PATHOBIOLOGY OF *BURENELLA DIMORPHA* JOUVENAZ AND HAZARD (MICROSPORA: MICROSPORIDA)

By

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Burenella dimorpha Jouvenaz and Hazard is a host-specific, dimorphic microsporidium that parasitizes the tropical fire ant, Solenopsis geminata (F.). The red and black imported fire ants, Solenopsis invicta Buren and Solenopsis richteri Forel, can be infected perorally by B. dimorpha, but the parasite survives only a few generations in colonies of these factitious hosts. Nine species of ants other than fire ants (representing eight genera) were refractory to infection.

Infected pupae develop pathognomonic signs (eye teratology and blister-like clearings in the occiput and petiole) that are due to destruction of the cuticle. In an advanced stage of disease, the pupa ruptures and is cannibalized by worker ants. The spores and other particulate matter are not ingested, but are diverted to the infrabuccal cavity, formed into a pellet,

expelled, and fed to fourth-instar larvae only. The intracolonial cycle of infection is thus from ruptured, diseased pupae to fourth-instar larvae via the adults, who are mechanical vectors. The binucleate, nonpansporoblast membrane-bounded (NMB) spores are infective perorally for larvae; the uninucleate, pansporoblast membrane-bounded (MB) spores are not infective, and their function is unknown. These spore types are tissue specific: NMB spores develop from disporous sporonts in the hypodermis; MB spores develop in octets from multinucleate sporonts in the fat body.

The development of MB spores is temperature-dependent. The lower thermal threshold for MB spore development is between 20 and 22.5 C; the upper thermal threshold is below 32 C. A hypothesis is advanced that these limits are set by the stability of an enzyme(s), and that the physiological function blocked in MB sporulation is meiosis.

Burenella dimorpha has two cycles of merogony (vegetative multiplication). The meronts of the first cycle were originally described as uninucleate cells that become binucleate and divide. However, these nuclei are actually pairs of nuclei in a diplocaryotic arrangement.

Surface structure has been seen for the first time in MB spores. The surface of NMB spores is smooth.

CHAPTER ONE

INTRODUCTION

The red and black imported fire ants, Solenopsis invicta Buren and Solenopsis richteri Forel, are medical and agricultural pests which infest ca 9.3×10^7 hectares (2.3×10^8 acres) in the Southeastern United States. Both species were apparently introduced into the United States with products shipped from South America to Mobile, Alabama, about 1918 and 1940, respectively. The tropical fire ant, Solenopsis geminata (Fabricius), may also be an introduced species; however, it has been a resident of this country for so long it is generally regarded as native. S. geminata is not important as a pest, except in Hawaii where it has been introduced.

Efforts to control the imported fire ants by chemical means have been the subject of serious controversy since before 1960. Consequently, the discovery of microsporidian infections in these ants in their native lands by Drs. G. E. Allen, W. F. Buren, and A. Silviera-Guido greatly stimulated interest in research on their possible use for biological control. Earlier, surveys by several investigators had failed to detect specific pathogens of fire ants in the United States.

In response to the reports of Allen, Buren, and Silviera-Guido, the Agricultural Research Service of the United States Department of Agriculture funded several trips to South America by federal and university scientists to search for potential biological control agents for fire ants. I was priviledged to participate in the first trip to Mato Grosso and Mato Grosso do Sul, the homeland of S. invicta, as a member of a team of five investigators. For five weeks we collected fire ants and laboriously examined them individually for evidence of infection. Despite the handicap of not knowing how to efficiently screen ants for disease, we collected spores of two species of microsporidia. Unfortunately, all of our attempts to infect healthy laboratory colonies of S. invicta with these spores failed.

Since we were unable to propagate and study the exotic microsporidia, a survey of pathogens of S. geminata in Florida was initiated to find a locally available host-pathogen model for studies of basic pathobiology, and to develop techniques for the detection and study of disease in ants.

The pathogen selected for study in detail was Burenella dimorpha Jouvenaz and Hazard, a microsporidium that is transmissible per os and which has the advantage of producing pathognomonic signs of infection in pupae of S. geminata. I have attempted to trace the history of this microsporidiosis form the transmission of spores to a healthy host, through the life cycle of the parasite and pathogenesis in the host, to the production of spores of the succeeding generation.

An understanding of the biology of the natural enemies of insects is fundamental to the development of strategies for their employment in pest management. The information gained in this study will contribute to our knowledge of the microsporidia in general, and, hopefully, in some small way to the development of effective biological control of the imported fire ants.

CHAPTER TWO

GENERAL LITERATURE REVIEW

As a general reference to the microsporidia, the reader is referred to the two volume monograph by Bulla and Cheng (1976, 1977). These two volumes constitute the only recent monograph on these protozoa. Earlier monographs on the microsporidia were published by Kudo (1924) and Weiser (1961); however, the development and recent extensive application of electron microscopy to the study of microsporidia has rendered these works largely obsolete. Since microsporidia are little known to most entomologists and even protozoologists, a brief synopsis of their systematics and biology is presented. A glossary of terms specifically used in the study of microsporidia and abbreviations used in this dissertation has been included.

Synopsis of the Microsporidia

Microsporidia are extremely small protozoa which are obligate (lacking mitochondria), intracellular parasites of invertebrate (primarily arthropod) and, less commonly, vertebrate animals. Their life cycles include the production of spores which contain a single sporoplasm and a long, coiled,

tubular organelle, the polar filament (or a rudiment thereof). Upon ingestion by a suitable host, the polar filament uncoils and extrudes with extreme rapidity from the spore, remaining attached anteriorly. The sporoplasm is expelled through the polar filament, which apparently evaginates as it extrudes from the spore. If the spore is near the gut wall and is properly oriented, the wall is penetrated by the violently extruding filament and the sporoplasm is injected into a host cell as though by a hypodermic syringe. There it multiplies and develops in direct contact with the host cytoplasm, there being no parasitophorous vesicle. After a large number of vegetative parasites have been produced by cycles of multiplicative development (merogony), they transform into sporonts, or cells which give rise to spores after a set number of divisions characteristic of the species (sporogony).

Sprague (1977), considering the protozoa a polyphyletic group, assigned the microsporidia to a new phylum, Microspora, consisting of two classes, Rudimicrosporea and Microsporea. The typical microsporidia, including the subject of this dissertation, are assigned to the order Microsporida, one of the two orders of Microsporea (Levine et al., 1980).

Microsporidia Parasitic in Ants

Only two species of microsporidia, Thelohania solenopsae Knell et al. (Thelohaniidae), and the subject of this dissertation, Burenella dimorpha Jouvenaz and Hazard (Burenellidae), have been described from ants.

Thelohania Solenopsae

T. solenopsae, the first specific pathogen known from fire ants and the first protozoan known from Formicidae, was discovered by Dr. W. F. Buren during a taxonomic study of S. invicta (Allen and Buren, 1974). While examining alcohol-preserved specimens from the state of Mato Grosso, Brazil, Buren observed subspherical, cyst-like bodies in the partially cleared gasters of workers. These cysts contained spores of the microsporidium, which was subsequently described from fresh material by Knell et al. (1977). Very soon after Buren's observation, Allen and Silviera-Guido (1974) reported similar microsporidia from S. richteri in Uruguay and Argentina, and from an unidentified Solenopsis species in Uruguay. T. solenopsae (or T. solenopsae and sibling species which cannot be differentiated) has since been detected in ca 22 described and undescribed species of fire ants in South America (Jouvenaz et al., 1977).

T. solenopsae infects fat body cells of workers and sexuals, and the ovaries of females. Infected cells hypertrophy, forming the cysts observed by Buren. Within the

cysts, the spores occur in octets bounded by a membrane (pansporoblast membrane). The infection is not rapidly fatal, but destruction of the fat body occurs which results in premature death of adult ants. Consequently, colonies are debilitated (Knell et al., 1977). Attempts to transmit T. solenopsae to healthy colonies in the laboratory have failed, and the mode of transmission of this parasite is unknown (Jouvenaz et al., 1981).

Burenella Dimorpha

Burenella dimorpha was described by Jouvenaz and Hazard (1978) as the type species of a new genus which represents a new family, Burenellidae. This family includes those species of microsporidia having two sporogonic sequences, one producing non-pansporoblast membrane-bounded (NMB) spores from disporous sporonts, and the other producing octets of spores bounded by a pansporoblast membrane (MB). Hazard et al. (1981) listed three additional genera as members of Burenellidae: Culicosporella Weiser and Hazardia Weiser (monotypic genera parasitic in mosquitoes) and Vairimorpha Pilley (parasitic in a variety of Lepidoptera). Certain genera of the family Thelohaniidae Hazard and Oldacre also have dimorphic (producing two morphologically distinct types of spores) species; however, their NMB spores arise from plasmodia rather than disporous sporonts.

Little is known of the pathobiology of B. dimorpha beyond those minimal aspects of spore morphology and life cycle

necessary for classification. This information will be reviewed in conjunction with the specific studies reported in this dissertation.

Other Microsporidia

In addition to B. dimorpha, at least three undescribed species of microsporidia infect S. geminata (Jouvenaz et al., 1977), and at least one undescribed species infects Solenopsis spp. in South America (Jouvenaz et al., 1980).

Other Pathogens of Ants

Because of the paucity of information on the diseases of ants, it is feasible to summarize briefly the literature on pathogens of ants other than microsporidia. The only remaining pathogen of ants which has been described is Mattesia geminata Jouvenaz and Anthony (Neogregarinida: Ophrocystidae), which also infects S. geminata in Florida. This protozoan develops in the oenocytes of the hypodermis, causing destruction of the hypodermis, melanization, and eye malformation in pupae. The infection appears to be invariably fatal in the pupal stage of development. Attempts to transmit the infection per os (using fresh, aged, and variously treated spores) and by placing infected pupae in healthy colonies (conspecific pupae are adopted) have failed (Jouvenaz and Anthony, 1979). A similar or identical neogregarine occurs in fire ants in Brazil (Jouvenaz et al., 1980).

Virus-like particles have been detected in an undescribed Solenopsis species from Brazil and in S. geminata from Florida (Avery et al., 1977). These particles are morphologically similar, being non-occluded, rod-shaped, and bound by double membranes. They occur in hypertrophied nuclei of fat body cells, but their pathogenicity is as yet undetermined. Virus-like particles have also been reported from apparently healthy wood ants, Formica lugubris Zett. (Steiger et al., 1969). These particles occurred in the cytoplasm of nerve cells, and were morphologically quite different from those found in fire ants.

Only one species of fungus, which remains unidentified, is known to be specifically associated with fire ants (Jouvenaz et al., 1977). Other fungi which have been reported as causing infections in various species of ants include Metarrhizium anisopliae (Metschnikoff) Sorokin, Beauveria bassiana (Balsamo) Vuillemin, and several species of Cordyceps. Allen and Buren (1974) summarized and discussed these reports. Broome et al. (1976) discussed the mechanism by which B. bassiana infection is initiated in S. richteri.

Solenopsis geminata

The literature on the tropical fire ant is very sparse; most studies of fire ants have been concerned with the imported species, which are pests. For information on the biology and behavior of S. geminata and fire ants in general, the reader is referred to the excellent review by Lofgren et al., (1975).

An outline of the life cycle of Solenopsis species is as follows: mating takes place during nuptial flights, after which the females (queens) return to earth, dealate, and secrete themselves in a closed, subterrainian chamber. During this "claustral" period, the queens, subsisting on their histolyzing flight muscles and food stored in their fat body and crop, lay eggs and rear their first offspring. As the number of workers increases, the queen stops caring for the immatures, but continues to lay eggs throughout her lifespan. The workers forage for food, extend and maintain the nest structure, defend the nest and territory, and care for the queen and brood. A mature colony may contain as many as 200,000 or more individuals.

The developmental stages of fire ants are egg, four larval instars, pupa, and adult. The development period is temperature dependent, but averages about three to four weeks.

CHAPTER THREE

GENERAL MATERIALS AND METHODS

Collection and Maintenance of Colonies

Laboratory colonies of fire ants were obtained by collecting queens and contingents of their workers from established field colonies, or (during the spring of the year) by capturing newly mated queens from under debris or as they wandered over the surface of the ground. Queens of other species of ants (used for host range studies) were collected exclusively by the latter method.

Ants were collected from field colonies by excavating the mounds with shovels and transporting the soil containing ants to the laboratory in plastic buckets, the inner walls of which were coated with Fluon GP-1 (ICI United States, Wilmington, Delaware 19897) to prevent escape of the ants. The soil in the buckets was slightly moistened (if necessary) and left undisturbed overnight to allow the ants to establish tunnels and to collect buried immatures. Water was then slowly dripped from medical intravenous fluids tubes into the buckets, forcing the ants to the surface of the soil. When the soil was completely submerged, masses of adult and immature ants floated or clung to the sides of the bucket, and were easily transferred with a ladle to Fluon-coated

trays (ca 52 x 40 x 7 cm) containing several layers of paper towels on their bottoms. After the wet masses of ants had dried and dispersed, the collections of ants were searched for queens. Those containing queens were transfered to laboratory nests in Fluon-coated trays held in metal racks (Fig 1).

Newly mated queens were placed in miniature nest cells or in glass culture tubes (150 x 17 mm) containing a mass of wet cotton and held in a Fluon-coated tray.

Nest cells for large colonies were constructed by pouring liquid Castone (Ransom and Randolph Co., Toledo, Ohio) to a depth of 10-12 mm in 25 x 150 mm disposable plastic petri dishes, allowing the Castone to solidify, and melting four exit ports in the sides of the bottom dish above the surface of the Castone (ports were unnecessary in the loose-fitting tops). Solidified Castone is too hard for the ants to tunnel through, yet remains slightly moist, providing adequate (but not excessive) humidity and substrate moisture within the cells for two to three months.

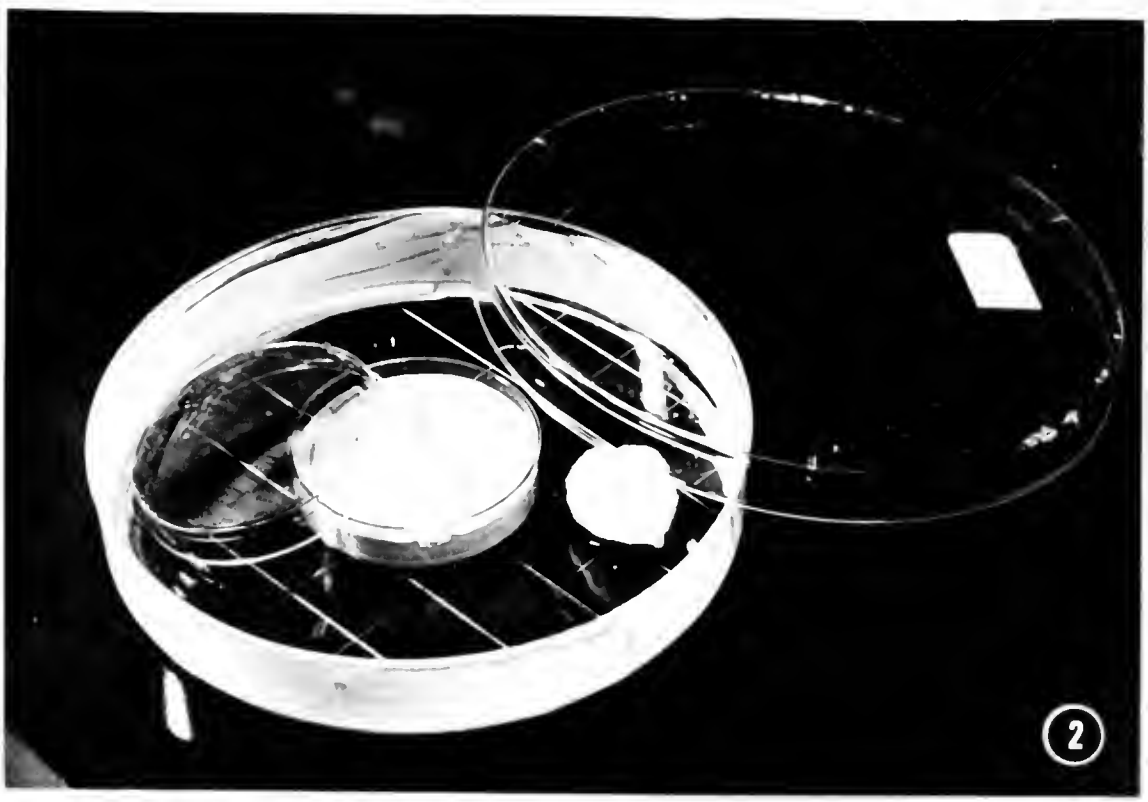
When the nest cells became dry and soiled, their tops were removed, and fresh cells were placed in the tray. The ants quickly moved into the new cells, and the abandoned old cells were discarded. Up to four cells were used simultaneously in each tray, and were replaced on a rotating basis.

Miniature nests for newly mated queens, very small colonies, or groups of experimental subjects held in isolation

Fig 1. A six-cell colony of S. geminata. Two glass culture tubes plugged with cotton and containing water or dilute honey, a dish of insect pupae, and part of a boiled egg are on the left. Alate sexuals are concentrated in the reat top cell.

Fig 2. A miniature nest cell. The nest cell has a layer of Castone (dental casting compound) in the bottom and an entry port in the side. A wad of wet cotton helps maintain high humidity. The wall of the large petri dish is coated with Fluon to prevent escape of the ants. The large petri dish top is normally taped securely to the bottom and labeled.

1



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from their parent colonies were constructed from 15 x 60 mm and 25 x 150 mm disposable plastic petri dishes. Liquid Castone was poured to a depth of ca 5 mm in the smaller dish, allowed to solidify and two or three exit ports were melted into the wall. This small nest, with lid in place, was placed inside the larger dish, the inner walls of which were coated with Fluon. A wad of wet cotton was also placed in the larger dish to help maintain high humidity (the much smaller mass of Castone in these cells dried within a few days) and the lid was taped to the petri dish bottom to provide additional security. Thus, a nest and a secure foraging area were combined in a single, compact unit (Fig 2).

Nest cells of both sizes were prepared in advance and stored in a refrigerator until needed. Prior to use, they were soaked in distilled water to saturation, then blotted with paper towels to remove excess water.

Temperature and Humidity

The colonies were maintained at a ca 28° C in a laboratory room having no humidity control; however, the Castone nest substrate and wet cotton balls (described above) maintained humidity within tolerable limits.

Diet

Laboratory colonies were fed live or dead (frozen) insectary-reared insects (cockroaches, mealworms, cabbage looper pupae), supplemented with boiled eggs, fruit, and 50%

aqueous honey solution. A diet composed of housefly or stable-fly pupae (1 liter) pureed with cooked ground beef (ca 250 g) in a blender and thoroughly mixed with whole eggs (1 dozen) and vitamins (5.0 ml Poly Vi-Sol, Mead Johnson & Co., Evansville, Indiana 47721) in hot liquid agar solution (50 g agar dissolved in 1.5 L distilled water) was also used as a supplement when insects were in short supply. The warm liquid diet was poured to a depth of ca 1.5 cm in shallow enamel pans and allowed to gel. It was then cut into cubes for dispensing to the ants. The excess diet was covered with plastic film and frozen or stored in the refrigerator.

Laboratory Propagation of Burenella dimorpha

Colonies of ants were infected with B. dimorpha by feeding them boiled egg yolk wetted with a suspension of spores to a consistency of paste. These colonies were then maintained and diseased pupae were harvested periodically as spores were needed. Prior to infection, colonies were carefully examined for other diseases using the methods described by Jouvenaz et al. (1977).

Harvest of Spores

Suspensions of spores free of cellular debris were obtained by density gradient centrifugation using Percoll (Pharmacia Fine Chemicals, 800 Centennial Ave., Piscataway, N.J. 08854). Pupae exhibiting signs of advanced infection

were homogenized in an equal volume of distilled water using a glass tissue grinder, and the homogenate was centrifuged at 10,000 g for ca 20 minutes through a discontinuous gradient consisting of layers of 25, 50, 75, and 100% Percoll. Over 97% of B. dimorpha NMB spores purified in this manner extruded their polar filaments when ingested by ant larvae (determined by phase microscopy of meconia), indicating that most immature spores had been removed. Slightly less highly purified suspensions suitable for use in routine propagation of the parasite were obtained by centrifuging the crude homogenate at 750 g for 20 minutes in a 50% solution of Percoll. After all centrifugations with Percoll, the spores were washed three times with distilled water, and the volume was adjusted to ca 10^7 spores/ml. Spore suspensions were stored at 4° C.

CHAPTER FOUR

TRANSMISSION AND INFECTIVITY OF SPORES OF *BURENELLA DIMORPHA*

Burenella dimorpha produces two morphologically distinct types of spores. Binucleate NMB spores develop from disporous sporonts in the hypodermis; uninucleate, MB spores develop in octets from multinucleate sporonts in the fat body. The infection is characterized by the development of clear, blister-like areas in the occiput and petiole which are due to destruction of the cuticle. As the infection progresses, the clear areas increase in size, the cuticle becomes extremely fragile, and eventually it ruptures. The pupa is then cannibalized by workers (I have observed this in laboratory colonies).

Suspensions of spores of *B. dimorpha* containing both types of spores are infective perorally for *S. geminata* (Jouvenaz and Hazard, 1978). However, attempts to separate the spore types by density gradient centrifugation were unsuccessful (the pansporoblast membrane is very delicate, rupturing on dissection of the host, and the free MB spores are nearly identical in bouyant density to NMB spores). Therefore, Jouvenaz and Hazard were unable to determine which or if both types are infective. They described this

parasite as a dimorphic species on the basis of light microscopy studies of the life cycle, and the statistical improbability of a dual infection occurring at high frequency, but they were not able to experimentally demonstrate by feeding tests that both spore types were produced by the same microsporidium.

Adult fire ant workers feed liquid food to larvae in all instars, but solid foods to fourth-instar larvae only (Petralia and Vinson, 1978). A pellet of solid food is expelled from the infrabuccal cavity and placed on the praesaepium of a larva by a worker, and the pellet is consumed by the pupa. This method of feeding larvae suggested a cycle of infection from ruptured pupae to fourth-instar larvae vectored mechanically by adults via the infrabuccal pellet. This study was conducted to 1) determine the mode of intracolonial transmission of infection, 2) to determine whether one or both spore types are infective, and 3) to verify that B. dimorpha is a dimorphic species.

Materials and Methods

Boiled egg yolk wetted to a paste consistency with a suspension of spores was offered to a small, healthy colony of S. geminata, which was allowed to feed for 24 hr. A sample of workers trapped immediately after feeding was held one hour, killed by freezing, sectioned into the three body parts, and examined by phase-contrast microscopy to

determine whether spores were ingested or diverted to the infrabuccal cavity (microsporidian spores are visible in slightly compressed, intact fire ant body segments at a magnification of 300X). Heads of these workers were fixed, stained with heavy metals, embedded in Spurr-Quetol resin, sectioned, and examined by phase-contrast microscopy. The details of these procedures are given in the section on host pathology. Eight fresh infrabuccal pellets were removed from the praesaepia of larvae and examined by phase microscopy for spores.

After 24 hours, 109 fourth-instar larvae were removed from the nest and held in a miniature nest cell with conspecific workers that had not been exposed to B. dimorpha. These workers functioned as nurses (immatures held in isolation from adults are quickly attacked by fungi). After 21 days these immatures (now pupae) were examined for infection. A group of 79 prefourth (primarily third) instar larvae were also removed from the same nest and held in a similar manner until they eclosed as adults.

A suspension of mature spores of both types was prepared by homogenizing diseased pupae in distilled water with a glass tissue grinder and centrifuging the extract in a discontinuous Percoll gradient (100%, 75%, 50%, and 25%) for 20 minutes at ca 10,000 g (Jouvenaz, 1981). This procedure produced a clean suspension of spores of both types, almost all of which appeared to be mature. These spores were fed

to fourth-instar larvae in a paste of finely powdered (mortar and pestle) dry baby cereal and spore suspension. Infrabuccal pellet-sized quantities of this preparation were placed on the praesaepia of fourth-instar larvae with a flattened insect pin, and the larvae were held in a miniature nest cell until pupation. Adult workers were provided to care for these larvae ca 4 hours after feeding. When the larvae pupated, 20 meconia were recovered and examined by phase-contrast microscopy to determine whether spores had extruded their polar filaments.

A suspension of NMB spores only was obtained by selecting diseased pupae which, on the basis of pathognomonic signs, were estimated to harbor some mature NMB spores but no mature MB spores (NMB spore development precedes MB spore development). Wet squashes of these pupae were examined individually by phase-contrast microscopy, and those that were free of mature MB spores were washed from the slide, pooled, and cleaned and concentrated by centrifugation. An examination of 10,000 spores individually in a diluted aliquot (0-5 spores/field) and careful scanning of the concentrated suspension confirmed the absence of MB spores.

The suspension of NMB spores was mixed with boiled egg yolk and fed to a small healthy colony of S. geminata. After 20 days, 25 pupae in advanced infection were individually homogenized in ca 0.5 ml water and examined by phase-contrast microscopy (0-6 spores/field) to determine the ratio of the

spore types in the first 200 spores seen. Twenty-five infected pupae from a laboratory colony that had been infected with a crude suspension of mixed spores for routine propagation of the parasite were also examined.

Results

Solenopsis geminata adults did not ingest spores into the crop; instead, they diverted them to the infrabuccal cavity (Figs 3 and 4). One hour after exposure to food containing spores, 100 of the 160 workers that were examined contained spores in their infrabuccal cavities. Not one contained even a single spore in the digestive tract. All eight infrabuccal pellets removed from fourth-instar larvae shortly after deposition by workers contained numerous spores.

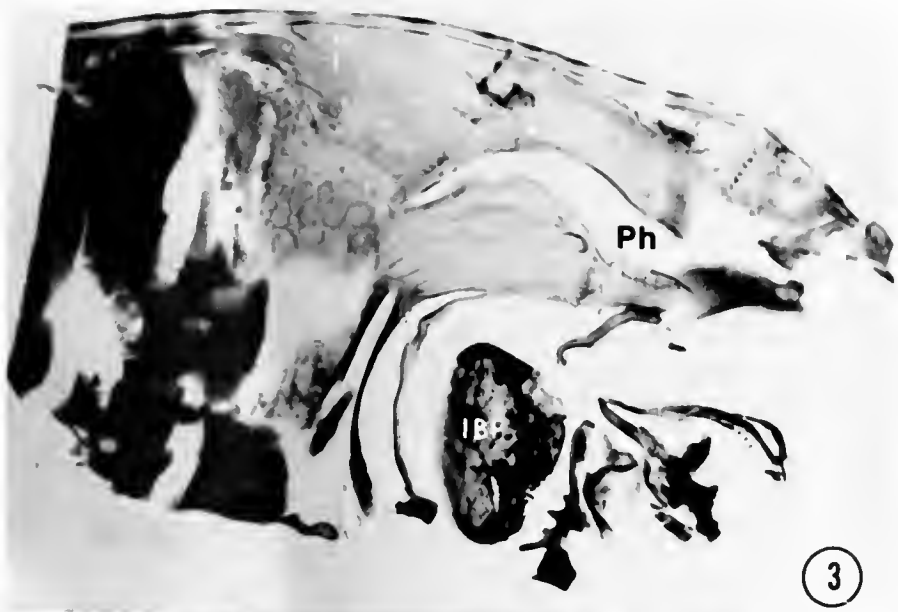
Only fourth-instar larvae became infected with B. dimorpha. Seventy-one of the 109 that were exposed as fourth-instar larvae became infected; none of the 79 immatures that were exposed to spores as prefourth-instar larvae became infected.

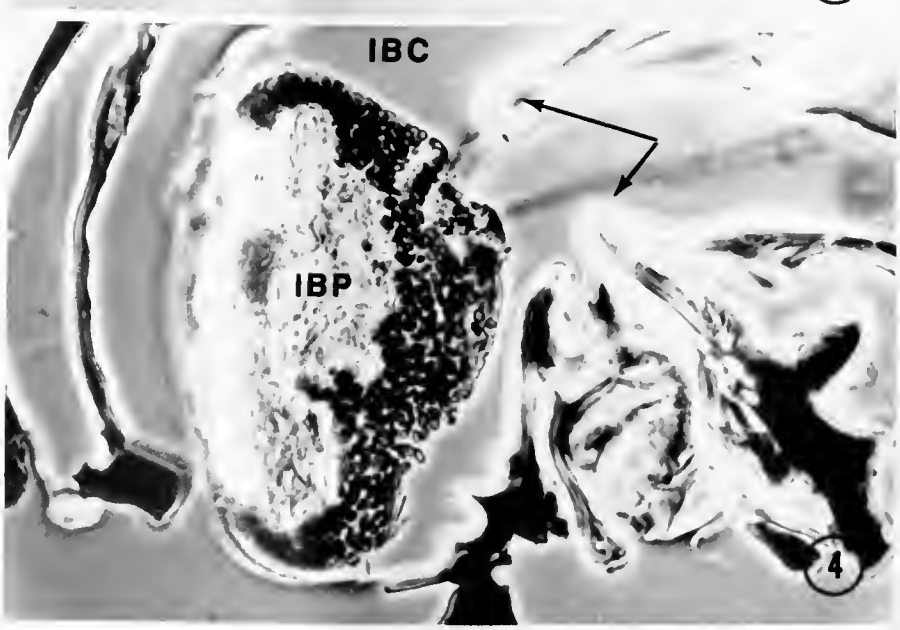
Nonmembrane bound spores were infective perorally for S. geminata and produced infections with pathognomonic signs and both spore types. Furthermore, the spore types were produced in normal ratios. Pupae that had been fed NMB spores only contained 28.6% ± 10.2 (range 16-48%) MB spores; those that had been fed both spore types contained 30.8% ± 7.8 (range 16-43%) MB spores.

In the meconia of larvae fed the suspension of mature spores of both types, 1,723 (97.1%) of the 1,774 spores found

Fig 3. Infrabuccal cavity. This medial section of an adult fire ant worker head shows an infrabuccal pellet in the infrabuccal cavity. P = pharynx; IBC = infrabuccal cavity; IBP = infrabuccal pellet.

Fig 4. Enlarged view of infrabuccal cavity and pellet. Numerous MB and NMB spores may be seen in the pellet. Arrows point to valves of the orifice of the infrabuccal cavity.


Ph
IBP
3


IBC
IBP
4

had extruded their polar filaments. The 51 (2.9%) NMB spores that had not extruded their polar filaments were probably not quite mature, as they were very slightly less refractile or less deep amber internally than mature spores.

Membrane bound spores did not appear to be infective per os for S. geminata. Examination of meconia showed that few if any MB spores had extruded their polar filaments while in the larval gut. A total of 274 mature, nonextruded MB spores were seen in the 20 meconia, but only one body was seen which strongly appeared to be an extruded MB spore. Five additional bodies were seen that resembled extruded MB spores but almost certainly were not. The meconia contained occasional empty walls of ingested or gut unicellular fungi, some of which superficially resembeled extruded MB spores; extruded NMB spores were distinct.

Discussion

The cycle of B. dimorpha infection within an ant colony may be summarized as follows: NMB spores develop in the hypodermis, producing clear areas in the heads, petioles, and gasters of pupae. As the infection progresses, the cuticle becomes very fragile and eventually ruptures. The adult ants cannibalize these ruptured pupae but do not ingest the spores. Instead, the spores, together with other particulate matter, are diverted to the infrabuccal cavity and formed into an infrabuccal pellet. This

pellet is expelled and placed on a specialized anteroventral area, the praesaepium, of fourth-instar larvae. The praesaepium, which bears spines specialized for holding solid food while the larva feeds, is absent from earlier instars, which are fed only liquid. Because of this method of feeding, the fourth-instar larva is the only stage which is vulnerable to infection. Both spore types are ingested, but only the NMB spore is infective. The MB spores are expelled unextruded in the meconium upon pupation.

It is evident that B. dimorpha is indeed a dimorphic microsporidium, since ingestion of NMB spores only resulted in typical infection in which both spore types were produced in normal ratios.

The function of the MB spore remains unknown. A most attractive hypothesis is that it either infects an alternate host or is primed in the gut of a mechanical vector for extrusion upon subsequent ingestion by ant larvae. Either would explain the mode of intercolonial transmission of the infection (fire ants are territorial and aggressive towards conspecific ants). Many candidate species exist for the role of vector; a large and varied arthropod fauna is associated with fire ants. Collins and Markin (1971) listed 52 species of insects that have been collected from fire ant nests; other invertebrates also occur. At least some of these organisms have symbiotic relationships with fire ants and are known to travel between fire ant nests (Wojcik, 1975).

CHAPTER FIVE

HOST SPECIFICITY OF *BURENELLA DIMORPHA*

Until recently, microsporidia were generally assumed to be highly host specific, and the discovery of an infection in a new host frequently led to the description of a new species. Now microsporidia are known to vary greatly in host specificity. Some species, particularly the parasites of lepidoptera, are able to infect numerous hosts in different genera, families, and even orders. Other species appear to be highly host specific; however, in some cases the difficulty of transmitting infection in the laboratory makes it impossible to determine host range. The host ranges of only a few species have been extensively investigated (Brooks, 1974).

The successful *per os* transmission of *B. dimorpha* infection to healthy colonies of *S. invicta*, *S. richteri*, and the southern fire ant, *Solenopsis xyloni* McCook, as well as the natural host, *S. geminata*, was reported by Jouvenaz and Hazard (1978). High rates of infection were obtained in colonies of these species by feeding them spores mixed with boiled egg yolk. The infected pupae exhibited typical pathognomonic signs, and Jouvenaz and Hazard concluded that "the course of the disease is identical in all four species." (p. 27)

After these initial transmission tests had been published, I attempted to propagate the parasite S. invicta. This species is not only the pest we ultimately hope to control, but I have found it easier to obtain and culture than S. geminata. Several colonies were fed spores and, as expected, successfully infected; however, after two or three months, these colonies were free of the parasite. It appeared then, that B. dimorpha might not be able to maintain itself in factitious fire ant hosts, even though these hosts were readily infected. The studies reported here were conducted to determine the degree of host specificity of B. dimorpha. In addition to fire ants, several species of ants of other genera were tested for susceptibility to infection by B. dimorpha.

The corn earworm, Heliothis zea (Boddie) (Lepidoptera: Noctuiidae) is susceptible to infection by Nosema algerae Vavra and Undeen, a microsporidium which is highly pathogenic to anophiline mosquitoes. Heliothis zea was being used for mass production of N. alterae spores at the Insects Affecting Man and Animals Research Laboratory, USDA, Gainesville, during part of the period in which I conducted my research. I therefore took advantage of the availability of this moth and tested it for susceptibility to infection by B. dimorpha.

Materials and Methods

Three small (ca 500 workers) colonies of S. invicta and one larger colony (ca 10,000 workers) of S. richteri (the only colony of this species that was available) were infected by allowing them to consume diseased pupae of S. geminata. The colonies of S. invicta were fed 15 selected pupae in the advanced stages of infection on each of the following days: Colony #1, days 1 and 3; colony #2, days 1, 2, and 3; colony #3, days 1, 3, and 5. The colony of S. richteri was fed 50 selected pupae on one occasion.

The colonies were examined 3 weeks after the final feeding to determine the initial rate of infection, and at monthly intervals thereafter. Pupae old enough (judged by eye development) to exhibit pathognomonic signs if infected were examined individually against a black background with a stereomicroscope illuminated by an annular fiber optic. All, or a maximum of 1,000, of these pupae were examined from each colony.

Ants of other species were gifts from colleagues or were collected after mating flights and reared as described in General Materials and Methods. Identifications of species were made or confirmed by Dr. Daniel P. Wojcik, Agricultural Research Service, USDA, Gainesville, or by Mr. James Trager, Department of Entomology and and Nematology, University of Florida. These ants were also fed selected diseased

pupae of S. geminata (ingestion of the pupae was confirmed by observation), and spores mixed with their regular diet. In addition to examining pupae for pathognomonic signs, aqueous extracts of pupae were examined by phase-contrast microscopy for spores in case external signs were not evident.

Larvae of H. zea and their rearing media were obtained from Mr. D. W. Anthony, Agricultural Research Service, USDA, Gainesville. The protocols for conducting infectivity tests with B. dimorpha included those used by Anthony et al. (1978) to infect H. zea with H. algerae for mass production of spores. Twenty larvae 4-5 days old were held in isolation without food or water for 24 hr, fed 0.1 ml of a suspension containing ca 10^6 spores per ml, and then placed on fresh media. Twenty larvae 8-10 days old were allowed to consume one diseased S. geminata pupa each, and an additional 20 larvae 8-10 days old were injected with 2-10 ul of spore suspension. The adult moths were examined by phase-contrast microscopy ca one month after they had ingested or been injected with spores.

Results

The B. dimorpha did not persist in S. invicta or S. richteri for more than a few generations (Table 1). Three weeks after the final feeding of B. dimorpha spores, the S. invicta colonies were infected at the rates of 6% (colony #1), 44% (colony #2), and 4% (colony #3). The colony of

Table 1. *B. dimorpha* infection rates in colonies of *S. invicta* and *S. richteri*.

Number of days after ingestion of spores	*S. richteri*	*S. invicta*		
		colony #1	#2	#3
21	5%	6%	44%	4%
49	0	0	8.5%	0
80	0	0	<1%[a]	0
110	-	-	0	-
140	-	-	0	-

[a]One pupa in the nest population of 786 was infected.

S. richteri had an infection rate of 5%. One month later (day 49), however, infection could be detected only in S. invicta colony #2, and the infection rate in this colony had declined from 44% to 8.5%. One month later (day 80), only one infected pupa (out of 786 pupae in the age group that exhibits pathognomonic signs) was found in the colony. By day 110, all of the 1,000 pupae that were examined were free of disease. All four colonies remained free of B. dimorpha on all subsequent examinations. In contrast, the colony of S. geminata from which the diseased pupae (those fed to the test colonies) were taken maintained an infection rate of ca 50% for 14 months.

None of the other species of ants tested were susceptible to infection by B. dimorpha (Table 2). The H. zea was not susceptible to infection either per os or by injection.

Discussion

The B. dimorpha is host specific for S. geminata. Although S. invicta and S. richteri may be readily infected, the parasite does not persist in these factitious hosts beyond a few generations. The 9 species (representing 8 genera) of ants other than Solenopsis spp. proved refractory to infection. While this is a very small sample of the living species of ants, it is highly improbable that a parasite that cannot maintain itself in factitious species of the genus of its host would be able to infect species of other genera.

Table 2. Susceptibility of selected species of ants and a moth to infection by *B. dimorpha*.

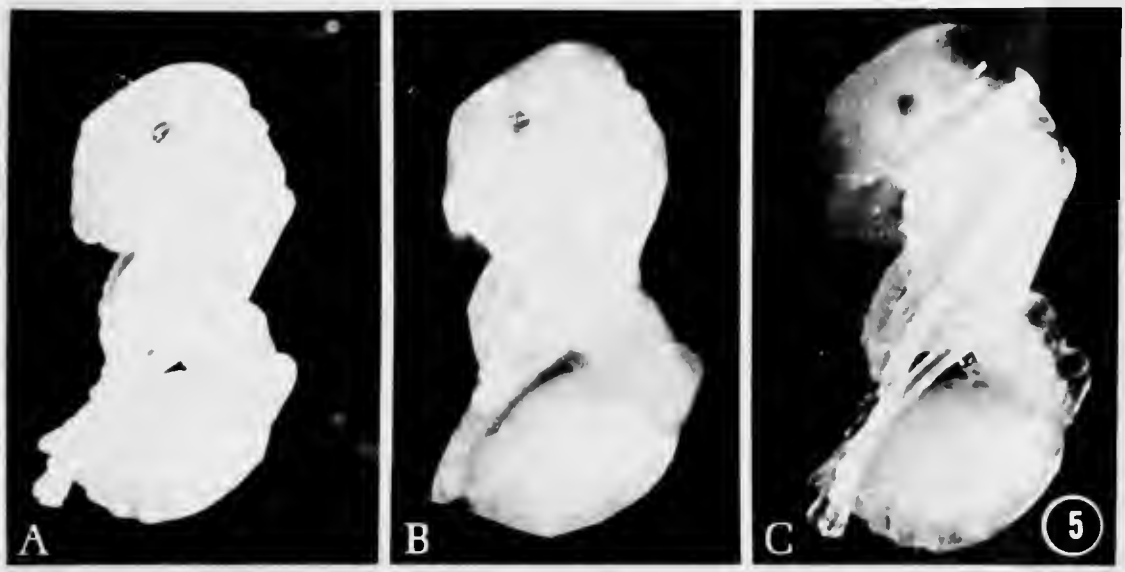
	Susceptibility[a]
HYMENOPTERA: FORMICIDAE	
Subfamily Myrmicinae	
Solenopsis geminata (Fabricius)	+
S. xyloni McCook	±?
S. invicta Buren	±
S. richteri Forel	±
Pheidole morrisi Forel	-
Monomorium minimum (Buckley)	-
M. floricola (Jerdon)	-
Crematogaster clara Mayr	-
Aphenogaster ashmeadi (Emery)	-
Ochetomyrmex auropunctatus (Roger) (= *Wasmannia auropunctata*)	-
Subfamily Pseudomyrmecinae	
Pseudomyrmex elongata Mayr	-
Subfamily Formicinae	
Paratrechina longicornis (Latreille)	-
Camponotus floridana (Buckley)	-
LEPIDOPTERA: NOCTUIDAE	
Heliothis zea (Boddie)	
per os	-
injection	-

[a] + = susceptible to infection
± = susceptible, but infection does not persist
- = not susceptible to infection

Following infection with spores of B. dimorpha from S. geminata, signs typical of infection in S. geminata develop in pupae of S. invicta and S. richteri. However, pupae that are infected subsequently in these colonies (by spores produced in siblings) develop atypical signs. The blister-like clearings in the head and gaster are not apparent, although faint clearing in the petiole may occur. Abnormal eye development typical of the disease was noted, however (Fig 5). Jouvenaz and Hazard were premature in their statement that the course of the disease is identical in all four species; they had examined only pupae infected with spores produced in S. geminata.

The reason(s) for the failure of B. dimorpha to cycle indefinitely in S. invicta and S. richteri is unknown. Obviously, they are "not well adapted" to these species, but this merely restates our observations. Perhaps the cuticle is less likely to rupture and cannibalization is less likely in these species due to dehydration or putrification in the intact state. The cadavers may then simply be discarded.

Fig 5. Diseased S. richteri pupa compared to healthy and diseased S. geminata pupae. The pupa in 5A is healthy. The signs exhibited by the S. richteri pupa (5B) are as pronounced as they will become. Note the faint clearing in the petiole and the slight blurriness and irregularity of outline of the eye. The diseased S. geminata pupa (5C) exhibits typical pathognomonic signs at maximal expression. X30.


A
B
C
5

CHAPTER SIX

ABUNDANCE OF *BURENELLA DIMORPHA* IN NATURE

As part of a survey for pathogens of native and imported fire ants in the United States, Jouvenaz et al. (1977) examined 307 colonies of *S. geminata* from 74 collection sites in Florida and Georgia. Twelve (3.9%) of these colonies from seven collection sites in Florida were infected with *B. dimorpha*. The infection rate at the type locality of *B. dimorpha* (which was not included in the survey) was obviously much higher than 3.9%. Therefore, I decided to investigate the infection rate at this specific site.

Materials and Methods

Sixty-nine colonies of *S. geminata* from the type locality of *B. dimorpha* (State Road #26, ca 1-3 km east of Interstate Highway 75, Alachua County, Florida) were screened for disease as described in General Materials and Methods. Forty of these colonies were examined in August, 1975, and the remaining 29 were examined in September, 1977.

Results and Discussion

The infection rate at the type locality of *B. dimorpha* was much higher than that of the 307 colonies examined by Jouvenaz et al. Seventeen (42.5%) of the 40 colonies in the first

group, and 4 (13.8%) of the 29 colonies in the second group (collected two years later) were infected. These limited data suggest that B. dimorpha may be quite common in specific localities, but not homogeneously distributed in nature. Unfortunately, surveys for disease are very expensive, time-consuming, and laborious, and the prospects of amassing data sufficient to draw conclusions about the distribution and abundance of B. dimorpha in nature appear remote. The S. geminata has now been almost completely displaced from the type locality of B. dimorpha by S. invicta.

CHAPTER SEVEN

PATHOLOGY OF *BURENELLA DIMORPHA* INFECTION

Very few protozoan diseases of insects may be diagnosed by simple observation of pathognomonic signs. Pebrine (infection of the silkworm, *Bombyx mori* L., by the microsporidium *Nosema bombycis* Naegeli) provides a classic example of pathognomonic signs in insects, being characterized by the appearance of dark, pepper-like spots on the integument. Infection of the tropical fire ant, *Solenopsis geminata* (Fabricius), by the neogregarine *Mattesia geminata* Jouvenaz and Anthony (1979), may be readily diagnosed by eye teratology and a pattern of abnormal melanization in pupae beginning with a "sooty" black discoloration of the legs. In the same host, infection by *Burenella dimorpha* Jouvenaz and Hazard is manifested by signs of a type that appears to be unique in insects. In general, however, specific manifestations of protozoan infections, if present at all, are related to particular tissues and may be detected only by histological examination (Brooks, 1974).

Insects infected by protozoa may exhibit nonspecific signs such as loss of appetite, diarrhea, sluggishness, irregular growth, stunted or malformed adults, reduced fecundity, and premature death of immatures or adults. Often,

however, there are no indications of disease other than premature death (Brooks, 1974). Individual fire ant workers infected with Thelohania solenopsae (Knell et al., 1977) or any of four undescribed species of microsporidia, for example, cannot be differentiated from healthy specimens by either appearance or behavior. Diagnosis of these infections must be made by microscopic examination or, in the case of T. solenopsae, by observing cysts in dissected gasters. Similarly, bees, Apis spp., infected by Nosema apis Zander are completely devoid of outward signs of disease, and even histological diagnosis is difficult (Bailey, 1981).

Pupae of S. geminata infected by B. dimorpha exhibit pathognomonic signs that are obvious, and, to the best of my knowledge, unique. Jouvenaz and Hazard (1978) attributed these manifestations to destruction of the hypodermis and described them as follows:

> A clear area in the occipital region of the head, which appears about the time the developing eyes become prominent, is usually the first noticeable change. Later, similar clear areas appear in the petiole and gaster, and the eyes become irregular in outline and appear sunken. Pupae having such changes do not mature or even melanize. Instead, the clear areas increase in size, and the cuticle eventually ruptures. (p. 27)

Jouvenaz and Hazard also noted that NMB spores develop in the hypodermis, and MB spores develop in the fat body.

This brief description of the appearance of diseased worker pupae and notation of tissue specificity of the spore types is all that has been published on the pathology of

B. dimorpha infection. The signs of infection in sexual pupae have not been described. The development and histopathological basis of the unique manifestations of this disease are described here.

Materials and Methods

The progressive development of pathognomonic signs was recorded by periodically photographing healthy and diseased pupae. Diseased pupae exhibiting the minimal degree of eye development necessary for diagnosis and healthy pupae of corresponding age and size were paired and held in miniature nest cells. The specimens were positioned on a plate of nonglare glass above a black parabolic background, illuminated by an annular fiber optic illuminator, and photographed through a stereomicroscope fitted with a polarizing filter. Photographs were made ca every 48 hours until the healthy specimen had eclosed and the diseased specimens had reached maximum expression of pathognomonic signs.

Specimens for both light and transmission electron microscopy were fixed and embedded in epoxy resin. Tissue specimens (heads, gasters, whole larvae, etc.) were prefixed in buffered 1% osmium tetroxide (0.1 M sodium cacodylate buffer, pH 7.5) for 30-60 minutes at room temperature, rinsed in the same buffer, and partially hardened in buffered 2.5% gluteraldehyde-1% acrolein (same buffer). Specimens were washed in buffer and usually stored in Histocon (polyvinylpyrrolidone, Tris-HCl, 2% chlorhexidine, distilled water;

Polysciences, Inc.) in the refrigerator overnight or for several days prior to postfixation. Specimens were washed in buffer, postfixed in buffered 1% osmium tetroxide for 2 hours at room temperature, washed in deionized water, and en bloc stained in 0.5% aqueous uranyl acetate overnight. Specimens were dehydrated with acidified 2,2-dimethoxypropane (Lin et al., 1977) and infiltrated and embedded in a Spurr-Quetol 651 resin (Ringo et al., 1979).

Blocks were sectioned with a LKB Huxley ultramicrotome. For light microscopy, 2-4 um sections were cut on dry glass knives, spread in a drop of 10% acetone, mounted in immersion oil, and studied by phase-contrast microscopy. Thinner sections, 0.5-1.0 um, were stained with 1% aqueous toluidine blue in 1% aqueous borax, rinsed in deionized water, mounted in immersion oil, and examined by either bright-field or phase-contrast microscopy. For transmission electron microscopy, gold sections were poststained with 2% aqueous uranyl acetate followed by lead citrate (Reynolds, 1963). Grids were examined and photographed at an accelerating voltage of 75 kV in a Hitachi H-600 electron microscope.

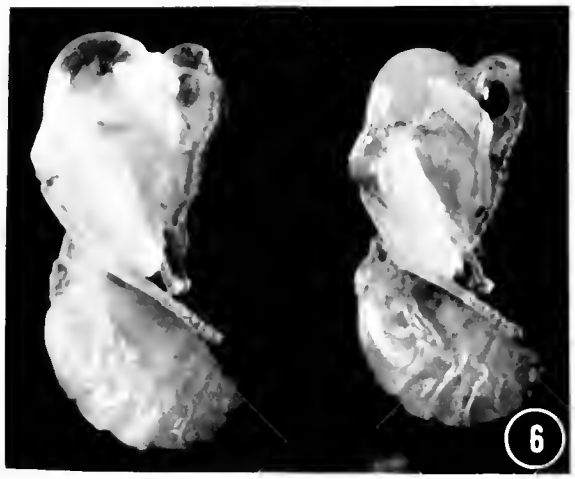
Results

Portraits of healthy and diseased male, female, and worker pupae are presented in Figs 6-8. The clearing in the dorsal thorax of the sexual pupae does not occur in worker pupae, and occasionally is reduced or absent in

Fig 6. Diseased (left) and healthy male pupae of S. geminata. Note the clearing of the occiput and dorsal thorax. The light color of the eye is due to destruction of the lens and a fluid space over the eye. X 10.

Fig 7. Diseased (left) and healthy female pupa of S. geminata. The apparent faint clearing in the head of the healthy specimen is a photographic artifact. X10.

Fig 8. Healthy (left) and diseased worker pupae of S. geminata. Note eye pathology and clearing in occiput and petiole of the diseased pupa. X 40.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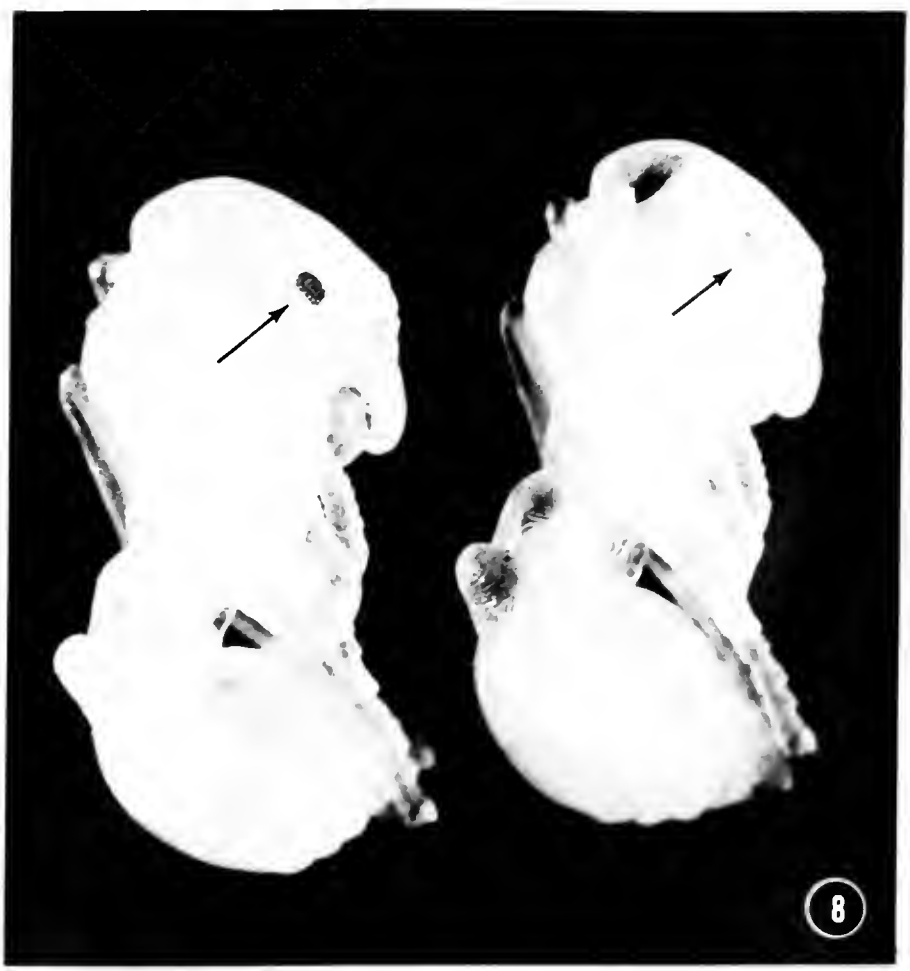
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sexual pupae. The eyes of the sexual pupae appear to be less affected than the eye of the worker pupa; this may be an artifact due to the larger (ca 16-20X in area) eyes of sexual pupae. The diseased worker pupa is in an advanced stage of infection, and exhibits typical pathognomonic signs. Note the irregular outline of the eye, the derangement of the facets, and its fainter color.

The development of pathognomonic signs is recorded in Fig 9. In the first pair of photographs, the healthy and diseased pupae (1H and 1D, respectively) are almost indistinguishable. The diseased pupa exhibits a faint clearing of the petiole, and in life--but not in the photograph--the earliest faint signs of eye teratology could be detected. In the photograph taken 48 hours later (3D), the clearing in the petiole is not evident due to a slight change in lighting. Subsequent photographs show the development of clearing in the head and petiole. The eye of the diseased pupa is rather blurry in these photographs. This is not due to focus, but to a fluid space over the eye.

The healthy pupa eclosed on day 18. The last photograph of the diseased pupa (3D) shows an indentation in the gaster, indicating dehydration. In a colony, rupture would probably have occurred by this time as a result of manipulation (being moved, groomed, etc.) by the workers.

Frontal sections of whole heads of younger and older diseased pupae are presented, respectively, in Figs 10 and 12.

Fig 9. The development of pathognomonic signs in pupae of S. geminata infected by B. dimorpha. The numerals indicate the number of days after the series began that the photographs were taken. The letters H and D indicate, respectively, the healthy and diseased specimens. X 30.

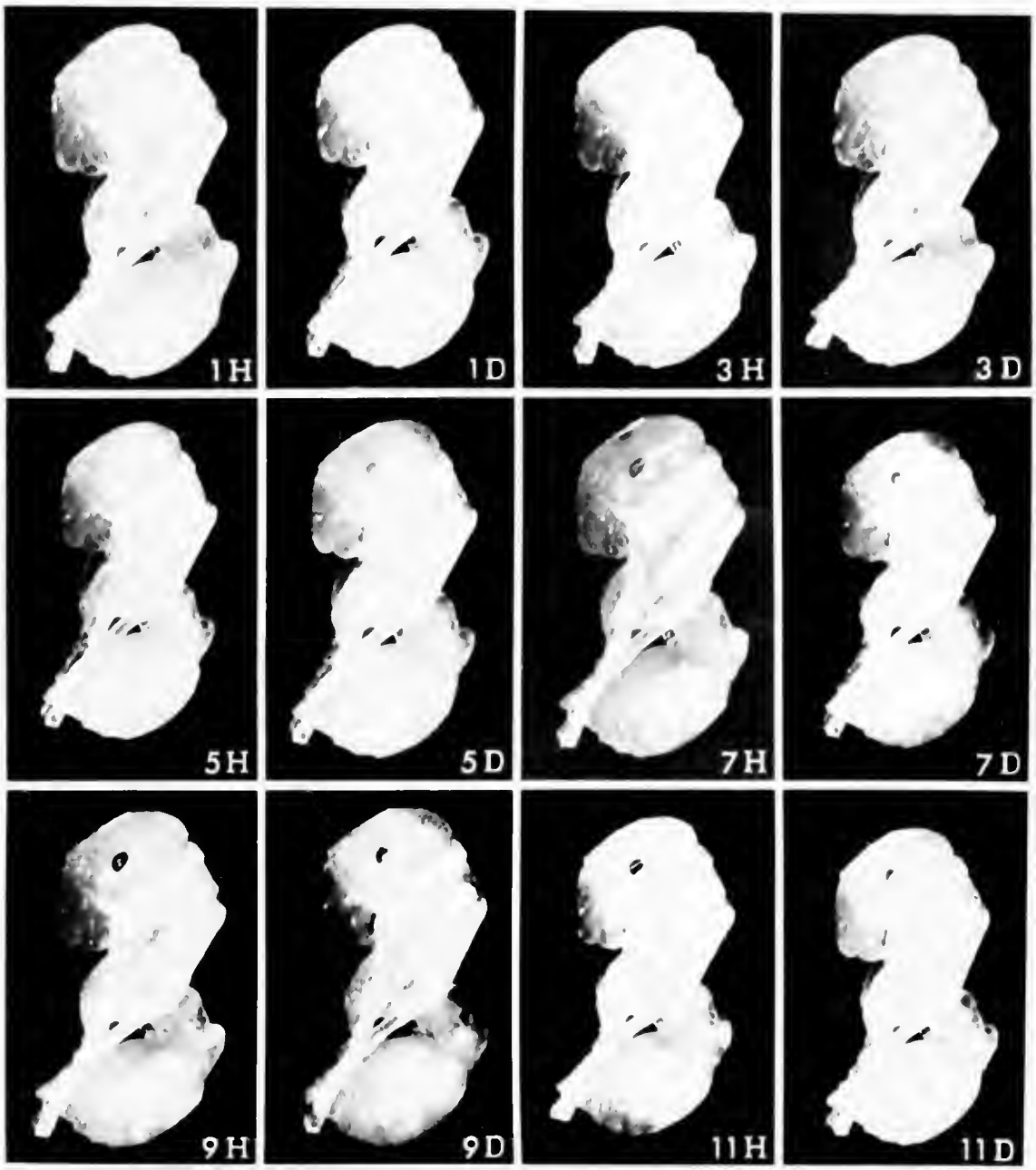

1H
1D
3H
3D
5H
5D
7H
7D
9H
9D
11H
11D

Fig 9 (cont.) The development of pathognomonic signs in pupae of S. geminata infected by B. dimorpha. The healthy pupa eclosed on day 18 (18H). X 30.

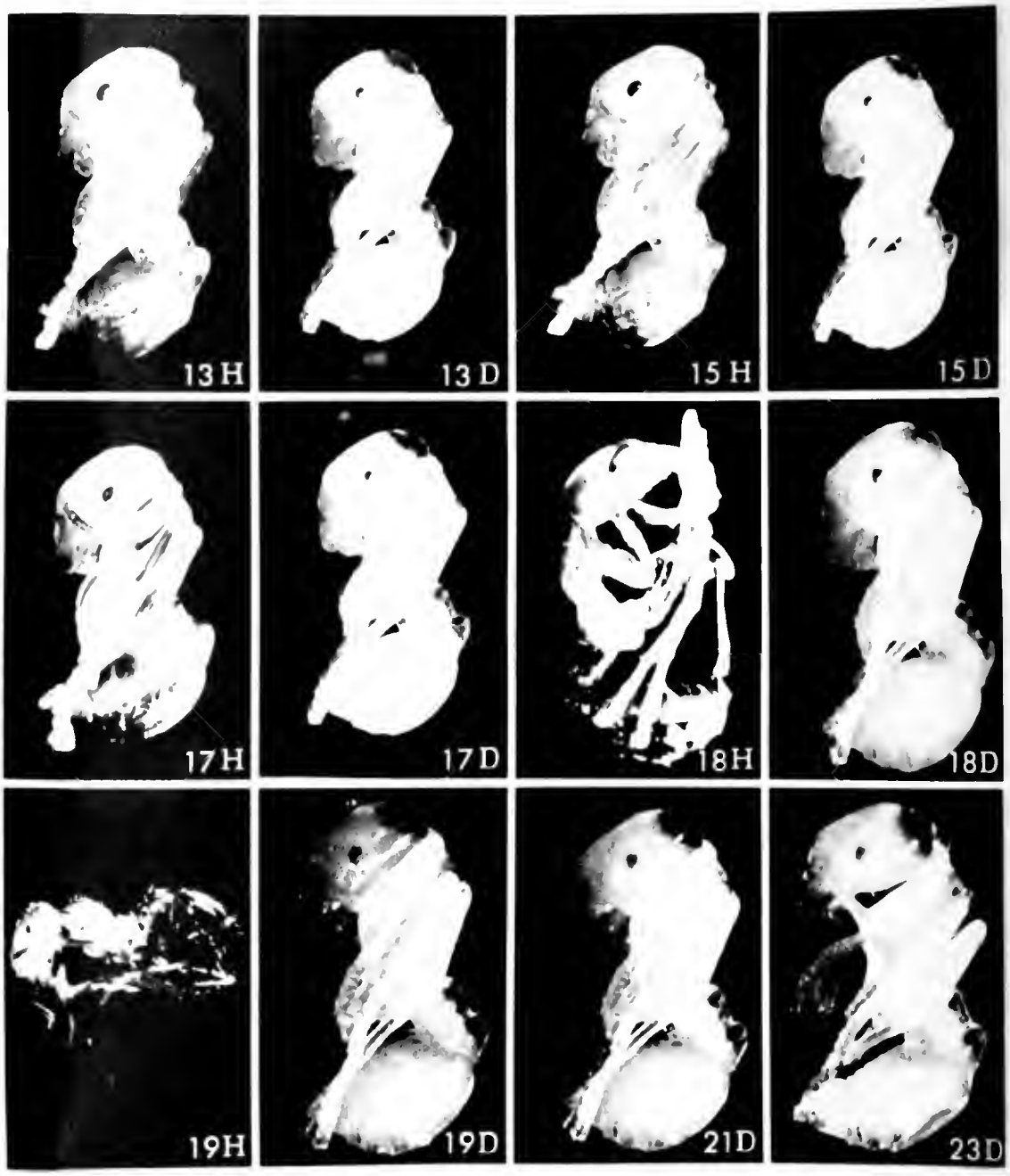
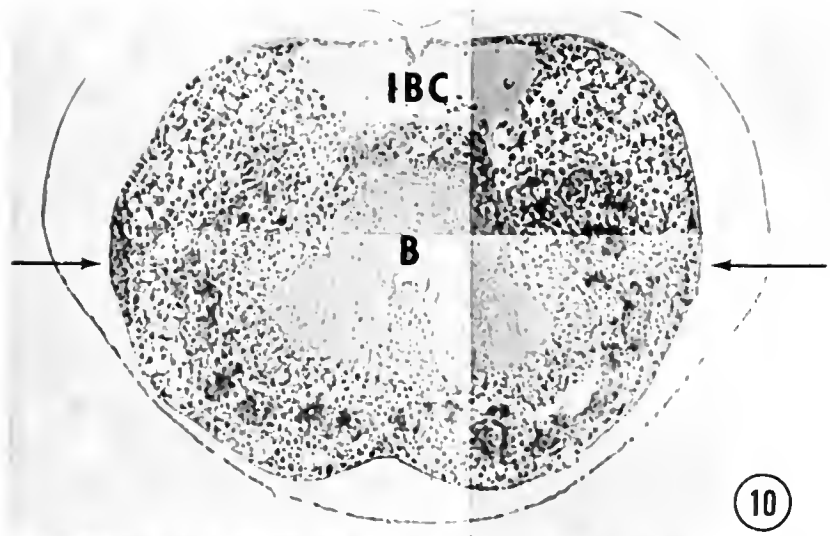

13H
13D
15H
15D
17H
17D
18H
18D
19H
19D
21D
23D

Fig 10. Frontal section through the head of a young (ca 10 days postpupation) pupa infected by B. dimorpha. Note the fluid space between the head and pupal sheath. B = brain; IBC = infrabuccal cavity; arrows point to the eyes. X 90.

Fig 11. Eye, optic nerve, and optic stalk of a young (ca 10 days postpupation) pupa infected by B. dimorpha. OS = optic stalk; ON = optic nerve; arrow points to eye. X 600.

Fig 12. Frontal section through the head of an older (ca 16 days postpupation) pupa infected by B. dimorpha. Note the shrunken, fiberous appearance of the brain and sunken, amorphous eyes. B = brain; IBC = infrabuccal cavity; arrows point to eyes. X 90.



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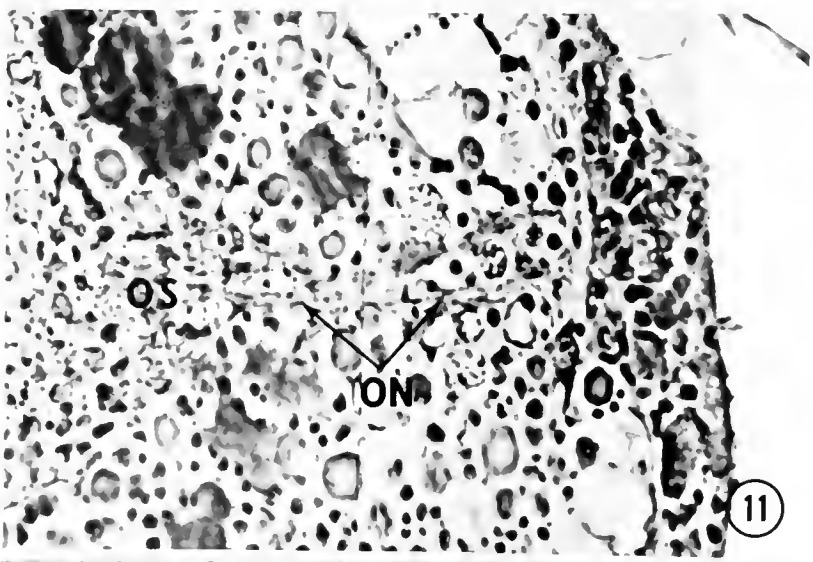

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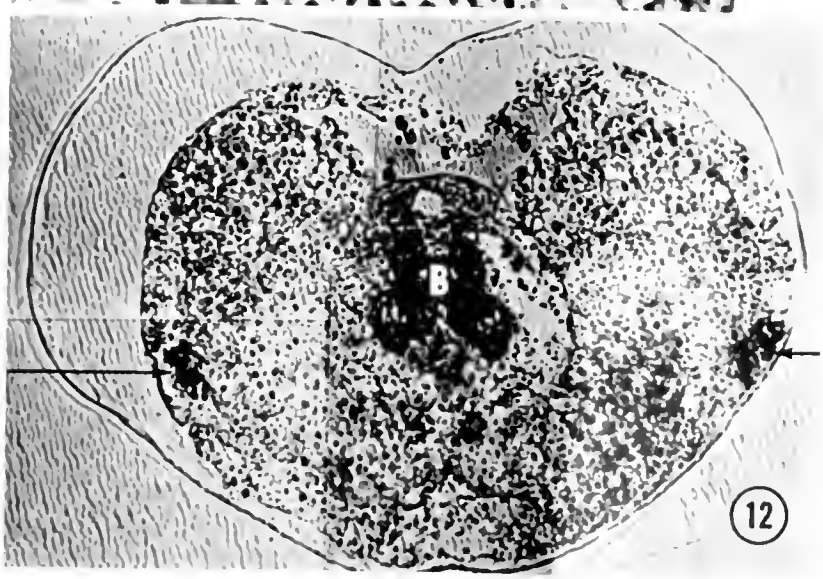

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Note the fluid spaces in both specimens, and the shrunken, fiberous appearance of the brain and the amorphous, sunken eyes of the older specimen. The optic stalk, optic nerve, and flattened, unstructured mass of developing eye tissue of a younger pupa (corresponding in age to the specimen in Fig 10) are shown in Fig 11.

The destruction or inhibition of development of the cuticle in infected pupae is shown in Figs 13-16. The healthy, developing cuticle with its trophic microvilli and two pores (Fig 13) is completely absent in the diseased specimen (Fig 14). The microvilli of the latter extend randomly into the fluid space. Details of the microvilli are presented at higher magnification in Figs 15 and 16. The tissues shown in these electronmicrographs are from the head where pathognomonic clearing develops. In tissues from the gaster, where little or no clearing occurs, some cuticle is present (Fig 26).

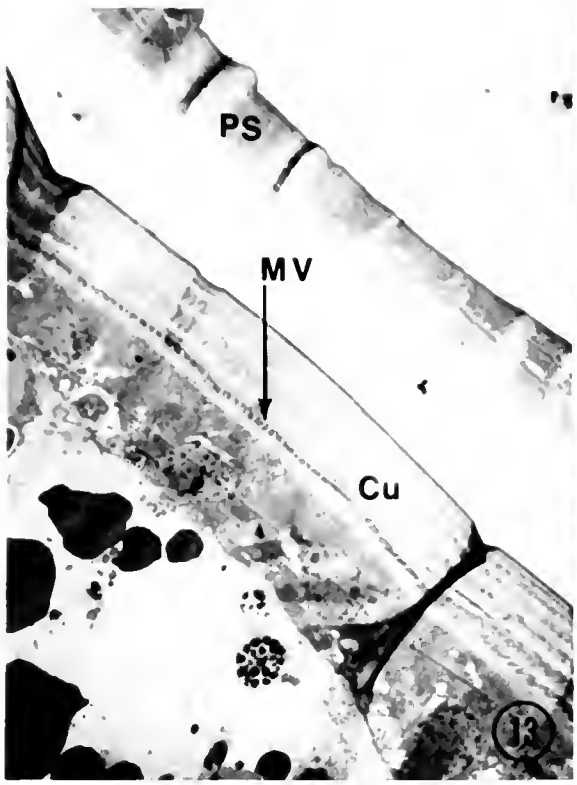
The structures of a healthy, developing pupa eye and a diseased eye of a similar age are compared at increasing magnifications in Figs 17-22. The lenses, composed of cuticle, are absent in the diseased eye, and the rhabdoms and associated cells are twisted, tangled masses. Also, the basement membrane is absent, and only remnants of the optic nerve may be seen. Pigment granules are present in the pigment cells of the diseased eye.

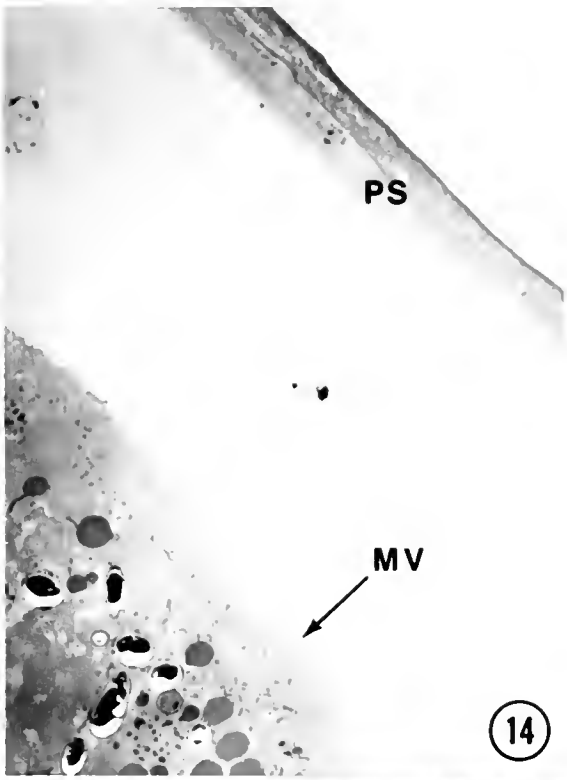
Fig 13. Cuticle of a healthy pupa. Note the trophic microvilli and two pores. PS = pupal sheath; MV = microvilli; Cu = cuticle. X 1,500.

Fig 14. Body surface of a pupa infected by B. dimorpha. There is no cuticle, and the microvilli that normally penetrate the cuticle extend into the fluid space. PS = pupal sheath; MV = microvilli. X 1,500.

Fig 15. Detail of the cuticle in a healthy pupa. Note the microvilli penetrating the cuticle. MV = microvilli. X 12,500.

Fig 16. Detail of microvilli extending into the fluid space of a pupa infected by B. dimorpha. X 12,500.


PS
MV
Cu
13


PS
MV
14


MV
15

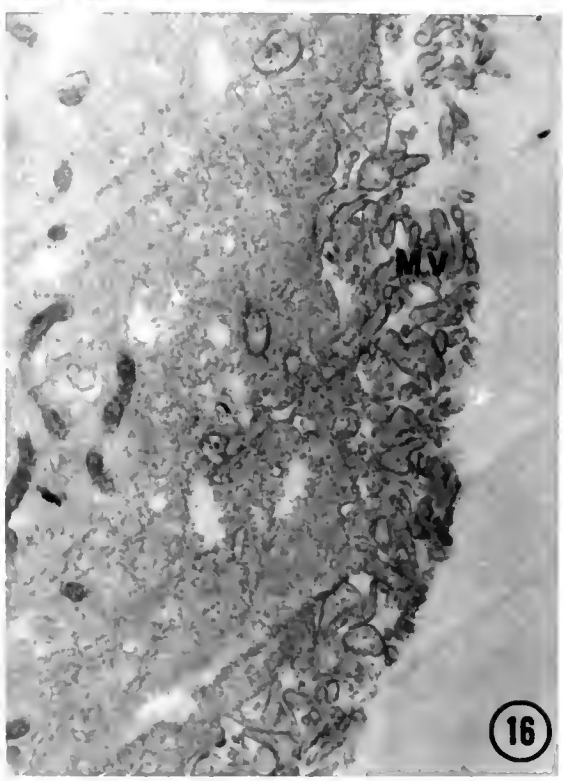

MV
16

Fig 17. Sagittal section through the developing eye of a healthy pupa. L = lens; Rh = rhabdom and developing associated cells; ON = optic nerve. X 450.

Fig 18. Sagittal section through the eye of a pupa infected by B. dimorpha. There is no lens, and the rhabdoms and the associated cells are twisted and tangled in an amorphous mass. Rh = rhabdom and associated cells; ON = optic nerve. X 450.

Fig 19. Sagittal section through the eye of a healthy pupa. X 1,500.

Fig 20. Sagittal section through the eye of a diseased pupa. Rh = rhabdom and associated cells. X 1,500.

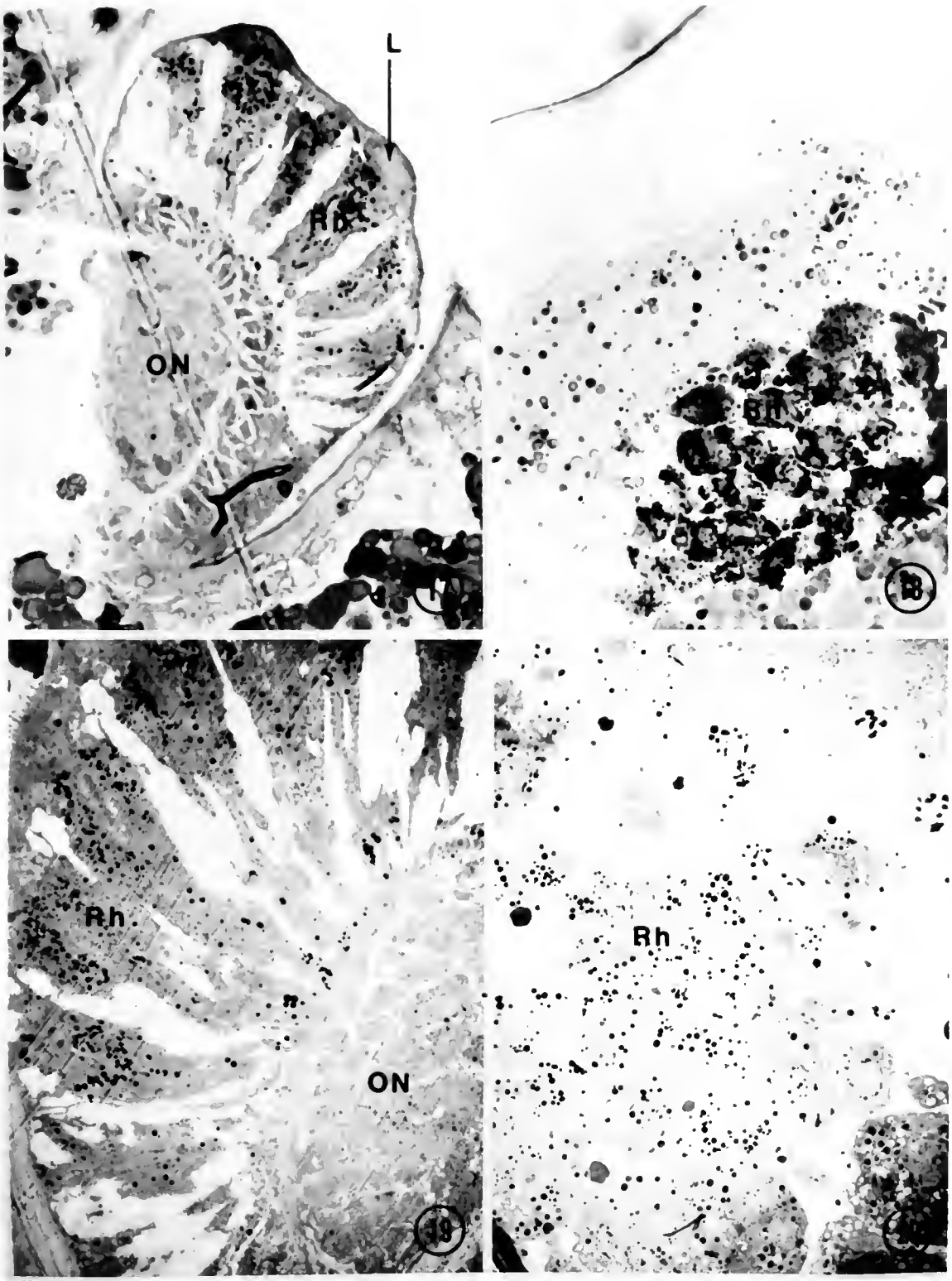
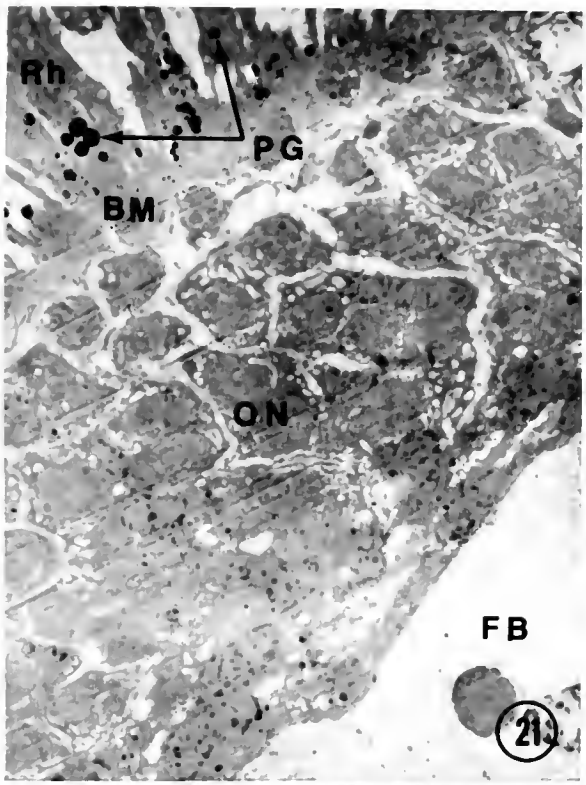

L
Rh
ON
Rh
B
Rh
ON
Rh
C

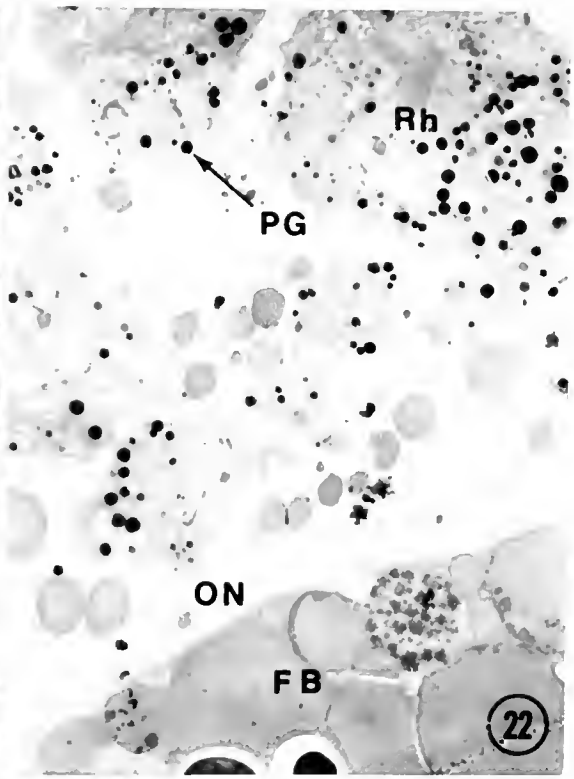
Fig 21. Sagittal section through the developing eye of a healthy pupa showing detail of optic nerve and base of the developing ommatidium. Rh = rhabdom and associated cells; BM = basement membrane; PG = pigment granules; ON = optic nerve; FB = fat body. X 3,000.

Fig 22. Sagittal section through the eye of a pupa infected by B. dimorpha. Only remnants of the optic nerve may be seen. The basement membrane area is destroyed. ON = optic nerve; FB = fat body; PG = pigment granules; Rh = rhabdom and associated cells. X 3,000.

Fig 23. Fat body of a healthy pupa. X 7,500.

Fig 24. Fat body of a pupa infected by B. dimorpha. Note the depleted appearance of fat body and the presence of several MB spores. X 7,500.


Rh
PG
BM
ON
FB
21


Rh
PG
ON
FB
22


23

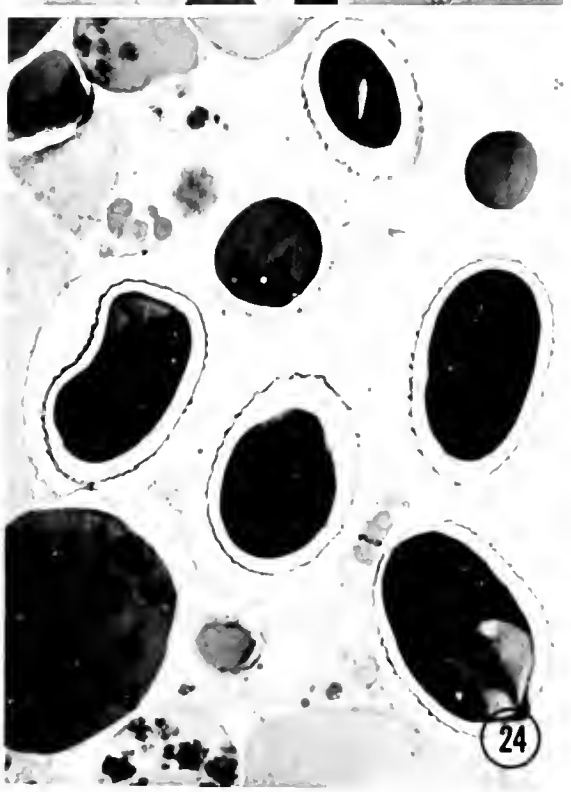
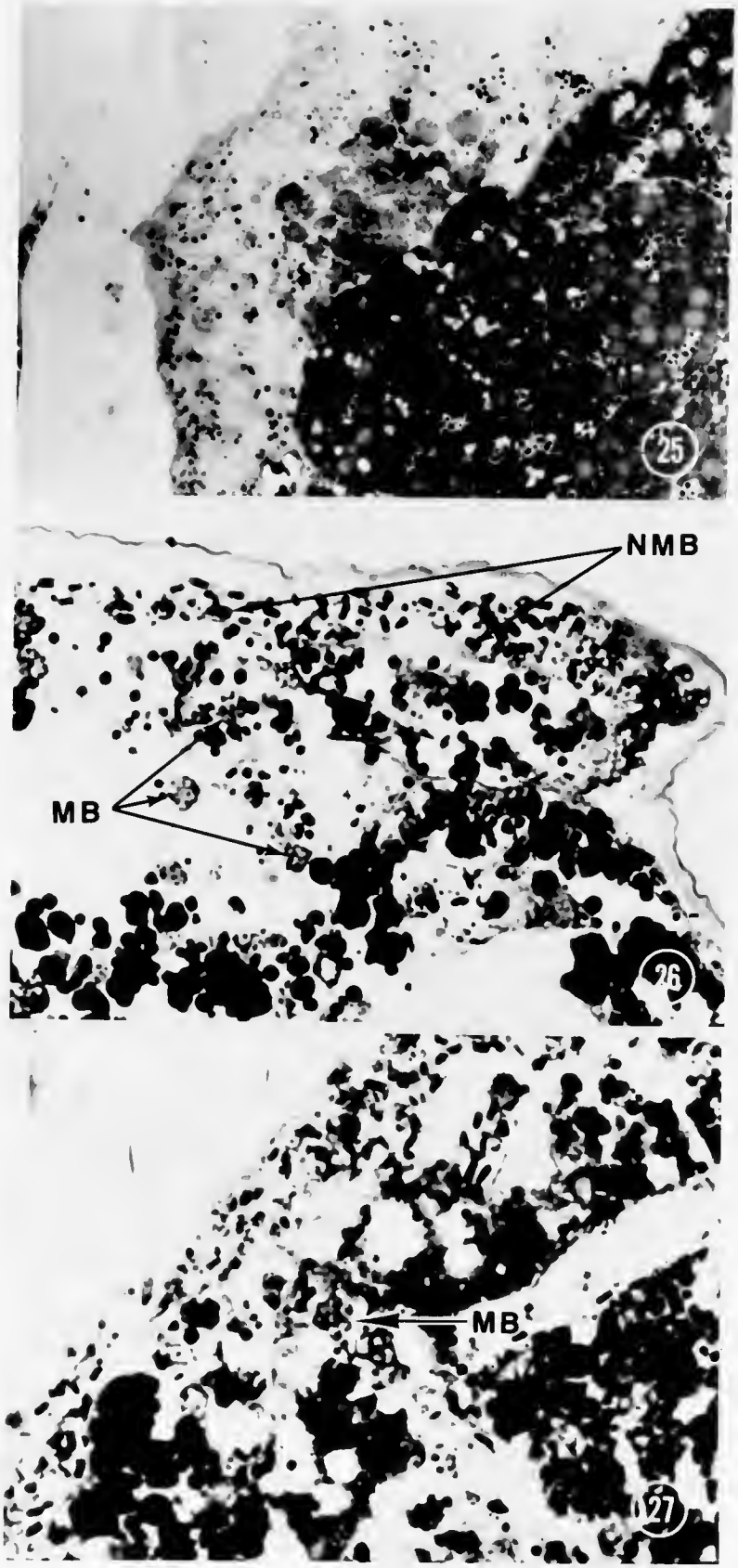

24

Fig 25. Tissue specificities of MB and NMB spores of B. dimorpha. The MB spores in the darkly stained fat body are seen in groups of less than eight because of the thinness of this section (o.5 mu). X 150.

Fig 26. Tissue specificities of MB and NMB spores of B. dimorpha. Nonmembrane bound spores are confined to the hypodermis; MB spores are confined to the fat body. The MB spores are seen in octets in this section because of its thickness (6-8 mu). Some cuticle is seen in this section from the gaster. X 240.

Fig 27. Tissue specificities of MB and NMB spores of B. dimorpha seen at higher magnification. X 375.


25
NMB
MB
26
MB
27

The fat body is diminished in infected pupae, as is evident in Figs 23 and 24. Several MB spores may be seen in the latter electronmicrograph.

The tissue specificities of the two spore types are shown at increasing magnifications in Figs 25-27. The MB spores are confined to the fat body; NMB spores are confined to hypodermal and connective tissues, including that in the deeper parts of the body such as the tissue encasing the brain. The tissue section in Fig 25 is only 0.5 mu thick; therefore, MB spores are seen in groups of 2-4 rather than octets. The tissue sections in Figs 26 and 27 are ca 6-8 mu thick, and thus show the octosporous pansporoblasts better.

Discussion

The pathognomonic signs of *B. dimorpha* infection in *S. geminata* reflect damage or destruction of the developing adult cuticle. In the pupal stage of development, the integument of the adult ant forms under the integument of the fourth-instar larva. During this period, the larval integument is transformed into a protective sheath that adheres closely to the developing adult integument and is moulted when the development of the latter is complete (eclosion). Over most of the body, the adult cuticle of pupae infected by *B. dimorpha* appears to be damaged or inhibited only to the extent that tanning is inhibited (Fig 26; also, the adult

morphology is essentially developed). In parts of the head and in the petiole, however, the cuticle is completely destroyed (Figs 13-16). In these areas, tissue fluids seep into the space between the denuded hypodermis and the pupal sheath. As the infection progresses, the fat body is diminished and the brain shrinks (due to loss of lipids?). The mass of these tissues, covered by hypodermis, decreases in size and recedes from the pupal sheath, which is extended by fluid. Jouvenaz and Hazard (1978) attributed the development of clear areas in the head and petiole to destruction of the hypodermis. More correctly, infection of the hypodermis results in destruction or inhibition of formation of the cuticle.

The malformation of the eyes is also due to destruction of the cuticle. The lens are part of the cuticle, and the remaining components of the ommatidium (rhabdom and pigment, retinular, and semper cells) extend between the lens and a basement membrane. The lens are nonexistent in eyes infected by B. dimorpha, and the rhabdoms and associated cells become twisted masses. Perhaps the surrounding connective tissue matrix is also destroyed. The eyes of pupae infected by B. dimorpha develop after cuticle destruction has begun, and are abnormal from the time they first become visible. Thus, the derangement of the eyes is teratologic in nature.

CHAPTER EIGHT

TEMPERATURE-DEPENDENT SPORE DIMORPHISM IN *BURENELLA DIMORPHA*

The effect of temperature on the relative abundance of MB and NMB spores was first studied by Maddox (1966) in the dimorphic microsporidium *Vairimorpha necatrix* (Kramer). This parasite was originally thought to be a mixed infection involving two species of microsporidia, *Nosema necatrix* Kramer and *Thelohania diazoma* Kramer, that produced NMB and MB spores, respectively, in lepidopteran hosts (Kramer, 1965). Maddox demonstrated that only NMB spores are produced if infected armyworm larvae, *Pseudaletia unipuncta* (Haworth), are reared at temperatures of 32° C or above. At lower temperatures (16° C) 40% or more of the spores are the MB type. Maddox infected armyworm larvae with an apparently pure suspension of NMB spores obtained from larvae reared at elevated temperature, and found that both types of spores were produced in larvae held at temperatures below 32° C. This led Maddox to suggest (as the more radical of two possibilities) that *N. nectarix* and *T. diazoma* were " . . .not two species at all, but rather one species with two distinct developmental cycles and resulting spore forms, the ratio of which is influenced by temperature" (p. 112).

The dimorphic nature of this microsporidium was experimentally demonstrated by Fowler and Reeves (1974) through the use of mechanical as well as thermal and temporal methods to separate the two spore types for transmission studies. They retained the name N. necatrix, suppressing the name T. diazoma. Pilley (1976), recognizing that N. necatrix does not conform to the definitive characteristics of the genus Nosema, reassigned it to a new genus, Vairimorpha. Jouvenaz and Hazard (1978) observed that V. necatrix and B. dimorpha are related and suggested that the genus Vairimorpha properly belongs in the family Burenellidae.

The temperature dependence of spore dimorphism in V. necatrix was also demonstrated by Maddox and Sprenkel (1975), who serially passed V. necatrix spores through eight generations of P. unipuncta held at 21.1° and 32° C. Maddox and Sprenkel also demonstrated temperature-dependence of spore dimorphism of "Nosema plodiae -Thelohania nana" Kellen and Lindegren, and in an undescribed microsporidium. Maddox (1966) described the V. necatrix infections of P. unipuncta reared at elevated temperature as "light."

The primary purpose of the experiments reported here was to determine whether spore dimorphism in B. dimorpha is temperature dependent. The effects of temperature on spore yield ("light infections", Maddox) and size were also measured. In addition, since the MB spores of B. dimorpha develop in the fat body while NMB spores have been found

only in the hypodermis (Jouvenaz and Hazard, 1978), I attempted to answer the following questions: 1) If MB spores do not develop at all temperatures at which NMB spores develop, is it because sporulation is inhibited in the fat body? Or, 2) do NMB spores develop in lieu of MB spores in the fat body? And 3) are the effects of heat and cold the same in this respect? Resolution of these questions was attempted by histological examination of diseased pupae reared at high or low temperatures.

Materials and Methods

Ratios of Spore Types

Fourth-instar larvae were removed from a heavily infected laboratory colony and held in miniature nest cells at the desired temperature (20, 22.5, 28, 32, and 35 C) overnight. The temperatures in the incubators were monitored by hygrothermographs and mercury thermometers. Humidity was maintained close to 100% by the design of the cells (see General Materials and Methods). Larvae that pupated during the first 16 hr were discarded, as were those that had not pupated during the next 24 hr. A contingent of nurses (young workers primarily care for the immatures) captured from the brood piles was introduced to care for the immatures. These were removed shortly before the end of pupal life (at that time they were no longer needed to groom the pupae) to facilitate the examination and harvest of specimens in advanced disease.

The uninfected pupae served as controls to monitor the rate of maturation at the different temperatures. Infected pupae were harvested only after eclosion of the controls, when the disease had progressed to near the point of cuticle rupture and spore production was essentially complete. The completeness of spore production was judged by the percentage of immature spores seen in phase-contrast microscopic examination. Spore ratios were determined only for those pupae in which > 98% of the NMB spores appeared to be mature.

The initial rearing study indicated that the development of MB and NMB spores of B. dimorpha is inhibited by both high and low temperatures. Therefore, an additional study was conducted to determine the maximum age of the host at which MB spore inhibition can be effected by raising or lowering the temperature. Immature workers were divided into five age groups and half of each group were incubated at 20 C and the remaining half at 32 C. The age groups were 1) fourth-instar larvae; 2) larvaform pupae; 3) early pupaform pupae (eye development not yet evident); 4) pupae whose eyes had developed sufficiently to permit diagnosis of infection; 5) pupae slightly older, having first evidences of clearing in the occiput. These groups were reared and examined as described above.

Spore ratios were determined by homogenizing individual pupae in ca 1 ml distilled water in a 3 ml glass tissue grinder, and examining this extract by phase-contrast

microscopy at a magnification of 600X. The concentration of spores in these extracts (0-6 spores/field) was low enough to facilitate counting and individual study of spores. The percentage of mature and immature MB spores was based on the first 200 spores observed.

Spore Measurements

Spore measurements were made using an A. E. I. Cook Image-Splitting micrometer calibrated for the microscope used. The spores were immobilized for measurement by trapping them between a layer of agar and the coverslip. This was accomplished by pipetting a small pool of warm, liquid Noble Agar (Difco Laboratories, Detroit, Mich.) (1.5%) on the slide with a dropping pipette and bulb, and withdrawing a drop of the agar just before gelling occurred. This produced an agar bed which was rather flattened on top. A very small drop of spore suspension was then placed on a coverslip, inverted and placed on the agar.

Spore Yield

Spore yield was determined by weighing groups of 25 pupae in advanced disease on an electric balance, homogenizing these in distilled water, adjusting the volume appropriately, and counting spores with a hemacytometer (phase-contrast microscopy, 300X).

Histology

Tissue specimens from gasters of pupae held at 20 or 32 C until infection was very advanced were fixed, stained with heavy metals embedded in Spurr-Quetol resin, sectioned, and examined by phase-contrast microscopy. The details of these procedures are given in the section on host pathology.

Results

The development of MB spores was inhibited in pupae held at high (32 or 35 C) or low (20 C) temperature. At 28 C, the temperature at which S. geminata colonies seem to live best in my insectary, an average of 35.9% ± 2.6 of the spores in 25 pupae were of the MB type (Table 3). Jouvenaz and Hazard (1978) stated that MB spores typically constitute ca 25-40% of the spores in diseased pupae collected in the field.

Only 10 of the pupae held at 35 C survived to an advanced stage of infection. These pupae were devoid of MB spores, and a small number (ca 2%) of the NMB spores from these pupae were morphologically aberrant (pairs of spores fused laterally or in tandem, Y-shaped or triangular spores, giant spores, etc.). Thirty-five degrees appears to be very near the upper thermal limit of survival for host and parasite.

Twenty-two of the 25 pupae held at 32 C were negative for MB spores. Only four immature MB spores were among the 600 spores from the remaining three pupae (200 spores from each specimen were examined).

Table 3. Relative abundance of B. dimorpha MB spores in pupae of S. geminata reared from larvae at various temperatures.

Temperature (degrees C)	Number of pupae	No. (%) positive for MB spores	% MB spores in positive specimens
20	25	0	-
22.5	5	5 (100)	3 (1-5)[a]
28	25	25 (100)	35.9 ± 2.6 (31-39)[b]
32	25	3 (12)	1 (0-1)
35	10	0	-

[a]Mean and (range)

[b]Mean and standard deviation (range)

Table 4. B. dimorpha spore yield of pupae of S. geminata reared from larvae at 20, 28, or 32 C.

Temperature (degrees C)	NMB Spores		MB Spores	
	Spores/pupa	Spores/mg	Spores/pupa	Spores/mg
20	6.7×10^5	3.9×10^5	0	0
28[a]	6.3×10^5	4.4×10^5	2.7×10^5	2.0×10^5
32	4.8×10^5	2.7×10^4	0	0

[a]Total spore production at 28 C = 8.9×10^5/pupa or 3.9×10^5/mg.

The development of MB spores was completely inhibited in 25 pupae held at 20 C. A few immature MB spores were found in pupae held at 22.5 C, indicating that the lower thermal threshold of MB sporulation is between 20 and 22.5 C.

Pupae reared from larvae at 20 and 28 C produced similar numbers of NMB spores (Table 4). Pupae reared at 32 C produced ca 75% as many spores per pupa and ca 60% as many spores per mg bodyweight as did those reared at 28 C.

The inhibitory effect of temperature on MB sporulation in pupae placed at high or low temperature at comparable ages was essentially the same (Table 5). Specimens placed at 20 or 32 C as larvae or larvaform pupae (a total of 40 specimens) were negative for MB spores. Of the 10 specimens placed at 20 C as early pupaform (lacking visible eye development) pupae, five had no MB spores, two had one MB spore each, two had two MB spores each, and the remaining pupa had seven MB spores among the 200 spores that were examined. Of the 10 specimens placed at 32 C as early pupaform pupae, nine had no MB spores and the remaining pupa had only one MB spore among the 200 spores. Thus, mean spore production by this age group was less than one percent at either temperature.

The production of MB spores in pupae that had developed eyes to the extent that diagnosis of disease was possible at the time they were placed at 20 or 32 C was lower than in those reared at 28 C for their entire life. Those held

Table 5. Relative abundance of B. dimorpha MB spores in pupae of S. geminata placed at 20 or 32 C at different ages.

Age Group	% MB Spores 20 C	32 C
larvae	0	0
larvaform pupae	0	0
lacking eye development	< 1	< 1
minimum eye development	10.3±7.6	16.8±6.8
slight clearing, occiput	18.7±14.0	24.4±3.7

[a]Mean and standard deviation

at 20 C produced an average of 10.3% ± 7.6 (range 2-25%) MB spores; those held at 32 C produced an average of 16.8% ± 6.8 (range 6-23%). Pupae exhibiting minimal clearing of the occiput produced averages of 18.7% ± 14.0 (range 1.5-40%) and 24.4% ± 3.7 (range 18.5-33.5%) at 20 and 32 C respectively. Approximately 90% of the spores in these latter two age groups appeared to be mature.

The measurements of NMB spores from pupae reared from larvae at 20, 28 or 32 C were, respectively, 3.0 ± 0.2 X 6.8 ± 0.3 mu, 3.0 ± 0.1 X 6.8 ± 0.3, and 3.0 ± 0.0 X 7.0 ± 0.4 mu. Obviously, there were no differences in NMB spore sizes (MB spores were not produced at 20 or 32 C).

Neither MB or NMB spores were seen in tissue sections of fat body of pupae reared at 20 or 32 C. Very few MB spores were seen in sections of fat body of pupae reared at 22.5 C.

Discussion

The development of NMB spores precedes the development of MB spores, and NMB spores predominate in number. In field colonies, MB spores typically constitute ca 25-40% (occasionally fewer) of the spores from pupae in advanced stages of infection (Jouvenaz and Hazard, 1978). The present study has demonstrated that MB sporulation also occurs in a more restricted range of temperature than NMB sporulation.

The lower thermal limit of MB spore development appears to be between 20 and 22.5 C. At 28 C production of MB spores

was consistently near maximum. The upper thermal limit of MB spore development is below 32 C. Thus, optimal MB spore production occurred near the upper thermal limit of spore production. This is reminiscent of the activity curve of heat-liabile enzymes and the growth curves of poikilotherms over a range of temperatures. Very possibly the upper thermal limit of MB spore development is the temperature of inactivation of an enzyme(s).

The B. dimorpha MB spore production is also inhibited by low temperature (20-22.5 C). In contrast, V. necatrix MB spores are produced at temperatures at least as low as 16 C. This difference in cold sensitivity may reflect different requirements for enzyme stability due to host behavior. Loss of quaternary enzyme structure at low temperature is relatively common in homeotherms and their microbial symbiotes. The homologous enzymes of poikilotherms do not, at least in some cases, lose their quaternary structure at low temperature (Hochachka and Somero, 1973). The hosts of both V. necatrix and B. dimorpha are, of course, poikilotherms; however, the host of the latter microsporidium is a subtropical, social insect that actively tends its brood, moving them in a subterranian environment to regulate temperature and humidity. The tumulus of the nest is a solar heating device, and there is evidence that metabolic heat production is significant (Seeley and Heinrich, 1981). The various lepidopteran hosts of V. necatrix, however, are exposed

to ambient temperatures as are most poikilotherms. Thus, the stability of quaternary enzyme structure may be more critical in this microsporidium.

We may only speculate as to the physiological function(s) that may be inhibited by extremes of temperature. However, the critical event in MB sporulation may well be meiosis. If the sister nuclei of the diplocaryon found in NMB spores and the vegetative stages of B. dimorpha are each diploid, meiosis would produce eight uninucleate (haploid) spores (MB spores occur in octets bound by a membrane). Evidence that meiosis does indeed occur in microsporidia has been published by Loubes et al. (1976) and Hazard et al. (1979).

The hypothesis that meiosis may be the critical event in MB sporulation inhibition by extremes of temperature is at least circumstantially supported by the essentially complete absence of MB spores in specimens placed at 20 or 32° C at an age before eye development becomes visible, and the partial and highly variable degree of inhibition in specimens exhibiting early eye development. The appearance of MB sporoblasts approximately coincides with the earliest visible eye development. At this stage, second cycle meronts enlarge to the full size of mature pansporoblasts, and the diplocaryotic nuclei become very diffuse. Successive nuclear divisions then produce plasmodia containing two, four, and finally eight nuclei. Endogenous cytoplasmic budding around these nuclei produces an octet of uninucleate

spores bound by a membrane. Thus, meiosis occurs at the age limit for thermal inhibition of MB spore development. The high variability of inhibition among pupae in this age group simply reflects the slight variability in age of individuals and the degree to which MB sporulation had advanced at the time of temperature change. Once sporogony is under way (meiosis has occurred?), it apparently cannot be stopped by temperatures at least as low as 20 C or as high as 32 C.

CHAPTER NINE

EMENDMENTS TO THE DESCRIPTION OF *BURENELLA DIMORPHA*

The vegetative stages of *B. dimorpha* were described by Jouvenaz and Hazard (1978) from light microscope studies as follows:

> *Burenella dimorpha* appears to have two sequences of merogony. The first involves uninucleate cells with deeply staining cytoplasm (Giemsa) and compact nuclei that become binucleate and divide. The second sequence involves binucleate cells with moderately staining cytoplasm and less dense nuclei that become tetranucleate and divide to produce two binucleate cells. (p. 27)

The accuracy of this description is challenged by the following considerations.

Studies on the transmission of *B. dimorpha* have demonstrated that only the NMB spores are infective for fire ant larvae (see Transmission and Infectivity of Spores). The NMB spores are diplocaryotic (Fig 32); therefore, the infecting sporoplasm is (presumably) also diplocaryotic. The formation of uninucleate meronts would require either fusion of the sister nuclei or separation of the nuclei followed by cell division. Studies of *Nosema* spp. (Sprague et al., 1968; Vavra and Undeen, 1970; Cali, 1971) and an *Amblyospora* sp. (Andreadis and Hall, 1979) indicate that in these species, the diplocaryotic condition persists throughout the life cycle.

Recently I collected an undescribed microsporidium (a parasite of S. invicta in Brazil) that appears to be closely related to B. dimorpha. This dimorphic species also has two sequences of merogony; however, its meronts are diplocaryotic. These observations and reports prompted me to reexamine the life cycle of B. dimorpha, with particular attention to the nuclear condition of early meronts. Additional studies of ultrastructure, including scanning electron microscopy, were also conducted.

The appearance of sexual castes in colonies of S. geminata is seasonal (unpublished), and Jouvenaz and Hazard were unable to examine diseased sexual pupae. Spores from sexual pupae have now been examined, and found to differ in one respect from spores from worker pupae.

Materials and Methods

Light Microscopy

Specimens were smeared on acid-alcohol cleaned, galss microscope slides, air-dried, and fixed in acetone-free methanol for 5 minutes. Slides were stained with 10% Giemsa stain in phosphate buffer, pH 7.41 (Fisher Gram Pac Buffer) for 12 minutes, rinsed in tap water or acidified deionized water (deionized water adjusted to pH 6.8 with acetic acid), blotted with filter paper, and examined under oil.

Scanning Electron Microscopy

Spores from a suspension in deionized water were attached to carbon-stabilized, formvar coated grids with polylysine (Mazia et al., 1975), fixed overnight in buffered 1% osmium tetroxide, and dehydrated in 2,2-dimethoxypropane. Specimens were critical point dried, sputter coated with palladium-gold, and examined in a Hitachi H-6010A high resolution scanning electron microscope at an accelerating voltage of 50 kV.

Results

Jouvenaz and Hazard (1978) were correct in their statement that B. dimorpha has two sequences of merogony; however, I am now convinced that these cells are diplocaryotic and bidiplocaryotic, rather than uninucleate and binucleate. The cells of the first merogonic sequence are very small, and their compact nuclei are very closely appressed; the cells of the second merogonic sequence are larger, and their nuclei are rather diffuse. The diplocaryotic condition of these nuclei is not readily apparent, and the published diagrammatic life cycle adequately reflects their appearance in stained smears.

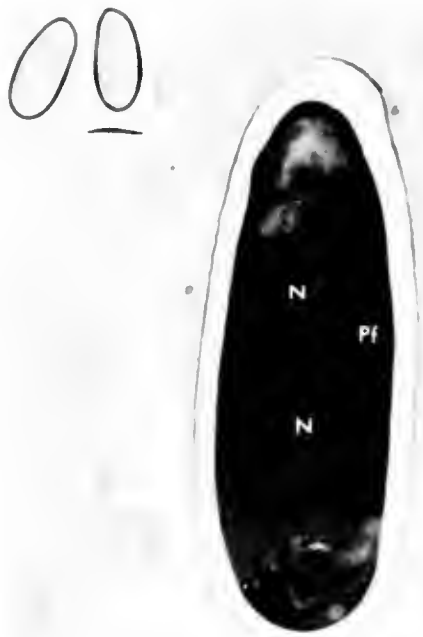
Jouvenaz and Hazard also stated that the surface of both spores is smooth. This is correct only with respect to the NMB spore (Figs 28 and 30). The surface of the MB spore has a reticular pattern of ridges that are evident

Fig 28. Transmission electronmicrograph of a mature NMB spore of B. dimorpha. Note smooth spore surface. N=nucleus; Pf=polar filament. X 17,100. Inset: NMB spores as they appear in phase-contrast microscopy. X 2,000.

Fig 29. Transmission electronmicrograph of a mature MB spore of B. dimorpha. Note the surface sculpture (arrow) and the polar cap (upper end of spore). N=nucleus; Pf=polar filament. X 18,000. Inset: MB spore as it appears in phase-contrast microscopy. X 2,000.

Fig 30. Scanning electronmicrograph of a mature NMB spore of B. dimorpha. Note the smooth surface and lack of a morphologically differentiated polar cap area. X 16,500.

Fig 31. Scanning electronmicrograph of a mature MB spore of B. dimorpha. Note the surface sculpture and morphologically differentiated polar cap area (bottom of spore). X 16,500.



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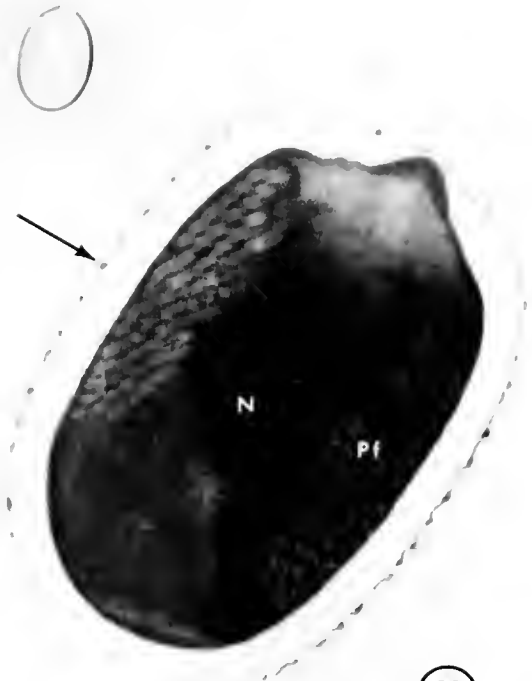

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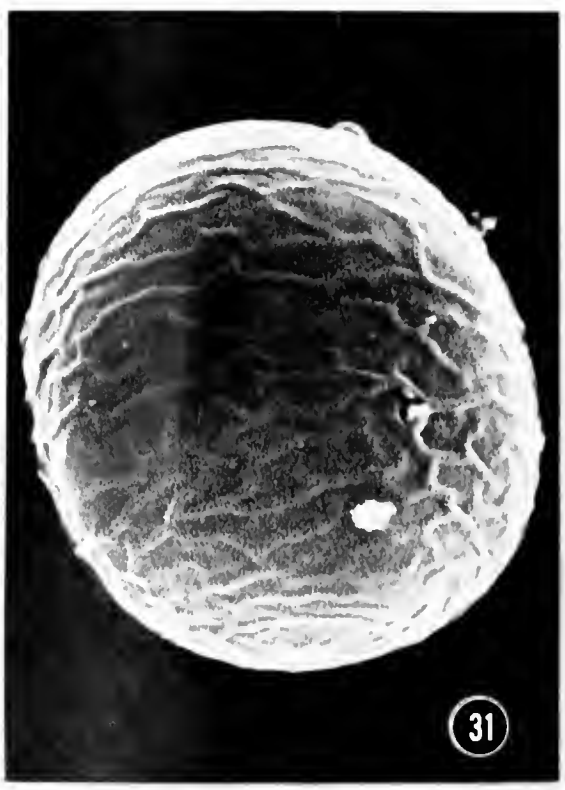

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in both the transmission (Fig 29) and scanning (Fig 31) electronmicrographs. The ridges may also be seen in the immature spores in Fig 33.

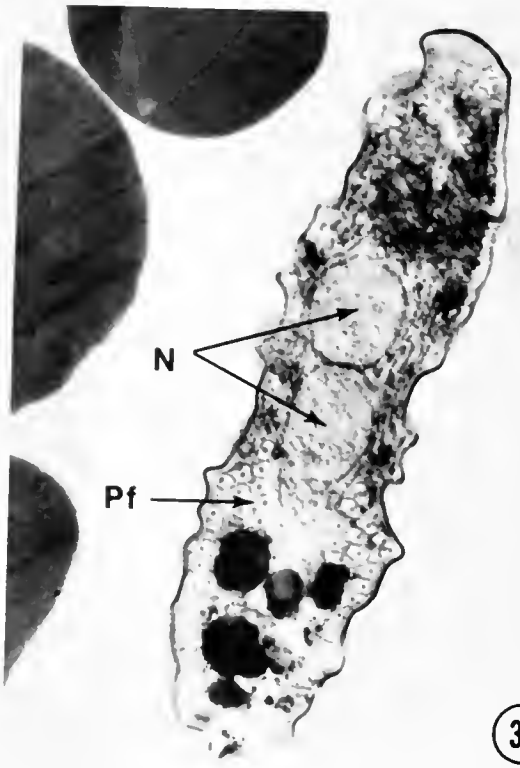
The pansporoblast membrane of B. dimorpha in worker pupae was described by Jouvenaz and Hazard (1978) as subpersistent and delicate, and ruptured during dissection of the host. Mature spores are never, and immature spores are rarely, seen in octets in smears. This is also true of B. dimorpha from male pupae; however, the pansporoblast membrane is quite persistent in specimens from female sexual pupae. In the latter, mature spores are commonly seen in octets in smears or aqueous extracts.

Discussion

The diplocaryotic condition of meronts of B. dimorpha is most difficult to observe. I have been unable to obtain convincing photomicrographs of diplocaryia in these cells, despite the use of high contrast technical pan film and manipulation of staining protocols. After studying a very large number of these cells with a microscope of excellent quality, focusing up and down to distinguish depth of staining and faint curvatures, I have become convinced that these cells are indeed diplocaryotic. Interestingly, I have consistently obtained much superior definition in stains of an undescribed, closely related microsporidium from S. invicta.

Fig 32. Transmission electronmicrograph of an immature NMB spore. The spore wall has not yet developed. N=nuclei; Pf=polar filament. X 12,000.

Fig 33. Transmission electronmicrograph of two immature MB spores within a delicate pansporoblast membrane. The surface sculpture is already prominent, although the endospore is not yet fully developed. PM=pansporoblast membrane. X 9,000.



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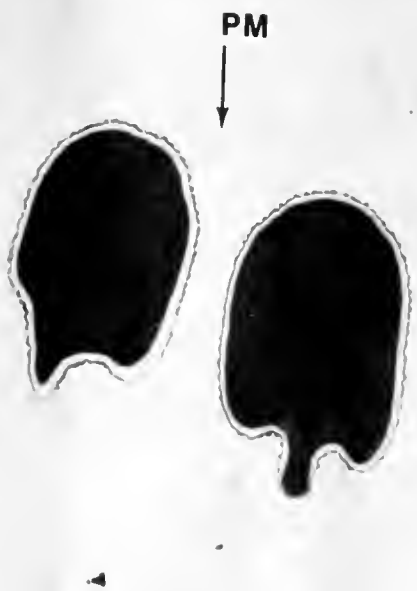

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CHAPTER TEN

SUMMARY AND CONCLUSIONS

Burenella dimorpha was described by Jouvenaz and Hazard (1978) as the type species of a new genus which represents a new family, Burenellidae. This family includes those species of microsporidia having two sporogonic sequences, one producing nonpansporoblast membrane-bounded (NMB) spores and the other producing octets of spores bounded by a pansporoblast membrane (MB). Jouvenaz and Hazard described this microsporidium as a dimorphic species on the basis of light microscope studies of the life cycle. They were unable to separate the two spore types, and therefore could not confirm by feeding tests that B. dimorpha is a single species. Neither, of course, could they determine which or if both spore types are infective. Also, the degree of host-specificity of the parasite was unknown. Three species of Solenopsis other than S. geminata were shown to be susceptible to infection, but later observations cast doubt on the ability of the parasite to persist in populations of these species.

Fire ant pupae infected by B. dimorpha develop pathognomonic signs--malformation of the eyes and blister-like clear areas in the occiput and petiole. These signs increase in severity as the infection becomes more advanced, and

eventually the pupa ruptures and is cannibalized by workers. The histopathological basis of these signs was not understood, nor was the exact mode of transmission of the parasite.

An effort was made to extend our limited knowledge of B. dimorpha in the areas outlined above. In addition, the effect of temperature on spore dimorphism was investigated, and certain aspects of the life cycle and morphology of the parasite were reexamined. The conclusions reached in this study are summarized below.

1. It was experimentally confirmed that B. dimorpha is a dimorphic species, producing two morphologically distinct types of spores, and not a dual infection of two species of microsporidia.

2. Only NMB spores are infective; unextruded MB spores are expelled in the meconium upon pupation.

3. The intracolonial cycle of transmission of B. dimorpha infection is from ruptured, diseased pupae to fourth-instar larvae via the adult workers, who act as mechanical vectors.

4. Adult workers do not ingest spores into the crop, but divert them to the infrabuccal cavity where they are formed into infrabuccal pellets with particulate food material.

5. Only fourth-instar larvae are vulnerable to infection.

6. Burenella dimorpha is host-specific for S. geminata. Infections in S. invicta and S. richteri do not persist in the colony.

7. Burenella dimorpha may be locally abundant, even though it is not common in the S. geminata population as a whole.

8. Spore dimorphism is temperature dependent. Membrane bound sporulation is inhibited by both low (20-22.5 C) and high (32 C) temperatures; NMB sporulation is affected little, if at all.

9. Nonmembrane bound spores do not develop in lieu of MB spores in the fat body at low or high temperatures.

10. The pathognomonic signs of B. dimorpha infection are due to destruction or inhibition of formation of the adult cuticle.

11. Meronts are diplocaryotic and bidiplocaryotic, not uninucleate and binucleate as originally described.

12. The NMB spore surface is smooth, but the MB spore surface has a complex pattern of ridges and a well defined polar cap area.

13. The pansporoblast membrane is extremely delicate and subpersistent in workers (as originally described), and in male pupae; in female pupae it is persistent.

GLOSSARY

Diplocaryon. Two nuclei in intimate contact, their membranes adhering to each other over a large area. These nuclei divide in a plane perpendicular to the plane of their physical contact, in synchrony.

Disporous. Producing two sporoblasts.

Endogenous sporogony. Sporogony within the limiting membrane of the sporogonial plasmodium.

Endospore. The chitinous inner spore wall.

Exospore. The proteinaceous outer spore covering or envelope.

MB. As used in this dissertation--bounded by a pansporoblast membrane.

Merogony. Vegetative multiplication. Synonym: schizont.

Meront. A cell that undergoes binary or multiple fission in the vegetative phase of the life cycle. Synonym: schizont.

NMB. As used in this dissertation--not bounded by a pansporoblast membrane.

Pansporoblastic membrane. A somewhat modified membrane that encloses a group of spores or sporoblasts.

Polar cap. An internal, chromophilic area at the anterior end of the spore.

Sporogenesis. The transformation of the sporoblast into a spore.

Sporogenesis. The transformation of the sporoblast into a spore.

Sporogony. The production of sporoblasts.

Sporont. A cell that gives rise to a sporoblast.

Sporoplasm. The nucleus (nuclei) and cytoplasm contained within the spore that is injected into a host cell.

Sporulation. Spore production; sporogony plus sporulation.

Vegetative stage. That phase of the life cycle when the parasite is actively feeding and multiplying prior to sporulation.

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BIOGRAPHICAL SKETCH

Donald P. Jouvenaz was born March 9, 1936, at Williston, Florida. He attended Florida public schools and graduated from Ocala High School in 1954. Following graduation he served as a medical specialist in the United States Army from 1954 to 1957. In 1957 he enrolled in Pensacola Junior College, Pensacola, Florida, and was employed as a surgical technician at Baptist Hospital of Pensacola.

He received the degree of Bachelor of Science with a major in biology from Florida State University in December, 1962, and began graduate studies in bacteriology in January, 1965. He transfered to the Department of Entomology, University of Florida, in September, 1966, after working the preceding summer as a laboratory assistant in that department.

He held both Graduate Research and Teaching Assistantships at Florida State University, and a Graduate Research Assistantship and Summer Research Fellowship at the University of Florida. In June, 1968, he received the degree Master of Science with a major in entomology and a minor in medical sciences (microbiology).

Upon his graduation from the University of Florida, Mr. Jouvenaz accepted an appointment as an Entomologist with

the Agricultural Research Service, United States Department of Agriculture. He has since been engaged in research on the biology and control of imported fire ants at the Insects Affecting Man and Animals Research Laboratory, Gainesville, Florida. From 1968 to 1973 he was assigned to monitoring fire ant eradication trials and to the development and improvement of toxic baits. In 1973 he became involved in studies of pheromones and pesticide residue monitoring. Since 1975 he has become progressively more concerned with insect pathology and biological control. He has been elected to the Phi Sigma Society and the Society of the Sigma Xi, and is a member of several scientific societies.

Don Jouvenaz is married to the former Judy Sandra Hughey of Kissimmee, Florida. They have three children.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Donald W. Hall

Donald W. Hall, Chairman
Professor of Entomology and Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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