance dominant. In juvenile snails, resistance is believed to be a polygenic trait, which is based on the complex interaction of other yet unknown genetic factors (Richards & Merrit, 1972). A more detailed understanding of both parasite and snail genes involved in this parasite-host interaction may lead to new methods for schistosomiasis control.

Compared to some other invertebrates that serve as intermediate hosts or vectors for major human diseases, relatively little is known about the molecular make-up of *B. glabrata;* no genetic or physical maps exist, and very few genes have been analyzed. A

[1]Biomedical Research Institute, Rockville, Maryland 20852, USA
[2]The Institute for Genomic Research, Gaithersburg, Maryland 20850, USA

search of sequences in GenBank showed only five *B. glabrata* sequences listed. Relatively few molluscs have been karyotyped (Burch, 1967; Patterson & Burch, 1978), and few studies have been conducted on the cytological analyses of chromosomes from different strains of *B. glabrata* chromosomes (Goldman et al., 1984). Based on the genome size of another closely related freshwater pulmonate gastropod, *Lymnaea stagnalis,* the *B. glabrata* genome is expected to be about 5.0×10^8 bp (Boer et al., 1977).

Using snails of different parasite susceptibility phenotypes, we previously described the identification of restriction fragment length polymorphisms (RFLP) and random amplified polymorphic DNA (RAPD) markers that can be used to distinguish some of our laboratory maintained, genetically selected resistant and susceptible snails (Knight et al., 1991; Larson et al., 1996). In the present study, we have used the isolation of ESTs to identify *B. glabrata* genes and to search for polymorphisms within some of these genes. This may be useful for conducting genetic linkage studies to identify genes associated with either resistance or susceptibility in *B. glabrata.* In this paper, we describe the identification of new *B. glabrata* ESTs from a survey of whole body and cerebral ganglia directional cDNA libraries from a *S. mansoni*-resistant snail (BS-90), and report the occurrence of RFLPs with some of these genes.

MATERIALS AND METHODS

Snails

The BS-90 snail line of *B. glabrata* is resistant at any age to *S. mansoni* infection (Paraense & Correa, 1963) and was made available to us by Dr. E. S. Loker (University of New Mexico). The M-line snail was selected for high susceptibility to *S. mansoni* infection by Newton (1955). Both snail lines were maintained as previously described (Miller et al., 1996).

RNA and DNA Extraction

Snails used for nucleic acid extraction were cleaned and kept overnight in sterile water containing 0.1 mg/ml ampicillin. RNA was extracted from either the whole body or cerebral ganglia. For whole body extraction, snails were crushed with a pestle into a fine powder under liquid nitrogen on dry ice in a chilled mortar. RNA was extracted with Rnazol as described by the manufacturer (Sinna Biotech). Cerebral ganglia were dissected from 10 individual adult snails (12–14 mm), plunged directly into Rnazol on ice, and extracted immediately. DNA was extracted from adult snails (10 mm) by a combination of the methods described by Knight et al. (1991) and Winnepenninckx et al. (1993). Briefly, snails were crushed into a fine powder as described above, and the powder was mixed, by inversion, into 10 ml pre-warmed (60°C) extraction buffer containing 2% cetyltrimethylammonium bromide (CTAB) (w/v), 1.4M NaCl, 0.2% (v/v) β-mercaptoethanol, 20 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0 and 100 μg/ml proteinase K (Boehringer Mannheim) and incubated for 1 h at 60°C. Samples were extracted sequentially with an equal volume of phenol, phenol/chloroform (1:1) and chloroform. The aqueous phase was digested with RNase A (20 μg/ml) for 1 h at 37°C, and extractions were repeated as described above. DNA was recovered by spooling after the addition of 2.5 volumes of ethanol (−20°C). Spooled DNA was washed in 70% ethanol (−20°C), air dried and resuspended in sterile dH_2O at a concentration of 0.1 mg/ml. Restriction enzyme digestions of DNA samples were performed as described by Knight et al. (1991), except that DNA was heated for 5 min at 65°C before enzymatic digestion. Digestions were done overnight at 37°C with buffer supplied by the manufacturer (New England Biolabs, Massachusetts).

Construction of cDNA Libraries

The whole body snail directional cDNA library was prepared from 5 μg of poly A+ selected mRNA in the phage vector λZAP using the λZAP-cDNA synthesis kit according to manufacturer's instructions (Stratagene, California). Briefly, first strand synthesis with reverse transcriptase was prepared by priming with *Xho* I-oligo-dT primer, followed by second strand synthesis with RNase H and DNA polymerase I. The final cDNA product was blunt-ended and, after ligation of *EcoR* I linkers and kinase treatment, was size selected on a Sephacryl S-400 column. The cDNA recovered was digested with *EcoR* I and *Xho* I and cloned directionally (*EcoR* I at the 5′ end and *Xho* I at the 3′ end) into the λZap vector *EcoR* I/*Xho* I phosphatase treated arms. Packaging was performed using packaging

extract (Gigapack gold) from Stratagene and plated out on *E. coli* strain XL1-blue MRF'. The library consisted of 1.1×10^6 independent recombinants with average insert size of 1000 bp.

The cDNA library of cerebral ganglia was constructed as described above, with the exception that first strand cDNA was prepared from total RNA (13.3 μg) extracted from cerebral ganglia from snails exposed for 5 h to 25 *S. mansoni* miracidia. The λZap cerebral ganglia directional cDNA library has 1.0×10^5 recombinants. Phagemids were prepared by mass excision of the libraries by co-infection with helper phage R408 and plating on *E. coli* (Sure strain) according to the manufacturer's instructions (Stratagene). Individual random colonies, selected by plating on IPTG/Xgal plates, were transferred in ordered array into Super broth (100 μl) in a 96-well microtitre plate. Phagemid DNA was prepared from each well after 37°C overnight incubation, with the mini-prep DNA isolation kit (Wizard, Promega, Wisconsin). Partial sequencing was on double stranded templates in the forward and reverse directions with fluorescent M13 universal primers and automated sequencers (Applied Biosystems 373A) (Adams et al., 1995). Nucleotide and protein sequence searches were conducted as described by using the algorithms BLAST (Altschul et al., 1990) and BLAZE (Brutlag et al., 1993), respectively.

Southern Hybridization

Restriction enzyme digested DNA was loaded (10 μg/lane) onto 0.8% TBE agarose gels and resolved by horizontal flat bed electrophoresis. Southern transfer onto nylon membranes (Nytran, Schleicher & Schuell) was performed using 10× SSC according to the standard method (Southern, 1975). DNA was immobilized by UV cross-linking and baking for 2 h at 80°C.

Hybridizations were performed overnight at 42°C in the presence of 50% formamide and 10% Dextran sulfate in 2× SSPE, 5× Denharts, 1% SDS and 100 μg/ml of sonicated salmon testes DNA. Probes were made from individual phagemid DNA by labeling with ^{32}P-dCTP (6000 Ci/mmole, Amersham) by the random priming method as described by Feinberg & Volgelstein (1983). Blots were washed at 55°C in 0.2× SSC and 0.1% SDS and set up for autoradiography at –70°C, for 2–5 days, with intensifying screens.

RESULTS & DISCUSSION

Sequence Analysis and Identification of *B. glabrata* ESTs

Of the 190 clones sequenced (95 from each library), 111 provided usable sequences. An average of 322 bases was obtained from either the 5′ or 3′ ends. The standard for elimination of unwanted and ambigous sequences (vector and poly A tail) was performed as described by Adams et al. (1995). Searches of peptide sequences were performed from all six possible reading frames. Table 1 shows the number of ESTs divided into categories which represent (1) sequences that show significant homology to database sequences, (2) sequences that show no significant matches, and (3) sequences of mitochondrial DNA. As indicated, significant matches were detected with 28% of the snail sequences, 54% showed no homology to existing database sequences, and 18% of the sequences corresponded to mitochondrial DNA. Because the RNA utilized to construct the cerebral ganglia library was not poly-A selected, we expected to identify large numbers of ribosomal RNA sequences, but none were detected among the clones we sequenced. On the other hand, the higher number of EST sequences corresponding to mitochondrial-related sequences in the cerebral ganglia library (14), compared to the whole adult library (5), may be the result of not using poly-A RNA as starting material in the construction of the former library.

The 31 EST sequences that showed significant matches with other genes in the databases are listed in Table 2. Several sequences identified corresponded to genes involved in protein/gene expression (ribosomal protein, translation elongation factor 1) or to genes involved in cell communication (acetylcholine receptor, ATP-dependent transporter). From the neural tissue library, we identified sequences with significant homologies (57%–

TABLE 1. Summary of *B. glabrata* ESTs

Libraries	Whole Snail	Cerebral Ganglia	Total
Database Match	15	16	31
Unknown	31	29	60
Mitochondrial	5	15	20
Total	51	60	111

TABLE 2. Database similarities of *B. glabrata* ESTs. ESTs with database matches are listed with their putative identification, the length, percent identity, and percent similarity of the match, and the accession number of the sequence matched. Match lengths are in nucleotides. In addition to those listed above, EST188741 had 83% nucleotide identity with GB:M69023, which is misidentified as a human globin gene. We counted this EST as unknown.

est#	putative ID	len	%id	%sim	acc#
Adult library					
EST188651	acetylcholine receptor	182	30.6	51.6	SP:P22770
EST188652	possible glycoprotein	233	34.2	51.9	GP:912490
EST188653	possible glycoprotein	233	34.2	51.9	GP:912490
EST188654	cystatin	155	36.5	57.7	PIR:S12913
EST188655	cystatin	239	30.9	48.2	PIR:A29632
EST188656	endo-1,3-beta-glucanase	296	45.5	58.6	GP:144808
EST188657	endo-1,3-beta-glucanase	242	47.6	59.8	GP:144808
EST188658	major secreted protein MPB70	119	42.5	60.0	PIR:A37195
EST188659	major secreted protein MPB70	119	45.0	62.5	PIR:A37195
EST188660	major secreted protein MPB70	119	45.0	62.5	PIR:A37195
EST188661	moesin	182	59.7	88.7	GP:623040
EST188662	ribosomal protein L13	119	50.0	65.0	SP:P26373
EST188663	ribosomal protein L17	344	93.0	95.6	PIR:JC1253
EST188664	ribosomal protein L17	242	91.4	95.1	PIR:JC1253
EST188665	ribosomal protein S20	218	93.2	95.9	GP:292443
Cerebral ganglia library					
EST188671	antigen HuD, neuronal	281	41.5	54.3	GP:179537
EST188672	ATP-dependent transporter	296	53.0	70.0	SP:P40024
EST188674	DNA topoisomerase II	104	42.9	60.0	SP:Q01320
EST188675	DNA topoisomerase II	104	42.9	60.0	SP:Q01320
EST188676	FMRFamide precursor	233	43.8	61.2	SP:P42565
EST188677	FMRFamide precursor	262	53.3	60.0	SP:P42565
EST188678	FMRFamide precursor	143	37.3	56.9	SP:P42565
EST188679	globin	389	30.8	49.2	SP:P02215
EST188680	heat shock protein 90	374	88.8	97.6	GP:256089
EST188681	heat shock protein 90	338	81.6	90.4	GP:256089
EST188682	proclotting enzyme precursor	242	35.7	54.8	SP:P21902
EST188683	ribosomal protein L41	74	92.0	92.0	GP:36136
EST188684	ribosomal protein S17	164	78.2	89.1	GP:337501
EST188685	ribosomal protein S17	101	82.4	82.4	GP:337501
EST188686	serine protease	500	29.2	47.0	GP:868212
EST188687	translation elongation factor 1, alpha	293	79.6	85.7	GP:214111

61% similarity) to the neuropeptide FMRFamide precursor. A number of sequences from both libraries were highly redundant. For example, the EST sequences for ribosomal proteins occurred frequently as did sequences for housekeeping genes, hsp90 and DNA topoisomerase. The high frequency of certain clones may either reflect the metabolic state of the tissue samples when RNA was isolated (abundant transcripts) or a bias created during the manipulation (amplification and mass excision) of the libraries. Using the partial EST sequence for globin, the corresponding full-length cDNA has been isolated and sequenced from the cerebral ganglia library (Dewilde et al., manuscript submitted). The 111 *B. glabrata* EST sequences discussed in this manuscript have been deposited in the dbEST database of the National Center for Biotechnology Information with the following accession number: dbEST:1193734 to 1193844; GenBank: AA547685 to AA547795.

Identification of Polymorphic ESTs

Hybridization patterns were compared between genomic DNA from parasite-resistant (BS-90) and -susceptible (M-line) snails, using the ESTs as probes. As shown in Table 3, RFLPs were detected with some ESTs. Most polymorphisms were observed with restriction enzymes *Hind* III and *Hinf* III. RFLP

TABLE 3. RFLPs identified between resistant (R) [BS-90] and susceptible (S) [M-line] *B. glabrata* snail lines with EST probes. The symbols show whether that an RFLP was detected (+) or not (–) for a particular enzyme.

		Bgl II		*Cla* I		*Dra* I		*EcoR* I		*EcoR* V		*Hae* III		*Hind* III		Hinf III		*Rsa* I	
PROBE	EST#	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
Acetylcholine receptor	EST188651	+	+	+	+	+	+	–	–	+	+	+	+	+	+	+	+	–	–
Ribosomal protein L13	EST188662	+	+	–	–	–	–	–	–	+	+	–	–	+	+	+	+	–	–
Cystatin	EST188654	+	+	–	–	+	+	+	+	–	–	–	–	+	+	+	+	–	–
Endo-1,3-beta-glucanase	EST188656	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–
Major secreted protein MPB70	ETS188658	+	+	–	–	–	–	–	–	+	+	+	+	+	+	+	+	–	–
Ribosomal protein	EST188663	+	+	+	+	+	+	–	–	+	+	+	+	+	+	+	+	+	+
FMRFamide neuropeptide precursor	EST188676	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–
ATP-dependent transporter	EST188672	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
Antigen HuD, neuronal	EST188671	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
Ribosomal protein S17	EST188683	+	+	–	–	+	+	+	+	+	+	+	+	+	+	–	–	+	+

analysis was conducted on either individual or pooled snail DNA samples. Linkage studies, using these EST RFLPs as probes, will be performed on progeny snail DNA derived from a cross between the resistant and susceptible snails as part of an ongoing study to identify sequences associated with either the resistant or susceptibility loci in *B. glabrata*.

Hybridization of parasite DNA using heterologous snail ESTs as probe, in some cases, demonstrated the occurrence of related sequences in the parasite genome. For example, Figure 1 shows the hybridization of the snail EST encoding acetylcholine receptor (EST188651) to *Hae* III digested DNA from the resistant snail (Lane 1), susceptible snail (Lane 2) and *S. mansoni* (Lane 3). As indicated, the snail probe detects a major fragment (590bp) in both the snail and parasite genomes. Considerable sequence homologies have previously been reported between parasite and snail genes (Dissous et al., 1990; Weston et al., 1994). Cross hybridization studies, using these snail probes, may be a useful strategy to identify and clone corresponding *S. mansoni* genes for which no sequence information currently exists. Conversely, ESTs that are currently being generated from the parasite (Franco et al., 1995; Neto et al., 1997), may serve as useful heterologous probes for genome studies of the intermediate host. Unlike the parasite, which shows an amino acid codon preference for A/T in the third base position (Meadows & Simpson, 1989), analysis of the partial amino acid sequences generated from the *B. glabrata* ESTs does not reflect a similar bias (data not shown). Full-length sequences will, however, be required in order to assess relative structural similarities and divergences between snail ESTs and related parasite sequences.

This study shows that the generation of *B. glabrata* ESTs is a useful approach that should quickly expand our knowledge on the molecular biology of this organism. Although most of the sequences identified in this study showed no homology to sequences listed in existing public data bases, indicating they represent novel snail-related sequences, the accumulation of such sequences will help in our collective efforts in this field towards the identification of genes involved in the genetic control of parasite infection in this snail host. The ability to identify RFLP EST sequences provides a useful method to limit the search of probes to known genes for which a biological function can be ascribed. These polymorphic

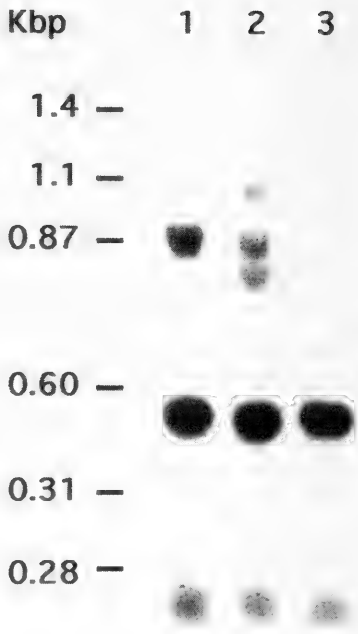

FIG. 1 Southern blot of *Hae* III digested DNA from resistant (1) and susceptible (2) *B. glabrata* snail lines and *S. mansoni* (3) hybridized with EST probe EST188651 (acetylcholine receptor).

EST markers can form the basis to begin to create a physical map of the *B. glabrata* genome.

The comparative analysis of the profile of transcripts obtained from the two libraries screened in this study suggests that for *B. glabrata,* the generation of ESTs from various tissues, rather than the whole snail, may be a more meaningful strategy to adopt in the identification of transcripts that may be relevant to a particular biological phenomenon. In this context, work being conducted in our laboratory is employing an EST strategy to compare the profile of transcripts, as described by Lee et al. (1995), between hemocytes of resistant *B. glabrata* snails, with and without parasite exposure.

In summary, ESTs have been generated by partial sequencing of clones from *B. glabrata* cDNA libraries. Most sequences showed no homology to sequences in existing data bases. Some snail ESTs may serve as useful probes to identify homologous genes in the parasite. RFLPs identified with ESTs provides useful markers to conduct genetic linkage

studies for the identification of the parasite resistance/susceptibility loci in this snail host.

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PHYLOGENETIC UTILITY OF THE 5′-HALF OF MITOCHONDRIAL 16S rDNA GENE SEQUENCES FOR INFERRING RELATIONSHIPS OF *ELIMIA* (CERITHIOIDEA: PLEUROCERIDAE)

Charles Lydeard[1], John H. Yoder[1], Wallace E. Holznagel[1], Fred G. Thompson[2] & Paul Hartfield[3]

ABSTRACT

Mitochondrial 16S rDNA sequences have proven useful for mesolevel phylogenetic questions. To date, most published studies have used primers that amplify the conservative 3′-half of the gene. We recently developed primers that amplify an approximately 550 bp portion of the more variable 5′-half of the gene. The primers work well for a wide range of gastropods tested. Because the 5′-half of the gene exhibits greater variation than the 3′-half, we wanted to determine whether there is sufficient phylogenetic signal for resolving relationships among closely related taxa. We examined the utility of the 5′ portion by assessing relationships within the pleurocerid genus *Elimia* of the Mobile River basin. Although the 433 bp data matrix possessed significant phylogenetic signal, and the resultant 320 most parsimonious trees had some aspects that were well resolved, most of the phylogenetic signal seemed to be partitioned among genera. Only 36 phylogenetically informative sites were found within *Elimia,* which is too few to resolve all nodes for such a diverse assemblage. We recommend the continued use of the 16S rDNA gene for studies examining intergeneric relationships of molluscs, and suggest researchers employ mitochondrial protein coding genes for interspecific studies.

Key words: mollusks, Cerithioidea, Pleuroceridae, *Elimia*, mitochondrial DNA, 16s rDNA.

INTRODUCTION

The introduction of conserved "universal" primers (Kocher et al., 1989), which permit amplification of specific regions of homologous DNA via the polymerase chain reaction (PCR) (Saiki et al., 1985), has offered tremendous opportunities for systematic studies. Many of the earlier molecular phylogenetic studies of molluscs have employed 18S or 28S nuclear ribosomal sequence data in an attempt to resolve relationships among distantly related taxa. For example, the relationships of molluscs to other metazoans (e.g., Ghiselin, 1988; Winnepenninckx et al., 1994), gastropod relationships (e.g., Emberton et al., 1990; Tillier et al., 1992; Rosenberg et al., 1994), and unionoidean bivalve relationships (e.g., Rosenberg et al., 1994). Although these genes appear useful for resolving some aspects of higher-level relationships among molluscs, they are too conservative to be of much use for studying relationships among more closely related genera or species.

Mitochondrial DNA has proven to be quite useful for studying evolutionary relationships of animals (Brown, 1985; Avise et al., 1987; Moritz et al., 1987). Sequencing the complete genome, although relatively labor intensive, has provided data for studying molluscan relationships based both on nucleotide homology and on mitochondrial gene order (Boore & Brown, 1994). In contrast, restriction fragment or site analysis of the mtDNA genome provides information for intraspecific population structure (e.g., Reeb & Avise, 1990; Liu et al., 1996). On the other hand, molecular phylogenetic studies employing mitochondrial DNA sequence data are only recently becoming more commonly conducted in malacology. The

[1]Aquatic Biology Program, Department of Biological Sciences, University of Alabama, Box 870345, Tuscaloosa, Alabama, 35487 USA
[2]Florida Museum of Natural History, Museum Road, University of Florida, Gainesville, Florida, 32611 USA
[3]U.S. Fish and Wildlife Service Endangered Species Office, Dogwood View Parkway, Suite A, Jackson, Mississippi, 39213 USA

delay is most likely due to the fact that many of the first universal primers reported in the literature do not work well if at all for molluscs (Spolsky et al., 1996). Thus, more effort is required on the part of the investigator to design primers that will work efficiently and yield sufficient variation for the question in hand. Folmer et al. (1994) developed primers that amplify a portion of the protein coding gene, cytochrome c oxidase subunit I from a wide range of invertebrate taxa, including molluscs. These primers have since been used to examine the evolution of gender-associated mitochondrial lineages in bivalves (Hoeh et al., 1996), and are now being used in a variety of other systematic studies of freshwater unionid mussels (Roe & Lydeard, 1998; Hoeh, pers. comm.; Liu & Mulvey, pers. comm.) and gastropods (Harasewych, pers. comm.). Primers for another protein coding gene, cytochrome b, have also been developed, which have proven useful for studying relationships within the gastropod genus *Nucella* (Collins et al., 1996) and for intraspecific relationships within the gastropod genus *Oncomelania* (Spolsky et al., 1996). Mitochondrial protein coding genes appear to be useful for a wide range of systematic questions. This can be attributed to rate variation among codon positions. For example, third codon positions are under fewer selective constraints and hence evolve faster than first and second codon positions. Thus, sufficient variation may be found to estimate relationships even among closely related taxa. In contrast, for deeper phylogenetic questions, substitutions in the third codon position can be downweighted or dropped (e.g., Lydeard & Roe, 1997).

Mitochondrial ribosomal genes have been of considerable use for systematics studies (Hillis & Dixon, 1991). The genes are thought to be more conservative than protein coding genes and are therefore more useful for meso-level phylogenetic questions. Recently, the mitochondrial 16S rDNA gene has been used successfully for estimating relationships among genera of North American freshwater unionids (Lydeard et al., 1996). The primers used by Lydeard et al. (1996) were designed by modifying the universal primers of Palumbi et al. (1991), which amplify a 550 bp fragment of the 3′ half of the gene. In another study, Lydeard et al. (1997) developed primers that extended into the 5′ half of the gene, which is rarely sequenced in phylogenetic studies. The combined data matrix of nearly 900 bp was used to study relationships among three genera of pleurocerid gastropods of the Mobile River basin. Interestingly, the 5′ half of the gene exhibits more variation than the 3′ half (Gutell et al., 1992; Lydeard et al., unpubl.), which lead us to believe the gene might be useful for assessing relationships among more closely related taxa. Here, we present our analysis of the utility of the 5′ half of the 16S rDNA gene for examining relationships within the gastropod genus *Elimia*. The primers used to amplify this region work well on a wide range of gastropod species, so our findings will be of interest to other malacologists interested in examining systematic relationships among similarly divergent taxa.

MATERIALS AND METHODS

Specimens Studied

Elimia were collected from various localities throughout the Mobile River basin, but with a particular emphasis on Coosa River species (Appendix). Twenty-three *Elimia* specimens representing eleven nominal species were included in the analysis, along with three *Pleurocera prasinatum* and one *Leptoxis taeniata* as outgroup taxa (Table 1).

Sequence Procurement, Alignment, and Analysis

Genomic DNA was isolated from frozen or 80% ethanol-preserved specimens (typically the proboscis or entire head of the snail; less tissue seems to yield better quality DNA) by standard phenol-chloroform extractions. Mitochondrial DNA sequences were obtained for an amplified segment of the mitochondrial 16S rDNA gene using primers SNL002 (5′-aaatgattatgctacctttgc-3′ and SNL-448 (5′-gaaatttcattcgcactag-3′). These primers were designed by Lydeard et al. (1997) specifically for pleurocerids and related gastropods, and amplify an approximately 550 bp fragment at the 5′ end.

Approximately 50 - 500 ng of genomic DNA provided template for double-stranded amplifications via PCR in 25 ul of Tris (67 mM, pH 8.8) containing 2 mM MgCl2, 1 mM of each dNTP, 1 uM of each primer, and *Taq* polymerase (1.25 units, Perkin-Elmer-Cetus). The amplification regime consisted of 30 cycles of denaturation at 92°C for 40 s, annealing at 52°C for 60 s, and extension at 72°C for 90 s. Single-stranded DNA was produced for se-

TABLE 1. Taxa and number of specimens included in the present study.

Pleuroceridae
Elimia catenaria group
E. crenatella (2)
Elimia carinocostata group
E. carinocostata (9)
Elimia gerhardtii group
E. gerhardtii (3)
Elimia haysiana group
E. alabamensis (1)
E. haysiana (1)
Elimia hydei group
E. hydei (1)
Elimia olivula group
E. cylindracea (1)
E. olivula (1)
E. showalteri (1)
Elimia vanuxemiana group
E. fascinans (2)
E. caelatura infuscata (2)
Pleurocera prasinatum (3)
Leptoxis taeniata (2)

quencing via asymmetric amplification (Gyllensten & Erlich, 1988) using low-melt agarose (FMC BioProducts) purified double-stranded PCR products as templates. Reaction conditions for asymmetric PCR were as above with the exceptions that one primer was held limiting, and the final volume of the reaction cocktail was increased to 50 uL. Asymmetric reactions were conducted for each specimen using both amplification primers in limited quantity in separate reactions. Thermal cycling was performed in a programmable heating block (Perkin-Elmer-Cetus) with negative (-DNA) controls included with each reaction set.

Following purification by centrifugal filtration (Millipore Ultra-free-MC 30,000), single-stranded DNA was sequenced by dideoxy chain termination using Sequenase Version 2.0 (United States Biochemical) and instructions supplied by the manufacturer. Both strands were sequenced using the appropriate amplification primer as a sequencing primer. The sequencing reaction products, which included ^{35}S to permit autoradiographic visualization, were run on 6% polyacrylamide gels (Long Ranger, FMC BioProducts) from 2 to 4.5 h. Following electrophoresis, all gels were vacuum-dried and exposed to X-ray film for 48 - 120 h.

Sequences were initially entered in the software program XESEE version 3.0 (Cabot & Beckenbach, 1989) and subsequently aligned using CLUSTALW version 1.5 (Thompson et al., 1994) and visual inspection. In addition, a consensus sequence of the ingroup taxa was superimposed over the hypothesized secondary structure of the fruitflies *Drosophila yakuba* and *D. melanogaster* (Gutell & Fox, 1988; Gutell et al., 1992) in an attempt to further refine the alignment and identify regions corresponding to hypothesized loops and stems. Pairwise percent sequence differences were calculated using Kimura's two-parameter model (Kimura, 1980), which corrects for multiple hits using the software package MEGA (Kumar et al., 1993).

The phylogenetic analyses were conducted using maximum parsimony of the orthologous sequences using the heuristic search option (10 replicates) of PAUP (version 3.1.1; Swofford, 1993). We employed the following options in PAUP: uninformative characters were ignored, only minimal trees were kept, gaps were treated as missing data, and zero-length branches were collapsed. A bootstrap analysis (Felsenstein, 1985) with 100 iterations was conducted to estimate the internal stability of the data matrix. Although we usually prefer to run more iterations, this particular data set tested the limits of memory for more than 100 replicates. A skewness test statistic (g1) was calculated based on the distribution of tree lengths of a random sample of 10,000 topologies. Data matrices with a strong phylogenetic signal are significantly more structured than random data (Hillis & Huelsenbeck, 1992).

RESULTS

Multiple sequence alignment of the amplified region from our selected taxa resulted in a data matrix consisting of 433 nucleotide positions (Fig. 1) including insertions and deletions. Several DNA sequences from conspecific specimens were identical, and therefore combined in subsequent analyses: *Elimia crenatella* (cren18 = 41A-2), *E. carinocostata* (5A-2 = 11A-1), *E. caelatura infuscata* (16-1 = 16-2), and *Leptoxis taeniata.* (2 individuals, 1 locality). Thus, 25 unique sequences from 28 individuals. Of the 433 nucleotide positions examined, 135 (31%) are variable among all taxa, including the outgroups, and 71 (16%) are potentially phylogenetically informative. Within the genus *Elimia,* 75 (17%) are variable and 36 (8%) are potentially phylogenetically informative.

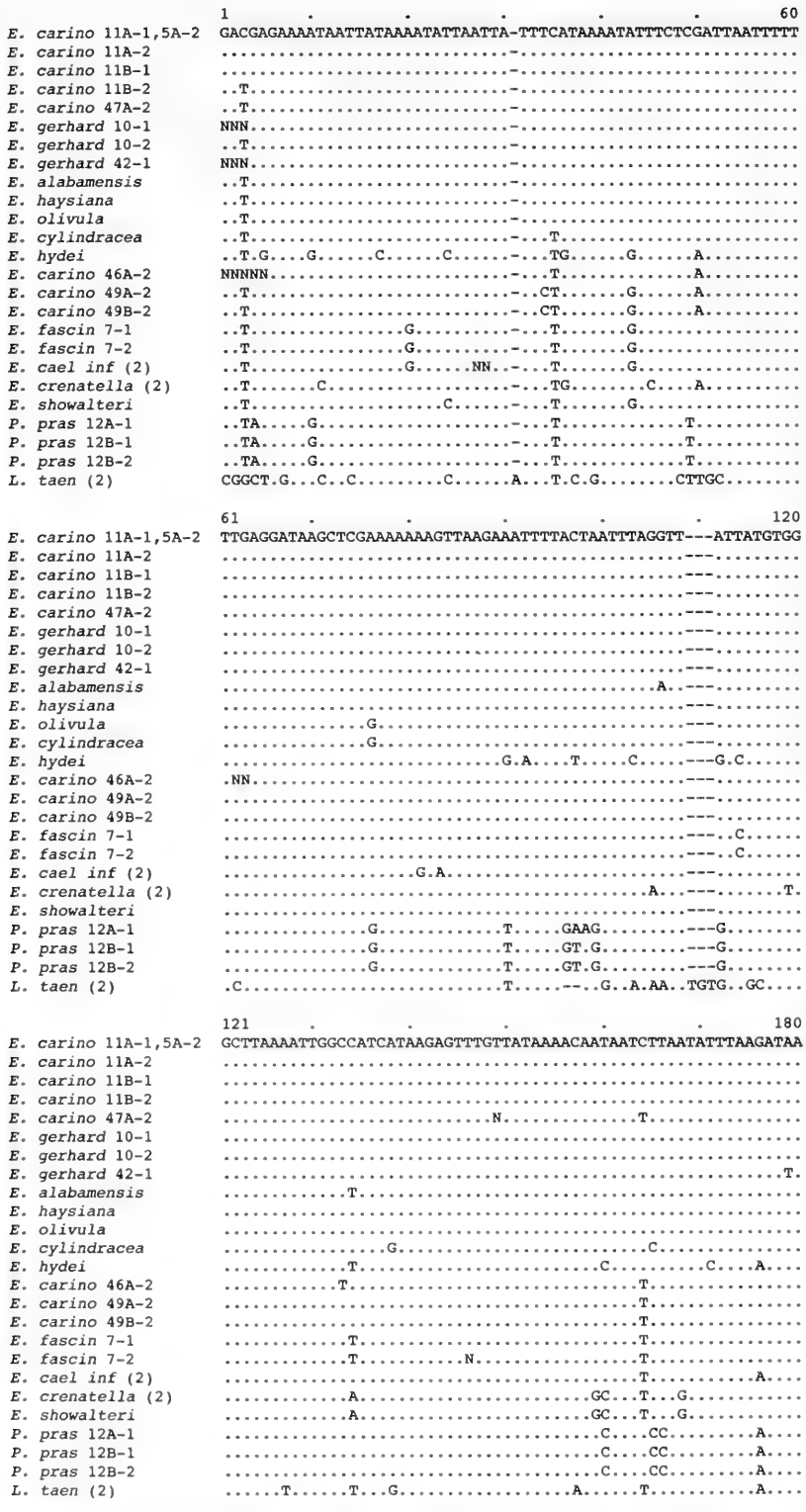

FIG. 1. An aligned data matrix of 433 nucleotide positions of mitochondrial 16S rDNA sequences for 25 pleurocerid specimens. Dashes correspond to gaps and N's are missing data. E. carino = *Elimia carinocostata;* E. gerhard = *E. gerhardtii;* E. fascin = *E. fascinans;* E. cael inf = *Elimia caelatura infuscata;* P. pras = *Pleurocera prasinatum;* L. taen = *Leptoxis taeniata.* Numbers following the name of the species is the specimen number. Locality information of the specimens can be found in the Appendix.

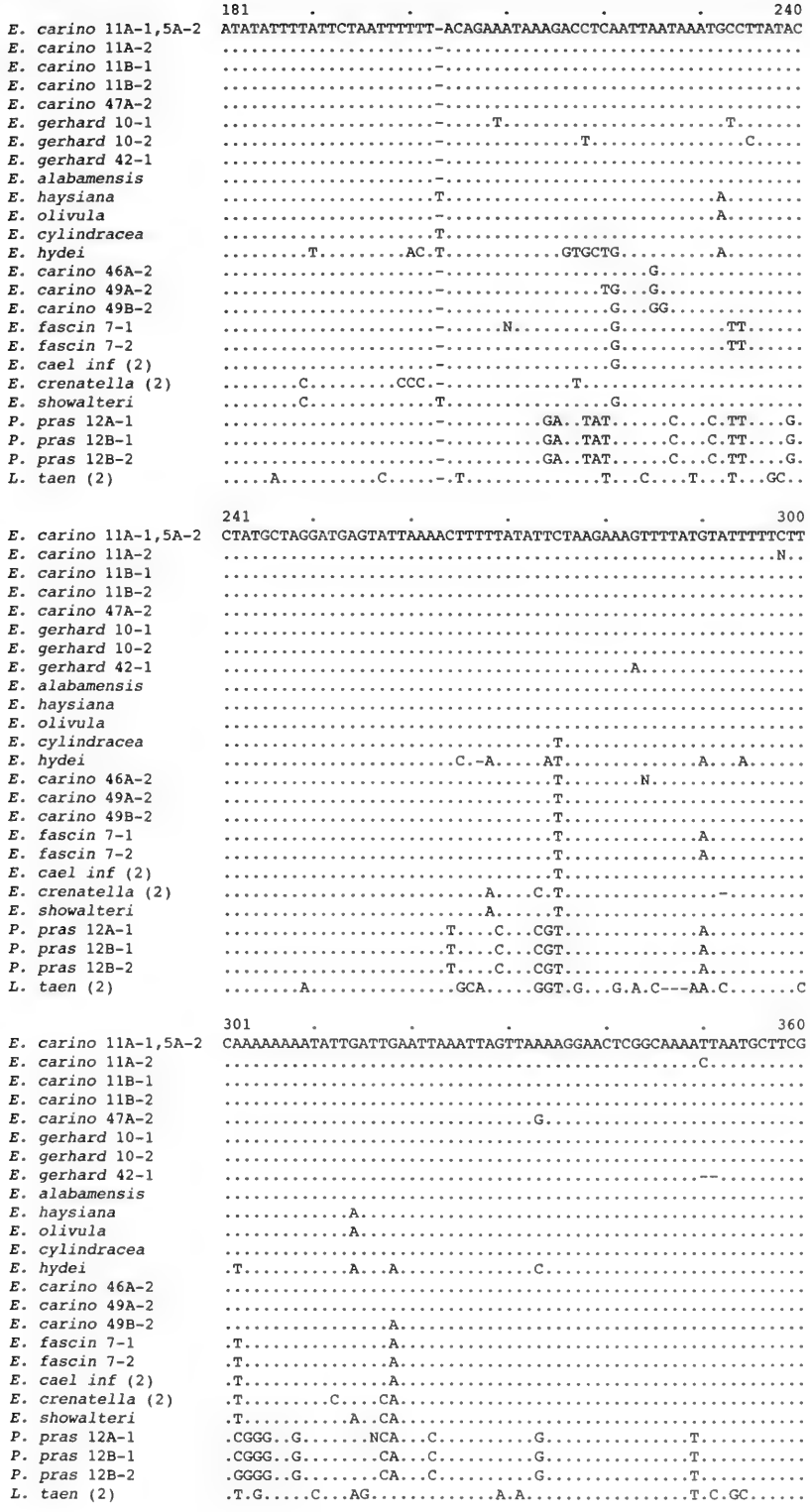

FIG. 1. (*Continued*)

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                          361       .         .         .         .         .       420
E. carino 11A-1,5A-2      CCTGTTT-ATCAAAAACATGGCTCTCTGAATTCATTTTTTATAGAGAGTCAGGCCTGCCCA
E. carino 11A-2           .......-.....................................................
E. carino 11B-1           .......-.....................................C...............
E. carino 11B-2           .......-.....................................C...............
E. carino 47A-2           .......-.....................................................
E. gerhard 10-1           .......-.....................................G...............
E. gerhard 10-2           .......-.....................................................
E. gerhard 42-1           .......-.....................................G...............
E. alabamensis            .......-.....................................................
E. haysiana               .......-.....................................................
E. olivula                .......-.....................................................
E. cylindracea            .......-.....................................................
E. hydei                  .......T.....................T...............................
E. carino 46A-2           .......-........NN.......T......T.....-.......................
E. carino 49A-2           .......-..................T......T.....-.......................
E. carino 49B-2           .......-..................T......T.....-...AG.................
E. fascin 7-1             .......-..................T...............................
E. fascin 7-2             .......-..................T..........G....................
E. cael inf (2)           .......-..................T...............................
E. crenatella (2)         .......-.....................G...A.....-....G.................
E. showalteri             .......-.....................A.....-....G.................
P. pras 12A-1             .......-..................T..C...T.....-...A.................
P. pras 12B-1             .......-..................T..C...T.....-...A.................
P. pras 12B-2             .......-..................T..C...T.....-...A.................
L. taen (2)               .......-..................T......-.........................

                          421      .433
E. carino 11A-1,5A-2      GTGAATAATATTT
E. carino 11A-2           .............
E. carino 11B-1           .............
E. carino 11B-2           .............
E. carino 47A-2           .............
E. gerhard 10-1           .........NNNN
E. gerhard 10-2           .............
E. gerhard 42-1           .............
E. alabamensis            .............
E. haysiana               .............
E. olivula                .............
E. cylindracea            .............
E. hydei                  .............
E. carino 46A-2           ..........NNN
E. carino 49A-2           .........NNNN
E. carino 49B-2           ..........NN
E. fascin 7-1             .............
E. fascin 7-2             .............
E. cael inf (2)           .............
E. crenatella (2)         .............
E. showalteri             .............
P. pras 12A-1             ...........NN
P. pras 12B-1             .............
P. pras 12B-2             .............
L. taen (2)               .....ATT.NNNN
```

FIG. 1. (*Continued*)

Pairwise percent sequence differences corrected for multiple hits by the two-parameter method of Kimura (1980) were from 0% to 3.93% within *Elimia* species, and 0.3% to 11.08% among *Elimia* species. Intergeneric values ranged from 10.64 to 15.33% for comparisons of *Elimia* and *Pleurocera* and 17.75% to 23.29% for comparisons of *Elimia* and *Leptoxis*. All pairwise genetic distances are shown in Table 2.

Maximum parsimony analysis was conducted treating all base substitutions as equally weighted based on a previous analysis of nucleotide substitutions within pleurocerids (Lydeard et al., 1997). Maximum parsimony analysis resulted in 320 equally most parsimonious trees (tree length = 207; consistency index = 0.619, excluding uninformative characters). The g1 statistical analyses showed the data were significantly skewed ($p = 0.01$), suggesting the data contain significant phylogenetic signal. Phylogenetic signal was stronger when outgroup taxa were included in the g1 statistical analysis ($g1 = -1.84$) than when they were excluded ($g1 = -0.583$). A strict consensus tree of the 320 equally most parsimonious trees and a phylogram of one of the 320 equally most parsimo-

TABLE 2. Estimated percentage nucleotide sequence difference (Kimura's 2-parameter) among pairwise comparisons of taxa based on mitochondrial 16S rDNA sequences

	cren	car11A1	car11A2	car11B1	car11B2	car46A2	car47A2	car49A2	car49B2
E. cren	—	6.99	7.27	6.99	6.72	6.67	6.74	7.33	7.02
E. car11A1		—	0.24	0.23	0.47	1.98	0.71	2.93	3.67
E. car11A2			—	0.47	0.71	2.23	0.95	3.19	3.93
E. car11B1				—	0.23	1.98	0.95	2.93	3.67
E. car11B2					—	1.98	0.71	2.68	3.42
E. car46A2						—	1.98	1.23	1.98
E. car47A2							—	2.68	3.42
E. car49A2								—	1.20
E. car49B2									—
E. ger10-1									
E. ger10-2									
E. ger42-1									
E. ala									
E. hay									
E. hydei									
E. cyl									
E. oli									
E. sho									
E. fas7-1									
E. fas7-2									
E. cael									
P. pra12A-1									
P. pra12B-1									
P. pra12B-2									

	ger10-1	ger10-2	ger42-1	ala	hay	hyd	cyl	oli	sho	fas7-1	fas7-2	cael
E.cren	7.10	7.26	7.06	6.99	7.26	11.08	6.99	7.52	4.16	7.29	7.02	7.04
E. car11A1	0.72	0.71	0.71	0.71	0.71	10.20	1.43	0.95	4.63	3.64	3.90	3.15
E. car11A2	0.96	0.95	0.72	0.95	0.95	10.50	1.67	1.19	4.90	3.90	4.16	3.41
E. car11B1	0.96	0.95	0.96	0.95	0.95	10.48	1.67	1.18	4.63	3.90	4.15	3.40
E. car11B2	0.96	0.71	0.96	0.71	0.71	10.20	1.43	0.95	4.38	3.64	3.90	3.15
E. car46A2	2.74	2.48	2.74	2.48	2.48	9.99	2.23	2.74	4.78	3.51	3.77	3.00
E. car47A2	1.21	0.95	1.20	0.95	0.95	10.22	1.67	1.19	4.39	3.40	3.65	2.91
E. car49A2	3.45	3.18	3.47	3.18	3.18	9.20	2.93	3.43	4.42	3.18	3.43	2.68
E. car49B2	3.71	3.92	3.71	3.92	3.92	9.99	3.67	4.18	4.15	3.42	3.16	2.92
E. ger10-1	—	1.20	0.96	1.20	1.20	10.94	1.94	1.45	4.71	3.70	3.44	3.71
E. ger10-2		—	1.20	0.95	0.95	10.20	1.67	1.18	4.89	3.90	4.15	3.40
E. ger42-1			—	1.20	1.20	10.88	1.93	1.44	4.68	4.19	3.93	3.69
E. ala				—	0.95	9.91	1.67	1.18	4.63	3.39	3.64	3.40
E. hay					—	9.33	1.66	0.23	4.37	3.90	4.15	3.40
E. hydei						—	10.17	9.64	8.81	8.30	8.57	8.87
E. cyl							—	1.43	4.62	3.64	3.90	3.15
E. oli								—	4.63	4.15	4.40	3.65
E. sho									—	3.89	3.64	3.65
E. fas7-1										—	0.24	1.92
E. fas7-2											—	2.17
E. cael												—

	P. pra12A-1	P. pra12B-1	P. pra12B-2	L. tae
E. cren	15.33	14.96	14.93	23.29
E. car11A1	12.03	11.70	11.72	19.72
E. car11A2	12.36	12.02	12.05	20.02
E. car11B1	12.03	11.70	11.72	20.06
E. car11B2	11.74	11.40	11.43	20.06
E. car46A2	11.78	11.50	11.53	17.75
E. car47A2	11.77	11.43	11.46	19.78
E. car49A2	12.10	11.82	11.85	19.44
E. car49B2	12.33	12.05	12.08	21.14
E. ger10-1	12.17	11.89	11.91	19.25
E. ger10-2	11.74	11.40	11.43	20.40
E. ger42-1	12.77	12.42	12.45	19.02
E. ala	12.33	11.99	12.02	19.06
E. hay	12.33	11.99	12.02	19.72
E. hydei	15.27	14.59	14.66	23.09
E. cyl	10.87	10.64	10.66	19.39
E. oli	12.03	11.70	11.72	20.06
E. sho	13.47	13.12	13.09	21.11
E. fas7-1	11.50	11.17	11.14	19.47
E. fas7-2	11.79	11.46	11.43	19.81
E. cael	12.12	11.78	11.76	20.21
P. pra12A-1	—	0.47	0.71	24.48
P. pra12B-1		—	0.24	24.09
P. pra12B-1			—	24.04
L. tae				—

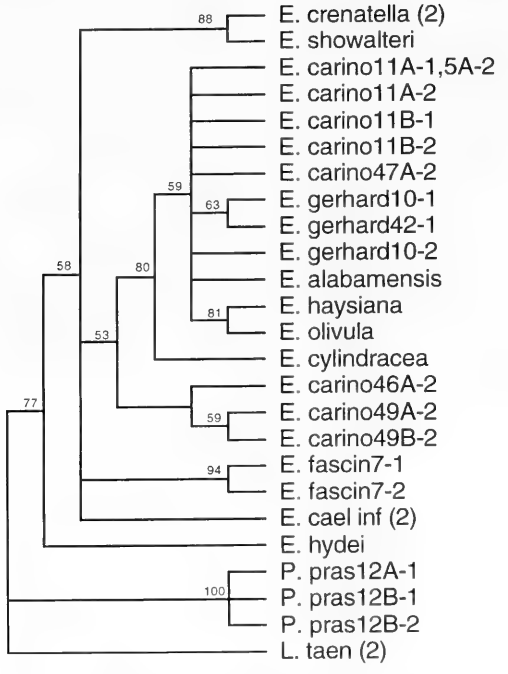

FIG. 2. A strict consensus tree of 320 most parsimonious trees obtained in the maximum parsimony analysis of the mtDNA sequence data using equal weighting for all substitutions. Bootstrap values are noted to the left of the corresponding node. Tree length = 207; consistency index = 0.619, excluding uninformative characters.

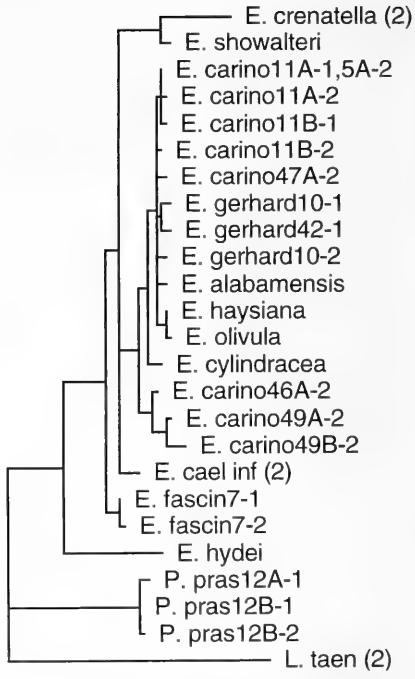

FIG. 3. A phylogram representing one of the 320 most parsimonious trees obtained in the maximum parsimonious analysis of the mtDNA sequence data using equal weighting for all substitutions. Tree length = 207; consistency index = 0.619, excluding uninformative characters. Branch lengths reflect total number of substitutions.

nious trees is shown in Figures 2 and 3, respectively. Bootstrap values are reported for each node of the strict consensus tree. Nodes without numbers have bootstrap scores of 50% or less.

DISCUSSION

The molecular phylogeny obtained here shows strong support for the monophyly of *Elimia* of the Mobile River basin. The basalmost species is *Elimia hydei,* which is sister to a large unresolved clade of remaining *Elimia* species. The unresolved polytomy is comprised of four clades: (1) *E. caelatura infuscata;* (2) *E. fascinans;* (3) *E. crenatella* + *E. showalteri;* and (4) *E. carinocostata* + *E. gerhardtii* + *E. alabamensis* + *E. haysiana* + *E. olivula* + *E. cylindracea.* There appears to be little congruence between the molecular phylogeny and the current classification scheme of pleurocerids (Burch, 1980). For example, the *E. olivula* group (*E. cylindracea, E. olivula,* and *E. showalteri*) and the *E. haysiana* group (*E. alabamensis* and *E. haysiana*) are paraphyletic. However, some of the most parsimonious trees depict *E. fascinans* + *E. caelatura infuscata* of the *E. vanuxemiana* group as sister taxa. Of course, it is worth noting that the classification scheme of pleurocerids is pre-Hennigian, and many of the groups are no doubt recognized by shared plesiomorphic characters. Within the largest of the four aforementioned clades, *E. carinocostata* (specimens 46A-2, 49A-2, and 49B-2) is depicted as being sister to a clade that contains *E. cylindracea* + *E. olivula* + *E. haysiana* + *E. alabamensis* + *E. gerhardtii* + *E. carinocostata* (specimens 5A-2, 11A-1, 11A-2, 11B-1, 11B-2 and 47A-1), rendering *E. carinocostata* paraphyletic. *Elimia cylindracea* is the next most-basal member of the largest of the four clades, and it is sister to the remaining *Elimia* species. The only resolved relation-

ships within this large clade are *E. haysiana* + *E. olivula* and *E. gerhardtii* (10-1 + 42-1). The relationship of the remaining *E. gerhardtii* (10-2) remains uncertain. The phylogeny obtained in the present study is consistent with the findings presented by Lydeard et al. (1997).

Of the five *Elimia* species that had more than one specimen sequenced, *E. crenatella* (identical sequences), *E. caelatura infuscata* (identical sequences), and *E. fascinans* are depicted as monophyletic. However, the monophyly of the aforementioned species should be tested with additional specimens and sequence data. Three *Elimia gerhardtii* specimens were sequenced from two separate locales. Interestingly, *E. gerhardtii* specimens from different locales are sister taxa, but this clade does not include the third *E. gerhardtii* specimen. Eight *E. carinocostata* specimens were sequenced. The three most-basal *E. carinocostata* specimens (46A-2, 49A-2, 49B-2) were collected in headwater streams of the Coosa River, whereas the remaining *E. carinocostata* specimens were obtained in sites located further downstream.

Genetic differentiation among *Elimia* species was generally low. This was underscored particularly in the *E. carinocostata* (in part) + *E. gerhardtii* + *E. alabamensis* + *E. haysiana* + *E. olivula* + *E. cylindracea* clade. Most species of this large clade differed by no more than 1.8%. Confounding this problem, is the presence of intraspecific variation that is equal to or exceeds the amount of variation present among several of the species. The low genetic variation detected among the aforementioned species can be interpreted in two ways. First, they represent a single evolutionary entity, and therefore should be synonymized or second, they represent valid species, but the gene is simply too conservative to detect any significant differences. We recommend a more detailed investigation of the genus using other more potentially useful genetic markers before any formal taxonomic decisions be made.

Elimia is the second most diverse genus of freshwater gastropods in North America. Burch (1988) lists 83 species within the genus, but this number is likely to change following more detailed studies. Support for this claim comes from Hershler's (1994, 1998) review of the hydrobiid genus *Pyrgulopsis,* which is now considered the most diverse genus of North American gastropods. Prior to Hershler's reviews, *Elimia* would have ranked first, and the hydrobiid genus *Somatogyrus* would have ranked second, with 35 species (Burch, 1988). This dramatic increase in the number of hydrobiid species underscores the need for detailed monographic studies of all freshwater gastropods (Hershler, 1996).

The 5′-half of the mitochondrial 16S rDNA gene seems to be of limited utility for assessing relationships among closely related *Elimia* species. Although it is evident that there is significant variation among more distantly related *Elimia,* there were only 36 phylogenetically informative sites, which is not very many when examining relationships among 20+ taxa. Despite exhibiting more variation than the 3′-half of the gene, most phylogenetic signal seems to be partitioned among genera. Although we recognize that evolutionary rate differences exist among taxa, and that pilot studies should be carried out before undertaking any major sequencing project, the 5′-half of the ribosomal gene is likely to be of use to investigators interested in resolving relationships among molluscan genera.

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APPENDIX

Elimia alabamensis –
Coosa River just below Mitchell Dam, downstream of fishing platform (east bank), Coosa Co., Alabama.

Elimia caelatura infuscata – Kahatchee Creek, on Co. Rd. 008 (Childersburg Parkway), Talladega Co., Alabama.

Elimia carinocostata –
(5A-2) Camp Branch Creek, 3 miles W of Shelby on Co. Rd. 42, Shelby Co., Alabama.

(11A-1, 11A-2, 11B-1, 11B-2) Waxahatchee Creek, 2.7 miles W of Shelby, Shelby Co., Alabama.

(46A-2) Beaver Creek, Co. Rd. 26 bridge, St. Clair Co., Alabama.

(47A-2) Shoal Creek, at Co. Rd. 21 bridge, 4.7 miles NW of Ragland, St. Clair Co., Alabama.

(49A-2, 49B-2) Little Canoe Creek, Etowah Co. line on St. Clair Co. Rd. 44, St. Clair Co., Alabama.

Elimia crenatella –
(cre 18) Cheaha Creek at Co. Hwy 005, 5.1 mile SSW of Eastaboga, Talladega Co., Alabama.

(41A2) Yellow Leaf Creek, 2 mile S of Westover on Co. rd. 51, Shelby Co., Alabama

Elimia cylindracea –
Noxubee River, 6 river miles above Alabama state line, Noxubee Co., Mississippi.

Elimia fascinans –
Shoal Creek, Pine Glen Recreation Area, Cleburne Co., Alabama.

Elimia gerhardtii –
(10-1, 10-2) Weogufka Creek, 0.8 miles NW of Moriah on Co., Rd. 15, Coosa Co., Alabama.

(42-1) Cheaha Creek, Co. Hwy 005, Talladega Co., Alabama.

Elimia haysiana –
Coosa River, main channel about 2.6 miles downstream of Jordan Dam, 4.0 miles NW of Wetumpka, Elmore Co., Alabama.

Elimia hydei –
Locust Fork of Black Warrior River at Warrior, 0.3 miles E of U.S. Hwy 31 on unnumbered Co. rd., Jefferson Co., Alabama.

Elimia olivula –
Alabama River ca. 1.5 miles downstream of US Hwy 84, ca. 300 m upstream from grain elevator, Monroe Co., Alabama.

Elimia showalteri –
Cahaba River at Booth's Ford, 4.7 mile NW of Pea Ridge, Shelby Co., Alabama.

Pleurocera prasinatum –
Coosa River, public boat ramp, just S of hwy 22 intersection with Coosa River S of Mitchell Dam, Chilton Co., Alabama.

Leptoxis taeniata –
Buxahatchee Creek, 5 miles ESE of Calera, Hiawatha Rd. off Co. Rd. 86, Shelby Co., Alabama.

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MOLECULAR SYSTEMATICS OF THE FRESHWATER MUSSEL GENUS POTAMILUS (BIVALVIA: UNIONIDAE)

Kevin J. Roe & Charles Lydeard

Aquatic Biology Program, Department of Biological Sciences University of Alabama, Box 870345, Tuscaloosa, AL 35487-0345, USA

ABSTRACT

Few explicit hypotheses for the relationships of unionid mussels exist. The absence of explicit phylogenetic hypotheses is problematic and is in part responsible for the lack of taxonomic stability seen in this group. In this paper we examine the relationships of mussels in the genus *Potamilus,* based upon the DNA sequences of a 600 base pair portion of the first subunit of the mitochondrial cytochrome *c* oxidase (COI) gene. We also examine the genetic distinctiveness of populations of the inflated heelsplitter *P. inflatus.* The molecular phylogeny indicates that *Potamilus* is paraphyletic with *Leptodea fragilis* and *Lampsilis ornata* nested between *P. capax* and the remaining *Potamilus* species. With the exception of *P. capax,* the remaining *Potamilus* species are depicted as monophyletic and form three distinct clades: (1) a reciprocally monophyletic *P. inflatus* clade; (2) a *P. ohiensis/P. amphichaenus* clade; and (3) a *P. purpuratus/P. p. coloradoensis/P. alatus* clade. While bootstrap values indicate a high degree of support for these three clades, relationships among these three clades are not as strongly supported.

The genetic distinctiveness of two populations of the inflated heelsplitter exceeds that seen between some other species in the genus. These populations represent geographically isolated, genetically distinct entities, and we therefore recommend the recognition of both the Amite and the Black Warrior populations of *P. inflatus* as separate species.

Key words: Unionidae, *Potamilus*, cytochrome *c* oxidase subunit I.

INTRODUCTION

The freshwater mussel genus *Potamilus* Rafinesque, 1818 (Bivalvia: Unionidae), currently contains six species: *P. alatus* (Say, 1817), *P. amphichaenus* (Frierson, 1898), *P. capax* (Green, 1832), *P. inflatus* (I. Lea, 1831), *P. ohiensis* (Rafinesque, 1820), and *P. purpuratus* (Lamarck, 1819) (Turgeon et al., 1988; Williams et al., 1992). In addition to these taxa, Simpson (1914) included *P.* (*Lampsilis*) *coloradoensis* (I. Lea, 1856), which is now generally considered a western form of *P. purpuratus. Potamilus* is distributed in the St. Lawrence and Mississippi drainages and in Gulf drainages from Alabama to Texas (Valentine & Stansbery, 1971; Burch, 1975; Clarke, 1981). The type species for the genus was designated as *Unio alatus* Say, 1817, by Morrison (1969).

The genus *Potamilus* in its current form was first recognized as a natural assemblage of species by Frierson (1927) in the synonymous genus *Proptera* Rafinesque, 1819. Several researchers have proposed classifications that render the genus paraphyletic (Simpson, 1914; Hoggarth, 1988; Burch, 1975) and have placed mussels currently assigned to *Potamilus* in the genus *Lampsilis* (*P. capax*) (Simpson, 1914), the genus *Leptodea* (*P. laevissima* [= *ohiensis*], *P. amphichaenus*) (Burch, 1975) or the resurrected genus *Lastena* (Hoggarth, 1988). Whereas *Potamilus* is generally perceived as a natural group by freshwater malacologists, it has not yet achieved taxonomic and nomenclatural stability, as evidenced by the continual change in generic assignments over the last 170 years. Even after successful petitioning by Bogan et al. (1990) of the International Commission on Zoological Nomenclature for the retention of *Potamilus* (BZN, 1992), *Proptera,* a junior synonym of *Potamilus,* appears in publications as late as 1993 (e.g., McMahon, 1993). While many descriptions of the genus include the presence of a posterior wing as diagnostic, this character alone does not discriminate members of *Potamilus* from their putative sister genus *Leptodea* (Ortmann, 1912; Valentine & Stansbery, 1971). Ortmann's (1912) statement that "this genus (*Potamilus*) stands in all characters except the glochidia, by that

of *Paraptera* [= *Leptodea*]," supports the similarity of these two genera. Valentine & Stansbery (1971) stated that the only unique feature that defines *Potamilus* is the possession of axe-head shaped or ligulate glochidium (Fig. 1), and Utterback (1915) noted that with the exception of the unique glochidia and the more developed hinge, "this genus (*Potamilus*) stands with *Lasmonos* [= *Leptodea*]." A phenetic analysis by Hoggarth (1988) of the utility of glochidia morphology for deducing the relationships among North American freshwater mussels indicated that *Potamilus* is not a monophyletic group and that *P. ohiensis* and *P. amphichaenus* are more closely related to mussels in the genus *Leptodea* than to other members of *Potamilus.* Hoggarth's analysis indicated two distinct groups of mussels within *Potamilus:* those with lateral hooks on the ventral valve edges (*alatus, capax, purpuratus*) and those without such hooks (*ohiensis, amphichaenus, inflatus*). He concluded that the glochidia bore only a superficial resemblance to each other, and implied that the axe-head shaped glochidia were not homologs.

The historic lack of taxonomic stability of *Potamilus* reflects the fact that no detailed or comprehensive cladistically based study has been conducted on this genus. Despite increasing interest in freshwater mussels, only a few cladistically based analyses have been published to date (Hoeh, 1990; Hoeh et al., 1996; Lydeard et al., 1996; Mulvey et al., 1997). The primary objective of this study is to test the monophyly of *Potamilus* using a molecular data set composed of the DNA sequences of a portion of the first subunit of the mitochondrial cytochrome *c* oxidase (COI) gene, and develop hypotheses for relationships within the genus.

Additionally, we wish to examine the level of intraspecific genetic variation in the inflated heelsplitter, *P. inflatus. Potamilus inflatus* was known from the Amite and Tangipahoa rivers in Louisiana, the Pearl River in Mississippi, and the Black Warrior, Coosa, and Tombigbee rivers in Alabama. Presently, it is limited to the lower and middle reaches of the Amite River, and a portion of the Black Warrior River. In 1990, the U.S. Fish and Wildlife Service listed *P. inflatus* as a threatened species, because of its diminished range and potential threats to its continued survival in those rivers where it still occurs (USFWS, 1992). Knowledge of how genetic variation is partitioned in *P. inflatus* will aid in making management decisions concerning this species.

MATERIALS AND METHODS

Twenty-four specimens representing ten species and five genera were included in the analysis (Table 1). Genomic DNA was isolated from fresh frozen or ethanol preserved tissues using the QIAamp Tissue Kit (QIAGEN #29304) following manufacturers instructions. Care was taken to use only somatic tissues as unionid mussels exhibit bi-parental inheritance of mitochondria (Hoeh et al., 1996; Liu et al., 1996b). Double-stranded and single-stranded DNA was generated via the polymerase chain reaction (PCR) using the primers LCO1490 and HCO2198 (Folmer et al., 1994). Approximately 100 ng of genomic DNA provided the template for double stranded reactions performed in a 25 µl solution containing each dNTP at 0.1 mM, each primer at 1.0 µM, 40 mM $MgCl_2$, 2.5µl 10X Taq buffer, and 0.6 units of AmpliTaq polymerase. Reactions were amplified for 32 cycles at 94° for 40 sec, 55° for 60 sec, and 72° for 90 sec. The amplified DNA was gel purified and then used as template for single-stranded amplification (Gyllensten & Erlich, 1988) using the same conditions and primer pair, with the H-primer used in limited quantity. Single stranded DNA was concentrated on Millipore Ultrafree MC filters, and sequenced using the Sequenase version 2.0 kit (U.S. Biochemical) and ^{35}S-labeled dATP following the manufacturers instructions. The heavy strand was sequenced using overlapping primers: HCO2198 (5′-taaacttcagggtgaccaaaaaatca-3′), UNICOIH (5′-tcagcaaccaacccaggag-3′), and HUNICOIC (5′-aacaacactctctaccaaag-3′).

DNA sequences were visualized via autoradiography, and aligned by eye using the software package XESEE (Cabot & Beckenbach, 1989). P-distances (uncorrected for multiple hits) and Kimura's "two parameter" distances (Kimura, 1980) were calculated using the software package MEGA (Kumar et al., 1993). Prior to phylogenetic analysis, the DNA sequences were examined for evidence of saturation by plotting the number of transversions and transitions at each codon position vs. p-distance. Trees were generated under maximum parsimony using PAUP version 3.1.1 (Swofford, 1993). Trees were rooted using *Fusconaia cerina* (Conrad, 1838) and

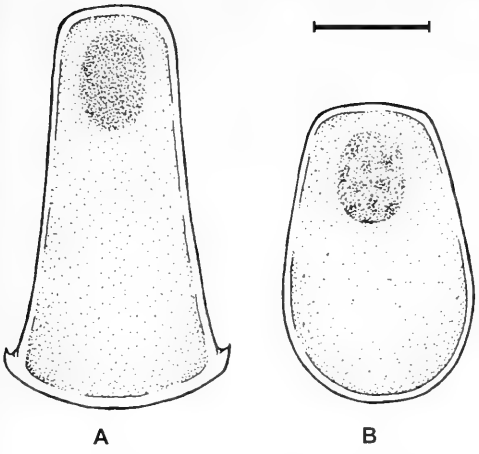

FIG. 1. (A) Glochidia of *Potamilus purpuratus,* showing the axe-head shape and lateral hooks. Redrawn from Surber (1915). (B) Glochidia of *Lampsilis cardium* for comparison. Redrawn from Surber (1912). Bar = 100 μm.

Obliquaria reflexa (Rafinesque, 1820). Bootstrapping (Felsentein, 1985) was employed to measure the internal stability of the topologies generated using 200 iterations. Skewness of tree-length distributions as a measure of phylogenetic signal (Hillis & Huelsenbeck, 1992) was estimated by generating 10,000 random trees.

RESULTS

Sequence Variation

DNA sequencing procedures yielded ~600 base pairs of COI sequence for 24 taxa for a total of 14,400 nucleotides (Genbank accession numbers AFO 49499-AFO 49522). Preliminary analysis of the sequence data revealed 182 variable sites, 151 of which were phylogenetically informative. Of those sites that were phylogenetically informative 16 were at the first position, 10 were at the second position, and 125 were at the third. Translation of codons into amino acids indicates 23 variable residues. Pairwise percent sequence differences corrected for multiple hits using the "two parameter" model (Kimura, 1980) ranged from 0 to 2.6% for intraspecific comparisons. Values for interspecific comparisons within *Potamilus* were between 1.2% and 14.5%. Pairwise comparisons for all taxa are presented in Table 2.

Scatterplots of pairwise genetic sequence differences versus the absolute number of transitions and transversions are presented for each codon position in Figure 2. Trends revealed by the scatterplots are typical for those seen in other protein coding genes (Roe et al., 1997a; Lydeard & Roe, 1997), transversions were relatively rare at first and second positions, not exceeding four and two substitutions respectively for any comparison. Transversions were considerably more common at the third codon position. A slight decrease in the number of transitions relative to the number of transversions at the third position provides evidence that some saturation is present. Saturation has the potential to affect phylogenetic analyses, therefore differential weighting of substitutions in the third codon position was employed.

Phylogenetic Analyses

Based on the analysis of nucleotide substitution patterns, phylogenetic analyses were performed under maximum parsimony using equal weighting and weighting transversions 2x transitions at the third codon position. The g^1 values (−0.362894, −0.625367) for weighted and equal weight analyses indicate the presence of significant phylogenetic signal ($p = 0.01$). Parsimony analysis of the data using equal weighting of transitions and transversions resulted in five equally parsimonious trees (CI = 0.636, RC = 0.517, 352 steps), the strict consensus of which is presented in Figure 3. Analysis of the data weighting transversions 2× transitions resulted in two equally parsimonious trees, which are presented in Figure 4. With the exception of the equivocal placement of *P. p. coloradoensis,* the two trees from the weighted analysis represent a single topology, identical to two of the five trees from the equal weight analysis. Whereas differences exist between the trees generated using transversion weighted and equal weighted parsimony analysis, all topologies depict *Potamilus* as paraphyletic. In addition, all topologies support the monophyly of all species with the exception of the *P. purpuratus* clade. All topologies also support the sister relationships of *P. ohiensis* and *P. amphichaenus,* and the reciprocal monophyly of the Amite and Black Warrior populations of *P.*

TABLE 1. Localities and number of specimens included in this study.

SPECIES	# INDIVIDUALS	LOCALITY
Potamilus alatus[1]	1	Elk River, Limestone Co., AL., 29 September 1994.
P. alatus[2]	1	Clinch River, Hancock Co., TN., 12 August 1994
P. amphichaenus[1]	1	B.A. Steinhagen Resevoir, Neches River Dr., Tyler Co., TX., 28 January 1996.
P. amphichaenus[2]	1	Sabine River, at US Highway 59, Panola Co., TX., 5 July 1995.
P. capax	2	Iron Mines Ck., ~1.25 mi. W. of AR. Highway. 140 and Red Oak Baptist Church, Poinsett Co., AR., 26 October 1994.
P. ohiensis[1]	1	St. Francis floodway, near Wittsburg, Cross Co., AR., 16 July 1995.
P. ohiensis[2]	1	Lake Arrowhead, Little Wichita River, Red River Dr., Clay Co., TX., 12 July 1994.
P. purpuratus[1]	2	Cahaba River, below Cooper Island, Bibb Co., AL., 15 September 1994.
P. purpuratus[2]	1	Cahaba River, ~1 mi. downstream of Hwy. 24, Bibb Co., AL., 30 June 1993.
P. p. coloradoensis	1	Twin Buttes Resevoir, Concho River Dr., Tom Green Co., TX., 30 August 1993.
P. inflatus	4	Amite River, above Port Vincent, Baton Rouge Pa., LA., 3–4 August 1994.
P. inflatus	4	Black Warrior River, (river mile 327.3), Tuscaloosa Co., AL., 15 October 1994.
Leptodea fragilis[1]	1	Cahaba, River, above AL. Highway 58, Centreville, Bibb Co., AL., 14 November 1994.
L. fragilis[2]	1	Elk River, upstream of AL Highway 127, Limestone Co., AL., 14 October 1996.
Lampsilis ornata	1	Cahaba, River, above AL. Highway 58, Centreville, Bibb Co., AL., 14 November 1994.
Obliquaria reflexa	1	Cahaba, River, above AL. Highway 58, Centreville, Bibb Co., AL., 14 November 1994.
Fusconaia cerina	1	Cahaba River, ~1 mi. downstream of Hwy. 24, Bibb Co., AL., 30, June 1993.

inflatus. Weaker support was found for some deeper nodes as evidenced by the low bootstrap values.

DISCUSSION

Phylogenetic Analysis

The COI data do not support the recognition of *Potamilus* as a monophyletic group. Whereas the majority of the species of *Potamilus* form a natural assemblage, the placement of *Lampsilis ornata* and *Leptodea fragilis* nested between *P. capax* and the remaining members of *Potamilus* renders the genus paraphyletic. The single morphological character that serves to unite members of *Potamilus* is the possession of axe-head shaped glochidia. Hoggarth (1988) suggested only a "superficial resemblance" between the glochidia of *P. amphichaenus, P. ohiensis* and those of *P. alatus, P. purpuratus* and *P. capax,* and recommended that mussels with axe-head shaped glochidia possessing hooks (*alatus, capax* and *purpuratus*) should remain in *Potamilus,* while those that lacked hooks (*amphichaenus, inflatus* and *ohiensis*) should be placed in the resurrected genus *Lastena* Rafinesque, 1820. Hoggarth had not examined the glochidia of *P. inflatus* and placed it in *Lastena* on the basis of the morphology of adult shells. His phenetic analysis indicated that *Lastena* was more closely allied to *Leptodea* than to *Potamilus.* Within *Lastena,* Hoggarth placed *P. ohiensis* and *P. amphichaenus* as sister to *P. inflatus.* However, recent examination of the glochidia of *P. inflatus* revealed the presence of large supernumerary hooks (Roe et al., 1997b). Based on Hoggarth's criteria, *P. inflatus* should have been placed in a group containing *P. alatus, P. purpuratus* and *P. capax,* all of which have glochidia that possess hooks. The molecular phylogeny (Fig. 4) agrees with the classification of Hoggarth (1988) in the recognition of *P.*

TABLE 2. Pairwise genetic distances based on Kimura's "two parameter" model. Values are percentages.

	P. inf.w1	P. inf.w2	P. inf.w3	P. inf.w4	P. inf.a1	P. inf.a2	P. inf.a3	P. inf.a4	P. purp1	P. purp.2	P. purp.c.	P. alatus1
P. inf.w1		0.00	0.00	0.34	2.46	2.44	2.62	2.08	9.68	10.16	10.55	10.16
P. inf.w2			0.00	0.34	2.12	2.29	2.47	1.93	9.55	9.51	9.70	9.88
P. inf.w3				0.34	2.29	2.45	2.62	2.09	9.49	9.82	10.39	10.00
P. inf.w4					2.46	2.08	2.26	2.07	9.26	9.72	10.10	9.72
P. inf.a1						0.35	0.35	0.17	9.36	9.32	9.49	9.10
P. inf.a2							0.17	0.17	9.13	9.58	10.17	9.18
P. inf.a3								0.34	9.49	9.82	10.19	9.39
P. inf.a4									9.09	9.53	10.32	9.34
P. purp.1										0.00	1.40	1.22
P. purp.2											1.55	1.38
P. purp.col.												1.20
P. alatus1												
P. alatus2												
P. capax1												
P. capax2												
P. ohien.1												
P. ohien.2												
P. amph.1												
P. amph.2												
L. frag.1												
L. frag.2												
L. ornata												
O. reflexa												
F. cerina												

	P. alatus2	P. capax1	P. capax2	P. ohien1	P. ohien.2	P. amph.1	P. amph2	L. frag.1	L. frag.2	L. ornata	O. reflexa	F. cerina
P. inf.w1	10.11	14.40	14.48	12.40	13.02	12.88	12.80	9.95	9.61	14.48	16.43	14.92
P. inf.w2	9.83	14.42	14.48	12.39	13.00	12.89	12.81	9.90	9.55	14.70	16.69	14.93
P. inf.w3	9.92	14.28	14.31	12.47	13.09	12.96	12.88	9.81	9.46	14.34	16.53	14.79
P. inf.w4	9.66	13.91	14.02	12.16	12.76	12.64	12.57	9.51	9.18	13.98	16.15	14.65
P. inf.a1	9.02	14.24	13.88	11.13	11.75	11.59	11.54	9.09	8.74	12.95	16.26	14.95
P. inf.a2	9.11	14.25	14.11	11.20	11.82	11.66	11.61	8.98	8.64	12.98	16.02	15.17
P. inf.a3	9.31	14.08	13.89	11.02	11.63	11.47	11.42	9.40	9.06	12.82	15.86	15.45
P. inf.a4	9.27	13.99	13.88	10.98	11.56	11.42	11.38	9.13	8.79	12.94	15.55	15.11
P. purp.1	1.23	13.79	13.62	10.18	10.56	11.64	11.61	7.16	7.21	11.74	14.51	14.37
P. purp.2	1.40	14.19	13.88	10.82	11.20	12.28	12.24	7.62	7.67	12.16	14.91	14.55
P. purp. c.	1.22	13.52	13.18	10.19	10.56	11.66	11.61	8.20	8.25	12.35	16.84	16.27
P. alatus1	0.00	13.12	12.99	9.98	10.37	11.27	11.22	7.24	7.29	11.33	15.75	14.79
P. alatus2		13.33	13.16	9.92	10.32	11.22	11.18	7.36	7.41	11.32	16.03	14.83
P. capax1			0.00	13.54	14.15	14.28	14.17	11.42	11.29	13.87	16.77	17.44
P. capax2				13.40	14.04	14.14	14.04	11.30	11.16	13.98	16.89	17.57
P. ohien.1					0.34	4.68	4.39	9.76	9.42	14.49	17.10	16.96
P. ohien.2						5.24	4.94	10.34	10.00	14.66	17.52	17.59
P. amph.1							0.17	10.78	10.44	16.65	17.84	17.37
P. amph.2								10.77	10.43	16.26	17.63	17.19
L. frag. 1									1.03	9.87	13.41	13.64
L. frag. 2										9.33	13.49	12.67
L. ornata											15.02	16.62
O. reflexa												13.44
F. cerina												

Note Taxon abbreviations: P. inf.w1–4, *Potamilus inflatus*-Black Warrior River; P. inf.a1–4, *Potamilus inflatus*-Amite River; P. purp.1–2, *Potamilus purpuratus;* P. purp. col., *Potamilus purpuratus coloradoensis;* P. alatus1–2, *Potamilus alatus;* P. capax1–2, *Potamilus capax;* P. ohien.1–2, *Potamilus ohiensis;* P. amph.1–2, *Potamilus amphichaenus;* L. frag.1–2, *Leptodea fragilis;* L.ornata, *Lampsilis ornata;* O.reflexa, *Obliquaria reflexa;* F. cerina, *Fusconaia cerina.*

amphichaenus, P. inflatus, and *P. ohiensis* as a natural group; however, it is not due to the shared absence of hooks. Clearly, given the homoplastic nature of hook development this character appears to be of limited phylogenetically utility.

The phylogenetic position of *P. capax* is problematic. In analyses of the molecular data, *P. capax* is depicted as the most basal member of the in-group in the weighted analysis, and is the most basal or second most basal member in the equal weight analysis.

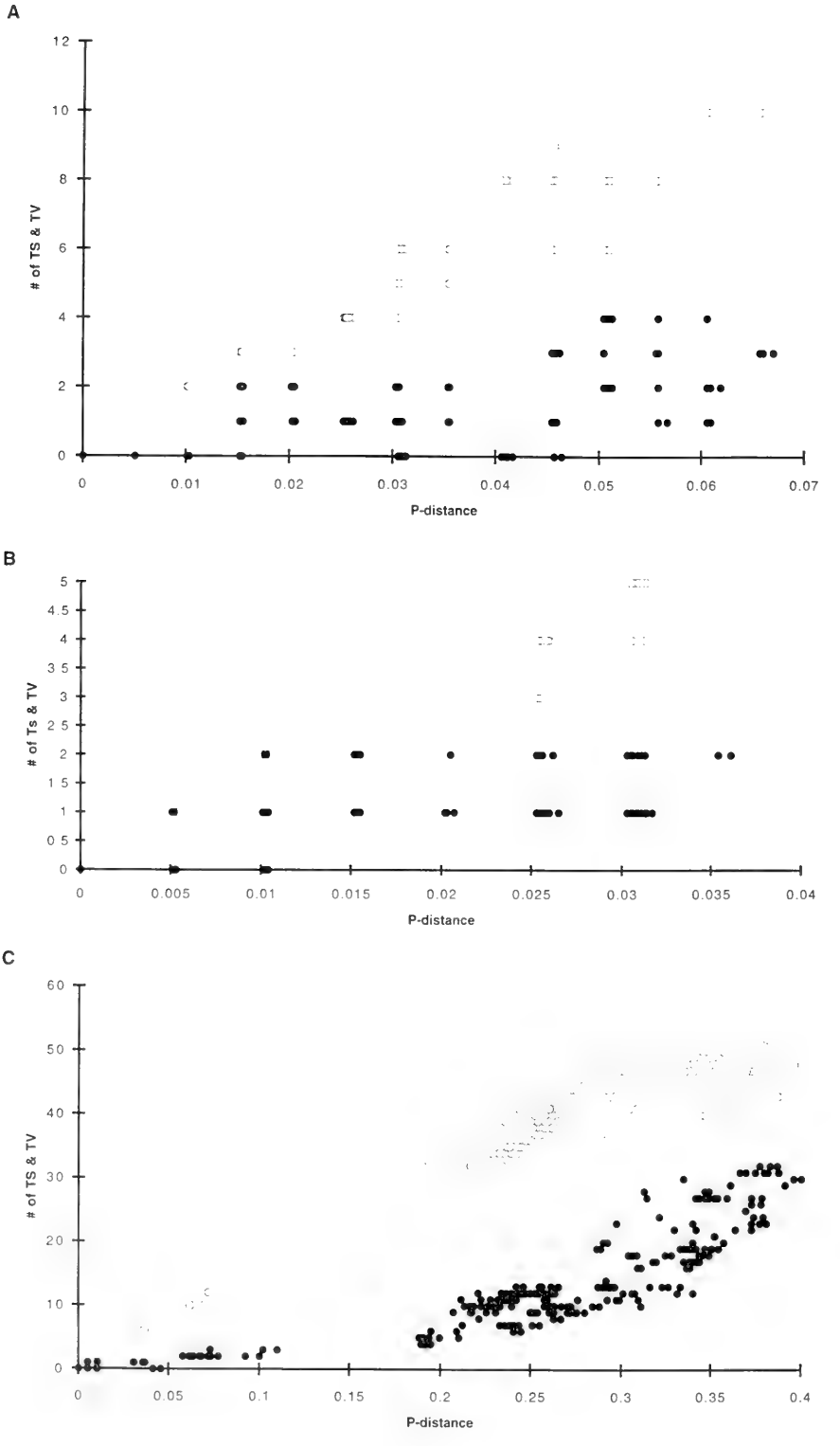

FIG. 2. Scatter plots of number of nucleotide substitutions (transitions (TS) = open circles, transversions (TV) = filled circles) versus genetic difference (p-distance) at (A) first, (B) second and (C) third codon positions.

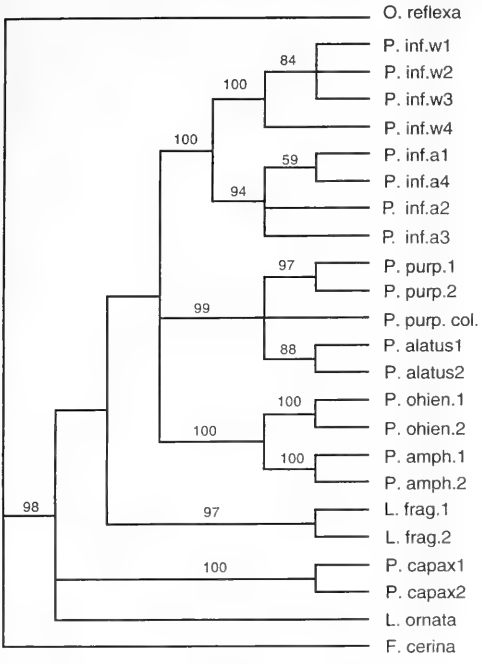

FIG. 3. Strict consensus tree for five equally parsimonious cladograms based on maximum parsimony analysis using equal weighting of all substitutions. Numbers correspond to the percentage of bootstrap replicates where the clade was found (200 total replications). Only values greater than 50% are shown. Taxon labels follow Table 2.

The placement of *P. capax* outside the remaining members of *Potamilus* indicates possible affinities with other genera. *Potamilus capax* had been placed in *Lampsilis* by Simpson (1914) based on similarities of the adult shells, particularly *L. satura* (I. Lea, 1852) (Valentine & Stansbery, 1971). Based on glochidia morphology, Coker & Surber (1911) indicated that *capax* was not a *Lampsilis* but a *Potamilus.* The molecular evidence presented here indicate no support for the placement of *P. capax* in *Potamilus;* for the present, we withhold a formal recommendation concerning the generic affinity of *P. capax* until a more inclusive analysis can be performed, including the type species of both *Leptodea* and *Lampsilis.*

Our analyses suggest that *P. p. coloradoensis* may represent a species distinct from *P. purpuratus* (Fig. 4B). Simpson (1914) also recognized *P. coloradoensis* (I. Lea, 1856) as a distinct species, although he admitted he was doubtful of its validity. The placement of the specimen referable to *P. coloradoensis* in our analysis is equivocal, either being sister to *P. purpuratus* or *P. alatus.* Examination of adult shells reveals differences in periostracum and nacre color between *P. p. coloradoensis* and *P. purpuratus* shells from east of the Mississippi River. Specimens of *P. alatus* are generally distinguishable from those of *P. purpuratus,* but examination of the glochidia of representatives of these taxa reveals no detectable differences. Based upon genetic distances *P. p. coloradoensis* is phenetically more similar to *P. alatus* (1.2%) than to *P. purpuratus* (1.5%). Genetic distances between these taxa exceed the intraspecific variation observed in all other species included in the study, with the exception of *P. inflatus.* Further research involving representatives of *P. purpuratus* and *P. alatus* from throughout their respective ranges is necessary to resolve the relationships of this clade. For the present, we recommend caution in treating *P. p. coloradoensis* and *P. purpuratus* as the same evolutionary entity.

Both *P. ohiensis* and *P. amphichaenus* were placed in the genus *Leptodea* by Burch (1975), however no support for the sister relationships of *Leptodea* and these taxa is found in this analysis. The molecular data do provide strong support for the sister relationships of *P. ohiensis* and *P. amphichaenus,* and indicate they represent distinct evolutionary entities, more closely related to other members of *Potamilus* than to *L. fragilis.*

The paraphyletic nature of *Potamilus* raises questions about the monophyly of other closely related unionid genera, such as *Leptodea. Leptodea* contains three species: *L. fragilis, L. ochracea* and *L. leptodon.* Of these, *L. ochracea* was assigned to *Lampsilis* by several authors (Simpson, 1914; Johnson, 1970; Burch, 1975) because of similarities in appearance of adult shells, particularly to *Lampsilis cariosa.* Morrison (1975) placed it in *Leptodea* because it lacked the mantle flaps often seen in species of *Lampsilis.* Hoggarth (1988) found the glochidia of *L. ochracea* to be more similar to *L. fragilis* and recommended retaining it in *Leptodea.* The type species, *Leptodea leptodon,* was originally assigned to *Leptodea* by Rafinesque (1820). It was also placed in *Lampsilis* by Simpson (1914). This species has always been considered rare (Oesch, 1984) and has become very difficult to find recently. Ultimately, any taxonomic revision of these taxa must include type species. Future phylogenetic analyses including these and other allied taxa are needed in order to more fully resolve relationships among these genera.

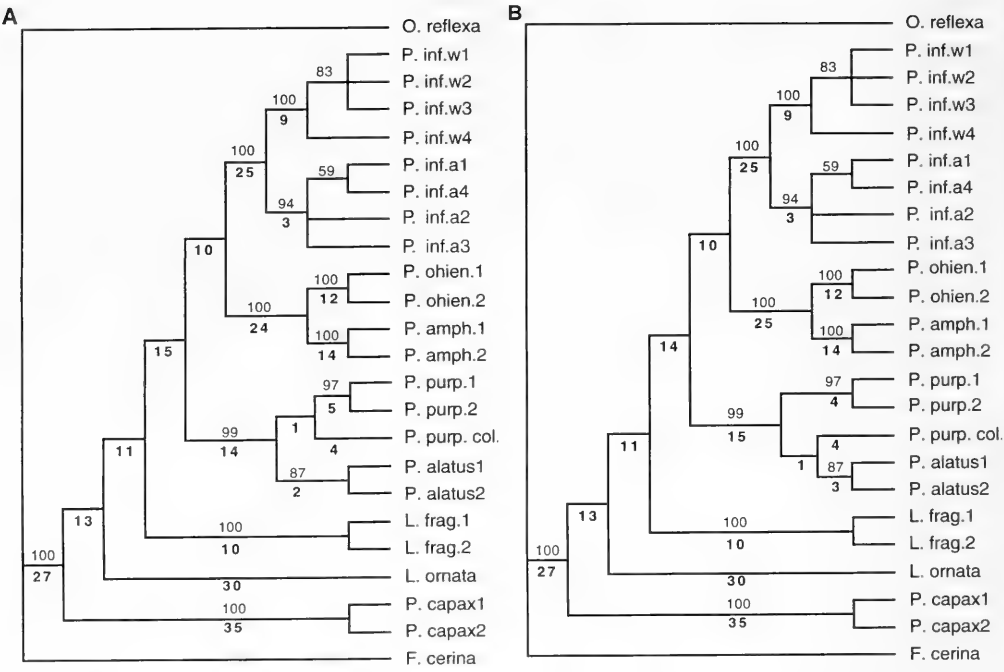

FIG. 4. (A, B). Two equally parsimonious cladograms based on maximum parsimony analysis weighting transversions 2× transitions at the third codon position. Numbers above the branches correspond to the percentage of bootstrap replicates where the clade was found (200 total replications). Only values greater than 50% are shown. Boldface numbers below the branches correspond to the number of nucleotide substitutions at those nodes. Taxon labels follow Table 2.

Conservation Genetics of *Potamilus inflatus*

DNA sequence data have been used to clarify relationships both between and within species for a large variety of organisms from whales (Milinkovitch et al., 1993) to hermit crabs (Cunningham et al., 1992). However, very few intraspecific comparisons of DNA sequences exist for studies involving unionids (Liu et al., 1996a; Mulvey et al., 1997).

Intraspecific studies are necessary for wise management decisions concerning endangered and threatened species. Phylogenetic analysis of sequence data of the COI gene indicates that populations of *P. inflatus* from the Amite River, Louisiana, and the Black Warrior River, Alabama, are reciprocally monophyletic (Figs. 3, 4) and represent distinct evolutionary entities (Moritz, 1994; Mayden & Wood, 1995). Genetic distances and the number of nucleotide substitutions that separate these two populations were compared with the number of substitutions that separate well-established species. Examination of genetic distances reveals that the two populations of *P. inflatus* are more distinct genetically than *P. purpuratus* is from *P. alatus* (Table 2). Examination of nucleotide substitution patterns reveals that a total of 12 diagnostic substitutions separate the two populations of *P. inflatus,* whereas *P. alatus* and *P. purpuratus* are separated from each other by eight substitutions. In another comparison of congenerics, *P. ohiensis* and *P. amphichaenus* are separated by 26 substitutions.

Nucleotide substitutions are considered by some researchers to accumulate at a similar rate for closely related taxa (Wilson et al., 1987; Vigilant et al., 1991; Wayne et al., 1991; Li, 1993). If this is true for *Potamilus,* it would indicate a more distant divergence time for the two populations of *P. inflatus* than that for some conspecifics. Alternatively, the differences observed could indicate an increased rate in nucleotide substitutions for the *inflatus* clade. In either case, based on these data, a strong argument can be made for the recognition of the Black Warrior and Amite popula-

tions of *P. inflatus* as distinct species. To date no conchological characters have been found that support the molecular data, and discrimination between these two species is based solely upon DNA sequence data. The recognition of cryptic unionid species is not without precedent. Davis (1983) identified allozymic differences for two phenotypically similar species of *Uniomerus.* The degree of genetic differentiation observed between populations of *P. inflatus* was greater than that seen in a comparison of two other morphologically distinct species of *Potamilus* and exceeded intraspecific values for all other species. The current geographic isolation of these two populations can only lead to further genetic differentiation of these entities and has serious implications for any plans to reintroduce *P. inflatus* in areas where it once occurred. Other studies involving mitochondrial DNA variation in unionids have come to similar conclusions regarding the protection of genetically distinct forms. For example, in a study of the conservation genetics of two unionid genera, Mulvey et al. (1997) confirmed the distinctiveness of *Amblema neislerii* (I. Lea, 1858) and *A. plicata* (Say, 1817) using allozyme and DNA sequence data. Mulvey et al. (1997) recommended additional protection for *A. neislerii* because of its restricted range and particular habitat requirements. In another study, Liu et al. (1996b) urged caution regarding any efforts aimed at re-establishing populations of the giant floater, *Pyganodon grandis,* in Colorado, because of observed mitochondrial DNA differentiation between different river drainages. Given the unique genetic status of the Amite and Black Warrior forms of *P. inflatus,* we recommend that each should be managed as a distinct evolutionary entity.

The utility of the COI gene for elucidating relationships at the species level in our study is based primarily on the relatively high number of substitutions at the third codon position. The relative lack of support, as measured by bootstrapping, for deeper nodes in the phylogeny is due in part to the smaller number of variable sites at the first and second positions. It is possible that sequencing a larger portion of the COI gene would result in higher support for these internal nodes. Lydeard & Roe (1997) found that the complete cytochrome *b* gene proved useful for diagnosing relationships of representative actinopterygian fishes, contrary to previous studies based on only a portion of the gene. These studies questioned the usefulness of this particular gene for resolving deeper phylogenetic relationships (Hillis & Huelsenbeck, 1992; Graybeal, 1993), but merely lacked sufficient data to address the question at hand.

Historically, much of the uncertainty surrounding the placement of particular unionid species in one genus or another can be attributed to the use of characters of unknown phylogenetic utility and the absence of any objective analysis. In the case of *Potamilus,* the phylogenetic analysis of an independent molecular data set indicates that such characters as glochidia shape and spines on glochidia may be homoplastic and thus not useful in diagnosing natural groups of mussels. Further investigations involving *Potamilus* and other genera are warranted and should include morphological as well as molecular characters. Davis (1983) recommended the use of multiple data sets for resolving relationships between unionid taxa. The use of multiple data sets, such as morphological and molecular characters, both independently and in a total evidence approach (Kluge, 1989) would provide a more accurate test of the phylogenetic utility of molecular and traditional morphological characters in an evolutionary context and provide much needed insight into the evolution of these traits.

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ALLOMETRIC GROWTH AND INSIGHT ON SEXUAL DIMORPHISM IN *POMACEA CANALICULATA* (GASTROPODA: AMPULLARIIDAE)

Alejandra L. Estebenet

Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, San Juan 670 - 8000, Bahía Blanca, Argentina

ABSTRACT

The shape changes associated with increase in size and sexual dimorphism in *Pomacea canaliculata* are described using bivariate and multivariate statistical analysis. Allometric growth was found in the studied population, the shell becoming relatively more globose and both aperture and operculum becoming rounder as shell height increases. Related to this ontogenetic change in shell shape is a relative increase in body dry weight. Adult snails show sexual dimorphism, males having both aperture and operculum rounder than females. Because juvenile snails do not exhibit these differences in aperture form, shell dimorphism seems to be associated with sexual maturity; that is, it is possibly related to the development of the penial complex. In summer, adult females of any given shell height weigh significantly more than males of equivalent size; this being possibly due to the remarkable development of the albumen gland in this period.

Shell dry weight shows great variability in similarly sized snails, this fact being partially ascribable to the seasonal growth pattern of *P. canaliculata* in waters showing thermal seasonality.

Key words: Sexual dimorphism, *Pomacea*, growth.

INTRODUCTION

Freshwater snails in the family Ampullariidae have a tropical and subtropical distribution. The literature describing shell shape in adult snails is extensive. Many species have been described according to external shell characters. However, shell shape changes associated with increase in size have not been explored. Allometric relationships between some linear shell measurements and snail weight have been investigated for adult *Pomacea canaliculata* (Lamarck 1822) (Guedes et al., 1981; Cazzaniga, 1990), but the values of proportionality constants cannot be related to specific or relative growth unless we are dealing with ontogenetic data (Shea, 1985).

Because young *P. canaliculata* have no developed sexual structures (penial complex and albumen gland) (Hylton Scott, 1957), their sex can be determined only by the microscopical analysis of the gonad. Cazzaniga (1990) reported external differences between sexes in adult *P. canaliculata;* however, shell shape in undifferentiated juvenile snails was not analyzed.

In this paper, ontogenetic changes in shell form and sexual dimorphism in *P. canaliculata* are analyzed using bivariate and multivariate statistics.

MATERIALS AND METHODS

Live *P. canaliculata* were collected from standing waters in an artificial pond at Paseo del Bosque, La Plata city, Argentina, in January and February 1989. Their shells were cleaned of adhering matter (mostly algae), and they were then starved in aquaria with tap water for 18 h to empty their guts. The snails were killed by immersion into warm water (75 °C) for 3–5 min.

The sex of each snail was determined by the presence of the penial complex or the albumen gland; the snails were classed as undifferentiated (or juveniles) when these sexual structures could not be clearly identified.

The shells, soft parts and opercula were

dried separately at 80 °C for 48 h and weighed on a scale to the nearest 0.1 mg.

The following seven measurements were taken on each specimen: shell height (SH), spire height (SpH), operculum height (OH), aperture height (AH), shell width (SW), aperture width (AW) and operculum width (OW). All measurements were made along straight lines either parallel or perpendicular to the imaginary axis shown on Figure 1. Shells <25 mm high were measured using a stereoscopic microscope fitted with a camera lucida. Some shell reference points were projected on a graph paper ruled in squared millimeters. From lines that joined these points, I obtained the linear measurements for each snail to the nearest 0.5 mm. Shells >25 mm high were measured also from a plane projection but obtained from X-ray images.

A total of 363 shells were measured (171 juveniles, 89 females, 103 males). Because some parts were spoiled while separating tissue from shell and operculum, only 226 snails were used to weight operculum and body (57 juveniles, 80 females, 89 males) and 233 snails to weigh shell (64 juveniles, 80 females, 89 males).

The power function $y = ax^b$ was used to describe the relationships among the linear and weight measurements. Least squares regression analyses were carried out for the whole population, and for males and females separately. The logarithm of SH was used as independent variable in all regressions. All the slopes of the regressions involving whole population were compared with isometry ($b = 1$ for linear variables or $b = 3$ for weight variables) using t statistics (Sokal & Rohlf, 1979). Analysis of covariance was used to test the equality of regression coefficients and intercepts between sexes (BMDP1V, Statistical Software, UCLA, 1982). Residual analysis was performed in all cases to test the basic assumptions of the linear regression analysis and to assess the adequacy of the linear model.

Stepwise discriminant function and canonical variate analysis were performed (BMDP7M, Statistical Software, UCLA, 1982) in order to reveal which variables contributed to discriminating the sexes. Only the linear, not weight, variables were used. Allometric growth exists in all characters measured (see results); all variables were therefore log-transformed before the analyses were carried out.

Twenty eight newly hatched snails from a single egg-mass were reared in the laboratory. They were placed individually in cylindrical plastic tubes submerged in an aquarium with aerated, warm (25 ± 1°C), tap water (hardness 90 to 96 ppm $CaCO_3$). The snails were fed with lettuce, and the water was changed periodically at the time the linear shell dimensions were measured. When the snails were approximately 25 mm high, their sexes were determined (14 males and 14 females).

RESULTS

Table 1 is a statistical summary of the 10 measured variables.

Analyses of residual plots showed no departures from the assumptions of the regression model; the linear log-log regression accurately describes the relationships between the pairs of selected variables, the only exception being log shell weight (SWt) - log shell height (SH) regression.

Table 2 shows the different allometric relationships calculated for the whole population. The null hypothesis that $b = 1$ was rejected in all regressions involving linear variables. The ratios of shell width, aperture width, operculum width and spire height to shell height (SW/SH, AW/SH, OW/SH and SpH/SH respectively) increase as the shell increases in size. The dry weight of the soft parts (BWt) and operculum (OWt) also increase relative to SH (the null hypothesis that $b = 3$ was rejected).

Table 3 shows the results of the ANCOVA on the two sexes of *P. canaliculata*. Except for log SW and log SpH, the slopes or intercepts of the regression lines were significantly different between the sexes. The values of b for aperture and operculum variables were significantly larger for males. The slopes of the log BWt-log SH and log OWt-log SH regressions did not differ between sexes, but the intercepts were larger for the females.

The linear log SWt - log SH regression was not appropriate to describe the relationship between both variables, because the ratio of specific growth rates changes during the ontogeny. Residual plot showed that residual variance increases along the independent variable.

In this study, the best discriminating model for separating sexes uses only three of the seven available measurements (SW, OH,

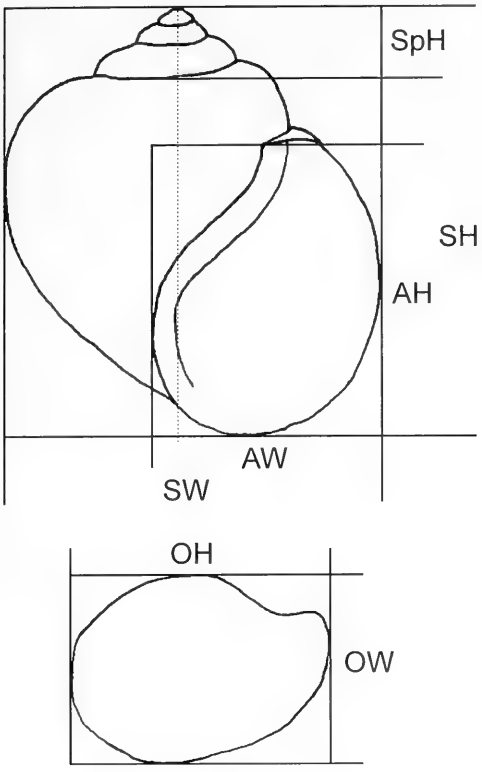

FIG. 1. Variables measured on the shell and the operculum of *Pomacea canaliculata* (SH: shell height, SpH: spire height, AH: aperture height, SW: shell width, AW: aperture width, OH: operculum height and OW: operculum width).

OW). The other four variables were not included, because they contribute no additional discriminating information. A discriminant score was computed for each snail by multiplying each of three characters by the corresponding coefficient and adding together these products (Fig. 2). There was a significant difference between the mean discriminant scores of the two sexes; that is, the function will discriminate, significantly, between the two groups (Wilk's lambda = 0.534 or χ^2 = 118, d.f = 3, $p < 0.0001$). The percentage of correctly allocated individuals by sex using this function was greater than 78% for the males and 85% for the females. The results of canonical variate analysis showed that OW had the greatest importance in separating sexes (Table 4).

The growth rate (measured as increase in SH) was similar in males and females reared in the laboratory, at least until sexual maturity was reached (Fig. 3). The form of the aperture (AW/AH) was compared between sexes twice during growth. The first comparison was made when the snails were 7.42 ± 0.707 mm high (mean ± SD); there was no significant difference (male mean ratio: 0.7605 ± 0.0255 SD, female mean ratio: 0.7636 ± 0.0269 SD; $t = 0.313$, $p > 0.10$, d.f. = 26). However, when the snails were 23.51 ± 2.996 mm high (mean ± SD), the aperture was significantly rounder in males than in females (male mean ratio: 0.8042 ± 0.0302 SD; female mean ratio: 0.7698 ± 0.0427 SD; $t = 2.461$, $p < 0.05$, d.f. = 26).

DISCUSSION

Allometric growth occurs in *Pomacea canaliculata,* the shell becoming relatively more globose, and both aperture and operculum becoming rounder as the shell increases in size. Related to this ontogenetic change in shell shape, there is a relative increase in the dry weight of the soft parts. The positive allometry of AW and SW relative to SH may be related to a positive allometric growth of the foot.

Though SpH shows positive allometric growth with respect to SH, it is a widely variable character within populations. The shell of *P. canaliculata* usually has a short spire. However, in our material, snails range from those whose shell apex does not exceed the plane that delimits the superior edge of the last whorl, to snails with a much higher spire. This variation has been noted by earlier authors (d'Orbigny, 1847; Barattini, 1939; Hylton Scott, 1957) and prevents SpH be used as a diagnostic character.

Adults of *P. canaliculata* from Paseo del Bosque show sexual dimorphism, as shown by Cazzaniga (1990) for another population of the same species. The external differences between males and females are manifested by both the form of the aperture and the operculum. The AW/SH ratio increases with increase in size. However, in females AW increases less rapidly than in males. Another ampullariid, *Marisa cornuarietis* (L.), also exhibits positive allometric growth of AW (Demian & Ibrahim, 1972), and the differ-

TABLE 1. Statistic summary of the variables measured on *Pomacea canaliculata.* Linear measurements are in mm; weights are in mg.

Variable	Mean	SD	Minimum	Maximum	N
Shell height	35.21	15.61	8.84	80.00	363
Shell width	29.38	13.65	7.12	70.50	363
Spire height	4.10	2.10	0.88	10.00	363
Aperture height	24.69	10.45	6.28	54.00	363
Aperture width	19.01	8.79	4.80	42.50	363
Operculum height	21.88	9.53	5.64	49.00	363
Operculum width	15.03	6.95	3.64	33.50	363
Body weight	1480	1250	110	6130	226
Shell weight	3865	2795	253.8	14946	233
Operculum weight	90	70	6	370	226

TABLE 2. Values for different linear regressions for a population of *Pomacea canaliculata,* with tests of significance of deviation from the values of isometry (values of test t)

Regression	N	b ± SE	Isometry t b = 1	P	a	R^2
log SW/log SH	363	1.042 ± 0.005	8.13	<0.0001	–0.147	0.99
log SpH/log SH	363	1.070 ± 0.020	3.47	<0.001	–1.053	0.88
log AH/log SH	363	0.945 ± 0.004	12.08	<0.0001	–0.067	0.99
log AW/log SH	363	1.025 ± 0.006	4.17	<0.0001	–0.308	0.99
log OH/log SH	363	0.967 ± 0.005	5.61	<0.0001	–0.156	0.98
log OW/log SH	363	1.015 ± 0.006	2.5	<0.02	–0.395	0.97
			b = 3			
log BWt/log SH	226	3.456 ± 0.062	7.36	<0.0001	–2.590	0.93
log OWt/log SH	226	3.253 ± 0.068	3.68	<0.001	–3.470	0.91

TABLE 3. Values for different linear regressions in males and females *Pomacea canaliculata.*

Regression	Sex	N	b ± SE	a	R^2	F#
log SW/log SH	females	89	1.020 ± 0.021	–0.104	0.96	F_b NS
	males	103	0.984 ± 0.022	–0.051	0.95	F_a NS
log SpH/log SH	females	89	1.084 ± 0.103	–1.083	0.56	F_b NS
	males	103	0.987 ± 0.103	–0.912	0.48	F_a NS
log AH/log SH	females	89	0.886 ± 0.019	–0.032	0.96	F_b 13.70**
	males	103	0.983 ± 0.018	–0.127	0.97	
log AW/log SH	females	89	1.048 ± 0.021	–0.353	0.96	F_b 3.69*
	males	103	1.114 ± 0.027	–0.445	0.94	
log OH/log SH	females	89	0.924 ± 0.023	–0.083	0.95	F_b 5.44*
	males	103	1.003 ± 0.024	–0.209	0.94	
log OW/log SH	females	89	0.975 ± 0.030	–0.342	0.92	F_b 8.51**
	males	103	1.123 ± 0.040	–0.545	0.88	
log BWt/log SH	females	80	3.229 ± 0.154	–2.145	0.86	F_b NS
	males	89	3.081 ± 0.112	–2.015	0.90	
	females		common slope	–2.034		F_a 59.63**
	males		3.164 ± 0.086	–2.151		
log OWt/log SH	females	80	3.076 ± 0.149	–3.230	0.85	F_b NS
	males	89	3.365 ± 0.172	–3.590	0.82	
	females		common slope	–3.326		F_a 34.77**
	males		3.204 ± 0.113	–3.443		

#F test for null hypothesis that $b_{males} = b_{females}$ F_b or $a_{males} = a_{females}$ F_a
NS: not significant ($p > 0.05$) *$p < 0.05$ **$p < 0.01$

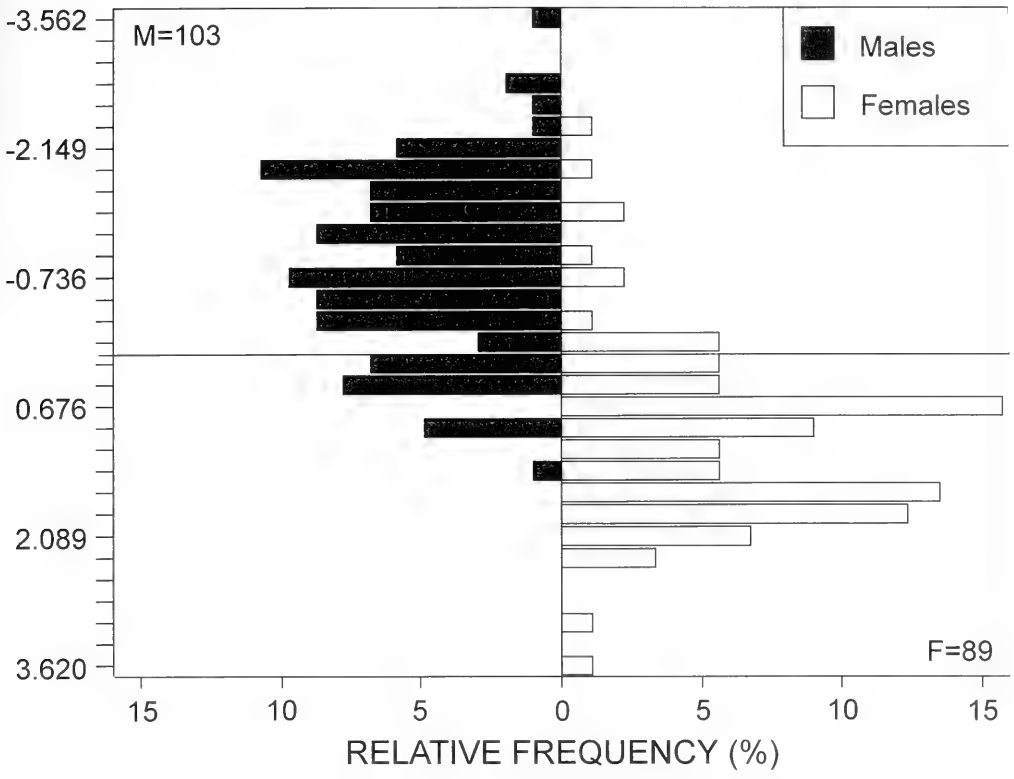

FIG. 2. Frequency histograms of discriminant scores (DS) for male and female *Pomacea canaliculata.* DS = –35.42(logOW) + 22.91(logOH) + 19.31(logSW) – 18.07

ences between the sexes in this species are greater than in *P. canaliculata.*

Multivariate analysis showed OW is a better discriminant between sexes than AW, because OW grows with positive allometry respective to SH in males, whereas in the females the growth is isometric.

In *P. canaliculata,* the penial complex, which arises as a ventral outgrowth from the mantle edge, appears to develop at the same rate in both sexes until the gonad becomes active, when its growth is arrested in females. This occurs when the females are 8 mm in diameter (Andrews, 1964). Around this point, the aperture form is still similar between sexes. The sexual dimorphism in the aperture form seem therefore to be associated with the sexual maturity, possibly with the posterior development of the penial sheath in the males.

About 50% of the males of *P. canaliculata* greater than 40 mm in SH show a slight reflection of the free edge of the peristomal lip. This feature was only present in three of the 89 females analyzed. This other differential shell feature was present in some males of *M. cornuarietis* (Demian & Ibrahim, 1972).

TABLE 4. Results of canonical variate analysis for shape differences between sexes.

Standardized coefficients	
log SW	1.487
log OW	–3.655
log OH	2.188
Eigenvalue	0.871
Canonical correlation	0.682

Sexually dimorphic growth and/or survivorship patterns resulting in sexually dimorphic sizes are known for many freshwater prosobranchs (Browne, 1978; Aldrige, 1982;

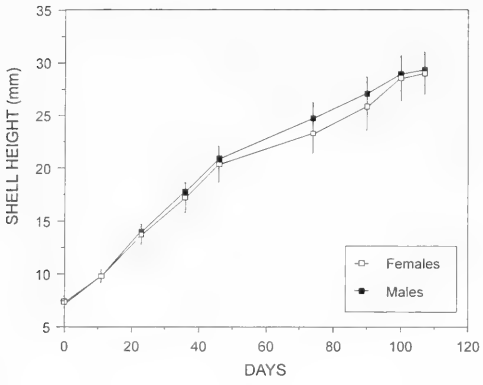

FIG. 3. Pattern of growth of *Pomacea canaliculata* reared under laboratory conditions (mean ± 95% confidence interval).

Jokinen, 1982; Jokinen et al., 1982; Brown & Richardson, 1992). Females reach sizes greater than males in several species of Ampullariidae: *Pila* spp. (Keawjam, 1987), *Pomacea urceus* (Müller) (Lum Kong & Kenny, 1989) and *Marisa cornuarietis* (Demian & Ibrahim, 1972). Burky (1974) reported that *P. urceus* males can attain a maximal size similar to that of females, but usually the proportion of males in the largest size classes is less than those of females. In my material, the maximal size attained by one male was 62.5 mm (SH) whereas around 12% of the females were larger, up to 80 mm. This size difference could be attributed to a sexually dimorphic growth pattern (Estebenet & Cazzaniga, 1997).

Females of any given size weigh significantly more than males. Because there was not marked shell form differences between sexes, this could be attributed to the sampling date (summer) that coincided with reproductive season (from late spring to late summer). The albumen gland grows up to represent 68% of the dry body mass in a reproductive active female, this being why Guedes et al. (1981) discarded reproductive active females to obtain reliable regressions for biomass estimation in *P. canaliculata.* Bourne & Berlin (1982) determined a similar weight difference between sexes of *Pomacea dolioides* (Reeve). It is therefore probable that the regressions involving soft parts weight are seasonally variable. This fact could be extended to other temperate ampullariid populations with seasonal reproductive patterns.

A linear model did not accurately describe the SWt-SH relationship. Great variability in SWt exists among similar sized snails that can be partly ascribed to seasonal pattern of growth showed by *P. canaliculata* in waters with marked thermal seasonality (Estebenet & Cazzaniga, 1992). Snails hatching at the beginning of the breeding season grow rapidly; they generally have a thin shell and are often similar sized as snails hatched at the end of the previous breeding season, the later generally having a heavier shell. Another source of variation in the SWt could be the sex of the snails, because Cazzaniga (1990) determined that shells of *P. canaliculata* males are significantly heavier than the shells of females.

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LETTERS TO THE EDITOR

TRANSLATING TREES INTO TAXONOMY WITHIN VENERIDAE (BIVALVIA): A CRITIQUE OF TWO RECENT PAPERS

Mary Ellen Harte

1180 Cragmont Ave., Berkeley, California 94708, USA

Cladistic analysis has been developed over the past two decades into a scientifically rigorous method for determining probable evolutionary relationships among taxa, and it is increasingly used throughout all biological disciplines concerned with evolution. In each case, the end result is a rooted or unrooted tree of taxa—commonly referred to as a cladogram—from which phylogenetic inferences are made. At the same time, various phenetic algorithms have been developed to create rooted or unrooted trees of taxa based on biomolecular data (e.g., Neighbor-Joining; Saitou & Nei, 1987), and these have been used to validate or refute existing taxonomies of organisms (e.g., Sibley & Ahlquist, 1990; Albert et al., 1992; Simon et al., 1993; Wainright et al., 1993; Halnych et al., 1995).

Two recent papers, Roopnarine (1996) in *Malacologia* and Canapa et al. (1996) published elsewhere, utilize such algorithms to study the bivalve family Veneridae and attempt to apply the resulting inferences to current taxonomy. An examination of these papers indicates some taxonomic and methodological misapplications to an already taxonomically controversial group.

A STUDY OF THE CHIONINAE

Roopnarine (1996) focuses on the venerid subfamily Chioninae, which represents a prolific evolutionary radiation centered in North America, judging by the number of current and extinct taxa. He collected data on 25 conchological characters from six extinct and ten Recent American chionine species, most recently classified into four genera and 11 subgenera (Keen, 1969; Fischer-Piette & Vukadinovic, 1977). Two of the four genera are represented by their type species; nine of the 11 subgenera are represented by their type species.

Character Definitions

Of the 25 characters, inadequately defined character states are present among the following seven: pallial sinus depth, spacing of commarginal sculptural elements, anterior development of commarginal elements, ventral margin crenulation, width of anterior cardinal tooth, posterior cardinal tooth shape, and width of central cardinal tooth. For example, the character of pallial sinus depth is defined as greatly reduced to absent for one state, and present but short for another. It is not clear where the demarcation is between these two states. Similarly, I disagree with the assignment of a character state in one of the taxa for spacing of commarginal elements, but the ambiguous definition of states ("close, widely spaced, widely spaced but narrowing later") allows independent workers to reach different conclusions. Such ambiguities exist in the other six characters, allowing for a significant amount of subjectivity in the analysis, and preventing other workers from replicating the results.

A second source of ambiguity arises because Roopnarine fails to define what percentage of examined specimens must have a character state in order for it to be considered present in the species. For example, in defining types of sculpture present, he asserts that "radials are absent in *Mercenaria*" (p. 140; presumably *M. mercenaria,* the only species included in his study), countering the observations of previous workers (Jones, 1979; Harte, 1992), who note the characteristic presence of radial sculpture in that species.

Representation of Genera

Inadequate representation of some of the genera weakens his analysis. The taxonomic placement of *Chione (Puberella)* based on the consensus tree is problematic, but its very presence is weakened because it was not represented by its type species. Two other taxa in the analysis, *Protothaca (Leukoma)* and *Timoclea (Glycydonta),* are parts of much larger groups that would require more analysis of related taxa to adequately assess their taxonomic status. If Roopnarine represented all of the *Chione* subgenera solely by their subgeneric names on his cladograms, however, he should have treated *Leukoma* and *Glycydonta* in the same manner. Using their generic classifications implies relationships that might not be taxonomically valid to those much larger groups. To justify inclusion of *Protothaca* and *Timoclea* in the cladograms, type species of the taxa should have been used.

Not using type species of taxa could possibly influence the results. For example, in his resulting consensus tree, Roopnarine (1996: fig. 20) shows *Timoclea* grouped with *Protothaca* in a clade separate from that of *Chione s.s.* In the study, the species representing *Protothaca* and *Timoclea* were *P.* (*Leukoma*) *asperrima* (Sowerby) of the east Pacific, and *T.* (*Glycydonta*) *marica* (Linnaeus), of the west Pacific, respectively. The type species of *Timoclea s.s., Timoclea ovata* (Pennant), an eastern Atlantic species, has different character states for at least some of the characters. Whether these different character states might make it more closely related to the type species of *Chione s.s., Chione cancellata* (Linnaeus), a western Atlantic species, than to *P. asperrima,* remains untested.

Roopnarine said that specimens of some of the type species were not available to him, but these species are not rare. For example, *Timoclea ovata* is present in the UCMP collection, one of several he used in his study, and availability of specimens is adequate through the current museum loan system of the major collections in North America.

Classification

Assuming these two sources of ambiguity do not seriously interfere with the cladistic results, can one justify his proposed taxonomic revision based on his analysis and its minimal translation into the taxonomy? Here, I believe he is only partly successful.

Reducing taxonomic ambiguity is a major consideration for creating hierarchical, supraspecific taxa. Thus, the introduction of subgenera into a classification clearly aligns some generic groups with one genus and not another, reducing ambiguity in intergeneric relationships. While taxonomic hierarchy does not have the flexibility for incorporating precisely the hierarchical information offered in a cladistic network, major cladistic hierarchical elements can be incorporated into the taxonomic framework, which can significantly improve its information content and reduce its taxonomic ambiguity. By demonstrating that *Chione,* as previously defined, was paraphyletic, Roopnarine justifies its breakup, but not, on the basis of his results and the standard of minimizing taxonomic ambiguity, his creation of genera from almost all former subgenera of *Chione.* For example, Roopnarine (1996) (Fig. 1) illustrates that the clade containing *Chione* subdivides into two clades. If he had incorporated this information into current chionine taxonomy following the taxonomic seniority rule, he could improve chionine taxonomy by proposing that *Anomalocardia,* the senior taxon of one clade, remain a genus under which the other members of that clade—*Iliochione, Lirophora,* and *Panchione*—are subsumed as subgenera. He did propose that the senior taxon of the second clade, *Chione,* remain a genus, under which the other supraspecific taxon of that clade, *Chionista,* was subsumed as a subgenus. He also proposed, justifiably, that *Chionopsis* stand as a genus based on its solitary location in his cladograms. Taken in combination, these steps reduce ambiguity in chionine taxonomy, thereby justifying the revision, and they incorporate much, but admittedly not all, plausible evolutionary information derived from the analysis. But elevation of all subgenera except *Chionista* to generic rank indicates an underutilization of cladistic hierarchical information, creating unnecessary taxonomic ambiguity by decreasing the hierarchical information within the existing taxonomy.

A STUDY OF VENERID PHYLOGENY

The second paper, Canapa et al. (1996), utilized biomolecular data from a length of 16s rRNA to construct two Neighbor Joining trees for nine Mediterranean venerid species from

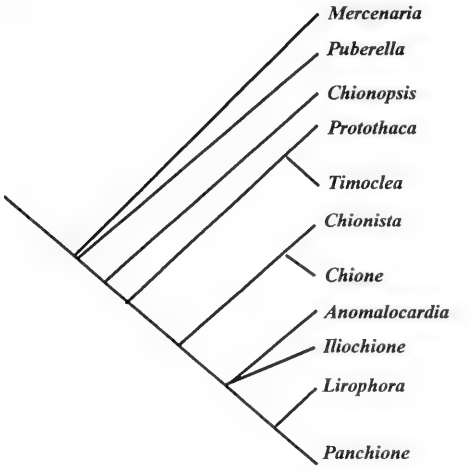

FIG. 1. Consensus tree after Roopnarine (1996: fig. 20, lower figure).

the five largest presently recognized subfamilies: *Chamelea gallina* (Linnaeus) [Chioninae], *Dosinia lupinus* (Linnaeus) [Dosiniinae], *Callista chione* (Linnaeus) and *Pitar rudis* (Poli) [Pitarinae], *Tapes decussatus* (Linnaeus), *Tapes philippinarum* (Adams & Reeve), and *Venerupis aurea* (Gmelin) [Tapetinae], and *Venus verrucosa* Linnaeus [Venerinae]. Three of the species, *Chamelea gallina, Callista chione* and *Venus verrucosa* are the type species for these genera, and the last is the nominal genus of the subfamily Venerinae and the Veneridae. The authors concluded that the resulting trees (Canapa et al., 1997) (Figs. 2, 3) support the results of traditional classification at the subfamily level but do not support the concept of a genus *Tapes.* Indeed, Chioninae and Venerinae, long considered to be closely related, fall within the same clade on both trees, and each of the other subfamilies forms a distinct clade, although the relationships among those clades differ substantially between their two trees.

Contrary to the paper's assertions, however, the data do not disprove the concept of the genus *Tapes.* The tapetine species used in this study do not represent true *Tapes,* but rather belong to the subgenus *Ruditapes* (Fischer-Piette & Métivier, 1971). In order to prove or disprove the concept of *Tapes,* both the species representing the genus *Tapes s.s.—T. literatus* (Linnaeus), an Asian species—and at least one of the 3–4 additional Asian species considered to be members of *Tapes s.s.,* should have been included in the study. If the tapetine species included in the study had fallen among these Asian species in the resulting trees, then this would indeed disprove the concept of *Tapes.* While the choice of species in this study reflects the accessability of material (local Mediterranean venerid species), this also limits what one can interpret, taxonomically, from the analyses.

As it is, a more accurate taxonomic interpretation of the analyses indicates some equally interesting insights and illustrates some of the limitations presented by the species used. For example, *Ruditapes decussatus* is the type species of *Ruditapes.* However, what malacologists identify as *Ruditapes decussatus* in the Mediterranean might not be the same species as from the type locality in the Indian Ocean, according to Fischer-Piette & Métivier (1971: 28).

Venerupis aurea is not the type species of *Venerupis;* that honor belongs to the British *Venerupis perforans* Montagu, 1803, which in turn is a synonym of *Venerupis corrugata* Gmelin, 1791 (Fischer-Piette & Métivier, 1971). Fischer-Piette & Métivier (1971) lumped several nominal taxa under *V. corrugata,* resulting in a geographic distribution from Norway to Natal, Mozambique, and beyond, and thus presenting the possibility that this is not one wide-spread species but a series of similar taxa. This, then, limits what can be inferred about the integrity of *Venerupis.*

What can be inferred about these taxa? Since *Venerupis aurea* falls among the *Ruditapes,* it calls into question the placement of *V. aurea* within *Venerupis,* the correct placement of the Mediterranean *Ruditapes decussatus* within *Ruditapes,* OR, if one assumes that the Mediterranean *Ruditapes decussatus* is indeed a valid representative of that taxon, the integrity of *Ruditapes* as a taxon. With respect to the last possibility, *Venerupis* Montagu, 1803, was established long before *Ruditapes* Chiamenti, 1900. Thus, if species of *Venerupis* are found to fall within *Ruditapes,* then *Ruditapes* would be a junior synonym of *Venerupis.* The data presented do not indicate which of the three possibilities might be correct, so no such inference can be made.

CONCLUSION

Careful study of these papers illustrates that the species chosen for analyses will limit

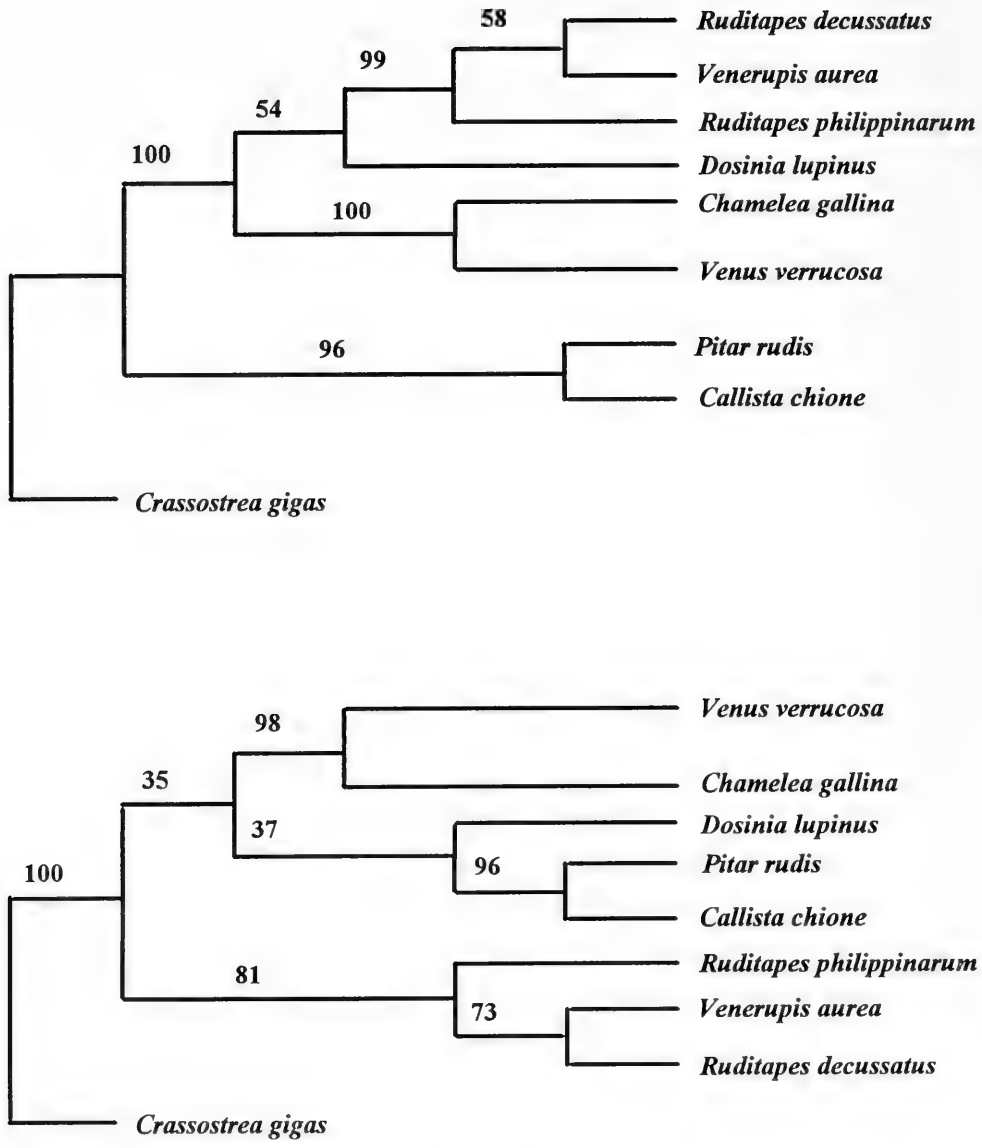

FIG. 2. Neighbor-Joining trees constructed with pairwise distances calculated following an application of two-parameter correction for multiple substitutions (upper), and calculated on the basis of the sole transversions (lower), modified after Canapa et al. (1996: figs. 2, 3). The numbers represent the percentage of 100 bootstrap replications in which a given node appeared.

what can be interpreted about higher taxa. In order to make significant inferences about generic and subgeneric taxonomy from the trees of cladistic or other types of analyses, studies must include at least the type species of those taxa, and preferably other species belonging to the same taxon or, in the case of larger taxa, that represent adequately the diversity of the taxon. In morphological cladistics, character states must be carefully defined. An initial review of the taxonomy of the group to be analyzed should be conducted, so as to optimize choice of species for insights into the taxonomy. To ensure that the material

is indeed the originally described species, the specimens should come from the same geographic locale as the originally described type specimens for the species. Conversely, if availability limits choice, this must be reflected in the limits of the resulting discussion of the taxa and presentation of cladograms.

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TRANSLATING TREES INTO TAXONOMY WITHIN VENERIDAE (BIVALVIA): A REPLY TO HARTE

Peter D. Roopnarine

Department of Biology, Southeast Missouri State University, Cape Girardeau, Missouri 63701, U.S.A.

Harte (this volume) has initiated, in comments on two papers (Capana et al., 1996; Roopnarine, 1996), an interesting and much needed discussion of the status of venerid systematics. The major focus of her commentary on Roopnarine (1996) is the selection and utilization of morphological characters for a phylogenetic analysis of the Chioninae, and the subsequent revision of the taxonomy of that subfamily on the basis of the phylogenetic analysis. In recognition of the fact that modern phylogenetic analysis is a dynamic process of hypothesis construction, testing and restructuring, I welcome her comments on my paper and below will admit shortcomings of my study but will also defend what I believe are valid and valuable contributions.

Character Definitions

Harte's first source of objection is the descriptive nature of several conchological characters employed in the phylogenetic analysis, for example pallial sinus depth. It should be made clear that, while the explanations of character states (for example, "greatly reduced to absent" versus "present but short") may be verbally ambiguous, morphologically they are not. Close examination of taxa involved, such as *Chione* (von Mühlfeld) and *Lirophora* Conrad, reveals that these character states are consistently identifiable and separable. Certainly a more robust method of presentation that would permit precise replication by other workers, besides productive interworker communication, would be the quantitative description of such characters. Attempts have been made to incorporate quantitative description of continuously varying characters into phylogenetic analyses, such as gap coding (Archie, 1985) and more recently thin plate spline decomposition of landmark data (Naylor, 1996; Fink & Zelditch, 1995; Zelditch et al., 1995), but much remains to be done in this area. In fact, salient arguments have been presented against the feasibility of such approaches (e.g., Bookstein, 1994).

One other reason for apparent character ambiguity is a less fundamental one. While many characters can be coded as dichotomously discrete states, several have additional states that represent single taxa. An example is ventral margin crenulation, which is normally either very regular and fine, or alternatively consists of significantly larger, coarser and more variable subunits (for example, *Lirophora* versus *Chione*). All taxa included in my analysis could be coded as one state or the other with the single exception of *Chionista* Keen, which has marginal crenulations intermediate in relative size and transitions posteriorly from regular to coarse and irregular. The trichotomous nature of this character then is based on one taxon out of sixteen. However, the potential ecomorphological importance of this character (Vermeij, 1987, 1993) justifies its inclusion in the analysis.

Another cited source of ambiguity is the failure to report the relative occurrence of a character state in a species. Only character states that were recognized as invariable with respect to relevant taxa were analyzed. Character conditions resulting from ontogenetic or environmental variation were purposefully excluded. Such an example would be the radials of *Mercenaria mercenaria* (L.) cited by Harte. These are typically obvious on the smoothed central portions of adult valves, and have even been used in the past as support of subspecific recognition (for example, *M. mercenaria subradiata* Palmer). The appearance of radial lines on the surfaces of smoothed, worn or eroded venerid valves that normally possess only concentric or commarginal sculpture is very common, but these radials should not necessarily be recognized as primary sculpture. They are perhaps indeed of phylogenetic importance, an issue not explored in my paper, but until they are examined in that context should not be considered homologous

with the prominent and primary radial sculpture of taxa such as *Chione, Chionopsis* Olsson and *Puberella* Fischer-Piette. Harte (1992) makes such an assumption (in addition to the possession of a rugose nymph) when arguing for the assignment of the species *Lirophora kellettii* (Hinds) to genus *Mercenaria.* As discussed in Roopnarine (1996), this species is more properly assigned to *Panchione* Olsson (Early Miocene-Recent) because of its obvious and overwhelming similarity to the numerous described extinct species also assigned to the genus, for example *P. mactropsis* (Conrad) and *P. ulocyma* (Dall). *Panchione kellettii* bears faint radial lines on its surface, but this is characteristic of all species of *Panchione,* but certainly not *Mercenaria.*

Representation of Genera

The absence of type species for some of the genera used in the systematic analysis does introduce a certain amount of ambiguity in the results if they are viewed at a level of phylogenetic universality beyond mere consideration of former genus *Chione.* The extent and effect of the ambiguity of course remains itself ambiguous until the data are reanalyzed with the inclusion of the type species. I do not believe that inclusion of the type or additional species of *Puberella* will alter the resulting cladograms in any way, because the species are morphologically very similar, being separated primarily by time, geography, and such labile characteristics as shell size (e.g., Stanley & Yang, 1987). Increasing the coverage of *Protothaca* Dall and *Timoclea* Brown in the analysis was unnecessary, because the major focus was to analyze the evolutionary histories of the tropical American *Chione* subgenera, and to place their histories in a testable phylogenetic framework. The phylogenetic relationships of these two genera, which range far beyond tropical American waters, to the former *Chione* subgenera is undoubtedly of great interest. That analysis would definitely require the examination of generic type species, as well as subgeneric types, a process that I am currently undertaking as an analysis of subfamily Chioninae. Moreover, varying degrees of relationship of say *Timoclea* (*Timoclea*) and *T.* (*Glycydonta*) to *Chione* would lead to much more than a simple change of *Timoclea*'s position on the current consensus tree. It would instead imply paraphyly of that genus and would require its entire taxonomic reconstruction.

Classification

In converting the results of my phylogenetic analyses to a revised hierarchical taxonomy of *Chione,* I relied upon two criteria: (1) changes to the existing taxonomy should be minimized, and (2) the revised taxonomy should be logically consistent with the underlying hypothesis of phylogeny (Wiley et al., 1991; deQueiroz & Gauthier, 1992). As Harte points out, the revised taxonomy is consistent with the phylogenetic results, but alternative revisions are available. Her major emphasis and point of contention seems to be the elevation of subgenera in the *Lirophora* clade (*Anomalocardia* Schumacher, *Iliochione* Olsson, *Lirophora* and *Panchione*) to generic status. She suggests instead that the hierarchical information implied by the topology of this subclade could be retained in a revised taxonomy by subsuming all subgenera under the historically senior taxon, in this case *Anomalocardia.* This alternative, while simpler and almost as informative, would unfortunately be inconsistent with the cladistic results. *Anomalocardia* and *Iliochione* spring from an unresolved polytomy, along with a branch bearing *Lirophora* and *Panchione.* The placement of *Iliochione* with respect to the other two branches is therefore unknown, and additional information may well place it outside of Harte's *Anomalocardia* (Fig. 1). The resulting *Anomalocardia,* while monophyletic, would conflict with the hierarchical structure implied by the phylogeny. Interestingly, the recent description of an extinct chionine genus (Roopnarine, in press) (Fig. 2) also from tropical America partially supports Harte's suggestion. It may now be reasonable to consider *Iliochione* a subgenus of *Anomalocardia.*

I would like to reiterate that the taxonomic revision of *Chione* suggested in Roopnarine (1996) merely gives phylogenetic and paleontological support to revisions implemented by previous workers. For example, Olsson (1961) regarded *Chionopsis* as a genus distinct from *Chione;* Keen (1969) treats *Panchione* as distinct from *Lirophora;* and Woodring (1982) and Ward (1992) treat *Lirophora* as a generic rank taxon. Finally, at the risk of portraying myself as an unrepentant cladist, I must point out that the maintenance of an artificial system of hierarchical categorization and classification is one of convenience (albeit a very in-

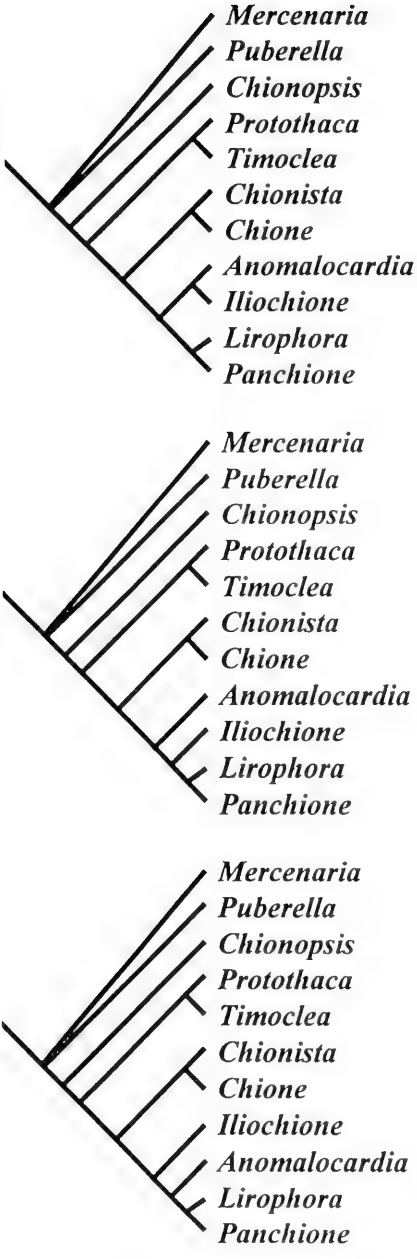

FIG. 1. Three possible solutions to the polytomy presented in Roopnarine (1996: figs. 4, 20). The upper two cladograms would support the inclusion of *Iliochione* within a new genus *Anomalocardia*, but the lowest cladogram would be incompatible with the hierarchical structure implied by such an inclusion (that is, genus *Anomalocardia* could then also include genera *Chione, Protothaca, Timoclea*, etc.).

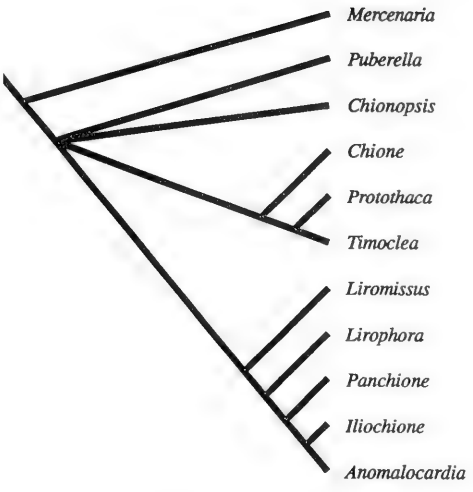

FIG. 2. Strict consensus tree of four equally most parsimonious cladograms illustrating the relationship of *Liromissus* Roopnarine (a recently described genus endemic to the late Neogene of Venezuela) to other chionine genera (consistency index = 0.426) (Roopnarine, in press). This solution supports the inclusion of *Iliochione* within *Anomalocardia* as a subgenus. Note the loss of resolution, with respect to *Chione, Chionopsis, Protothaca, Puberella* and *Timoclea* in the other portion of the tree. The correspondence between addition of a taxon and loss of resolution suggests the need for more characters, possibly non-conchological (although this would be difficult for the extinct *Liromissus*).

valuable one). The Linnean system of taxonomic classification does not necessarily lend itself to descriptions of history, and it was never intended to, nor will it ever be capable of capturing fully the depth of phylogenetic relationships implied by cladistic hypotheses.

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CORRECTIONS TO WHITE ET AL., 1996, MOLECULAR GENETIC IDENTIFICATION TOOLS FOR THE UNIONIDS OF FRENCH CREEK, PENNSYLVANIA MALACOLOGIA 38:181–202

Laura R. White

School of Forest Resources, The Pennsylvania State University, University Park, Pennsylvania 16802, U.S.A.

It has recently come to my attention that several errors appeared in the "Laboratory Techniques" section of the article "Molecular genetic identification tools for the unionids of French Creek, Pennsylvania" [White et al., Malacologia, 1996, 38(1–2): 181–202]. Specifically, the concentration of proteinase K in the standard phenol-chloroform nucleic acid extraction protocol should be 0.05 μg/μl (instead of 5 μg/μl); the concentration of $MgCl_2$ in the manufacturer-supplied amplification buffer at 1× final concentration should be 1.5 mM (instead of 15 mM); and the concentration of gelatin should be 0.001% (instead of 0.01%). I regret any inconvenience or confusion that these errors might have caused.

7 May 1997

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