

An Ultrastructural Study on Stages in the Life Cycle of a Microsporidian Parasite (Microspora: Nosematidae) in *Helicoverpa armigera* (Lepidoptera: Noctuidae)

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The ultrastructure of the developmental stages of a highly pathogenic microsporidium found in laboratory cultures of *Helicoverpa armigera* (Lepidoptera: Noctuidae) was investigated. The microsporidium caused infection of the gut epithelium, muscle, and Malpighian tubules. The life cycle included disporoblastic sporogony, but quadrinucleate meronts were not observed. All stages were lacking a sporophorous vesicle. Spores were monomorphic, measured 2.43 × 1.22 μm , and contained up to 13 coils of the polar filament. The ultrastructural and developmental features indicate that this is a microsporidium of the genus *Nosema*. It is tentatively suggested that this organism may be of potential value in the control of this insect pest. © 1992 Academic Press, Inc.

KEY WORDS: *Helicoverpa armigera*; *Nosema* sp.; Microsporidia; Lepidoptera; Noctuidae; ultrastructure.

INTRODUCTION

Many noctuid moths cause substantial damage to cereals and other crops. For example, *Helicoverpa* (*Heliothis*) *armigera* and *Heliothis* spp. are significant economic pests in Asia, Africa, the Americas and Europe and their effective control would lead to improved crop production. Numerous insects are known to have developed resistance to conventional insecticides and this, together with increased concern to reduce chemical pollution of the environment, requires alternative control measures to be found. Certain parasitic organisms may offer such an alternative approach. For example, microsporidia have been used to control the lepidopteran, *Lymantria dispar* (David and Novotny, 1990). Such organisms have the advantage of being fairly specific in terms of host "target" and infections occur relatively infrequently in vertebrates other than fish (Sprague, 1977). Microsporidian diseases in man are usually confined to reports of patients with immunodeficiency or stress related illness (Cali and Owen, 1988).

During the course of rearing laboratory cultures of *H. armigera*, major population "crashes" were noted

from time to time. Examination of light microscopical sections of larval midgut and associated tissues (e.g., gut muscle tissue) revealed that insects were dying due to the presence of an acute microsporidian infection.

The present study reports the presence of a nosematid parasite in larval *H. armigera*, describes the fine structure of various stages in its life cycle, and compares the morphology of the spore with that of similar microsporidian parasites.

MATERIALS AND METHODS

H. armigera (Sudan strain) were reared in an insectary at 26 ± 0.5°C and 60% relative humidity. The photoperiodic regime was 14 hr light and 10 hr dark.

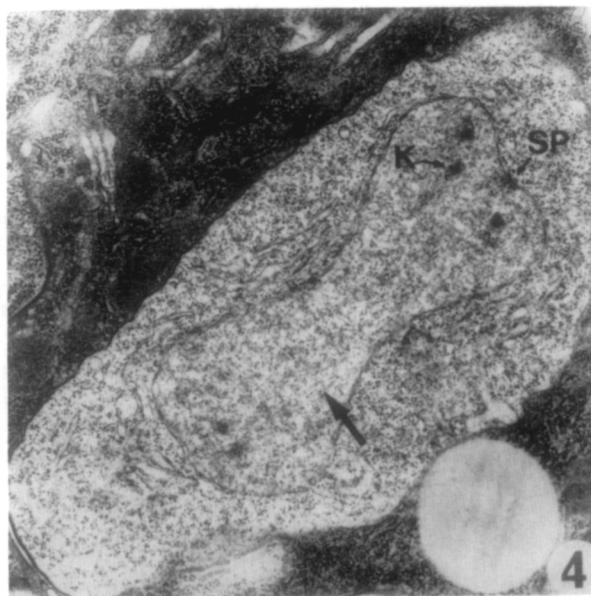
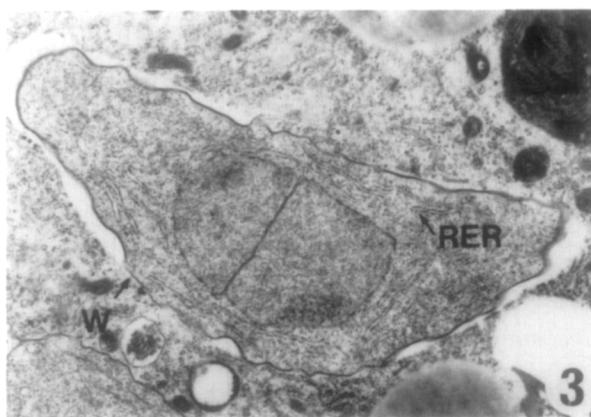
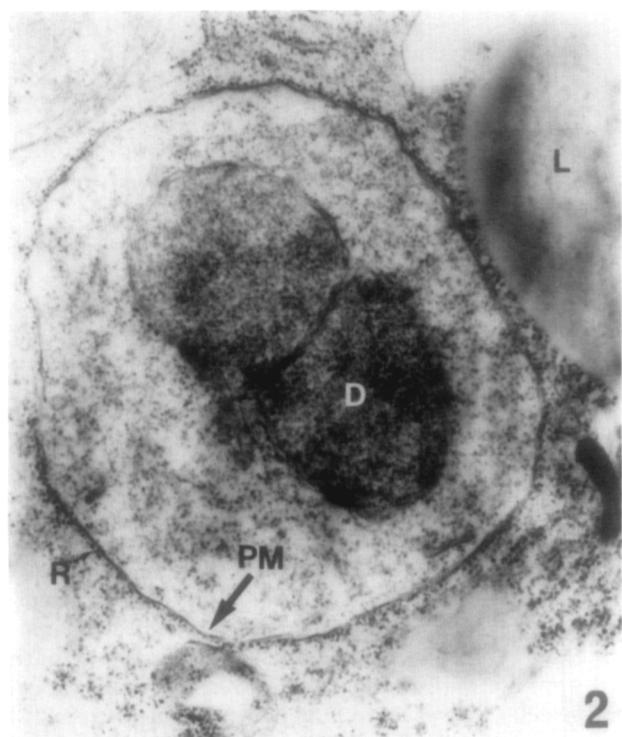
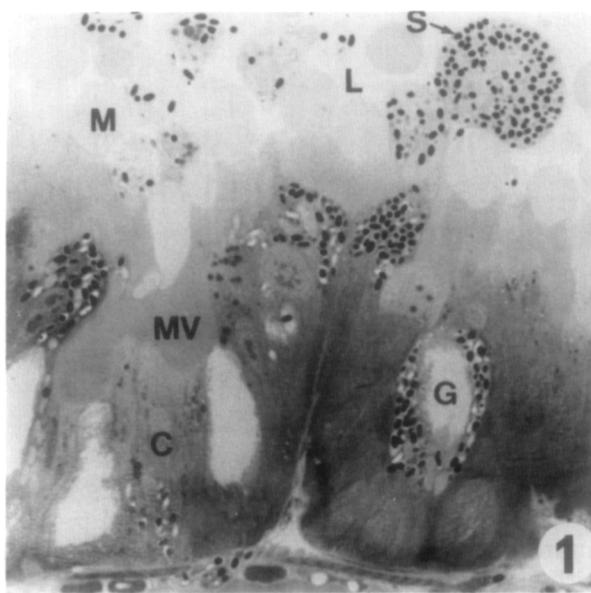
Electron Microscopy

The midguts and associated Malpighian tubules were dissected from newly moulted, actively feeding and nonfeeding fifth and sixth instar-infected larvae. The tissues were then placed for 60–90 min at 0–4°C in a Karnovsky's formaldehyde–glutaraldehyde fixative, followed by postfixation in 1% osmium tetroxide in sodium cacodylate buffer (pH 7.3) for 1 hr at 0–4°C. After dehydrating the tissues at room temperature through a graded series of ethanol solutions to acetone, they were embedded in Araldite epoxy resin. Silver/silver-gold sections were cut on a Reichert NK ultratome and stained with uranyl acetate and lead citrate before examination under a Philips EM 400T electron microscope.

Sections (1- μm thick) for light microscopy were also prepared and cut from Araldite-embedded tissue as described above. These were placed on glass slides prior to staining with toluidene blue and were then mounted in DePeX mounting medium.

RESULTS

The effects of the microsporidium on the stock population of *H. armigera* were not pronounced for about



two generations following their discovery in sectioned tissues. Later, larvae were more heavily infected, showed acute paralysis, and frequently died in the larval or pupal stages. Similar disease symptoms have been reported in *Spodoptera litura* infected with *N. mesnili* (Abe, 1989). Mass infestation of muscle tissue did occur in *H. armigera* which would be consistent with the lethargic behavior of the animals prior to death.

Light Microscopy

Examination of the midgut sections revealed the presence of massive microsporidian infestation with large numbers of oval spores prominent in both columnar and goblet cell cytoplasm (Fig. 1). In addition, spores were visible in mucous droplets in the gut lumen, in the musculature around the gut wall, and in cells of the Malpighian tubules.

Electron Microscopy

Micrographs have been arranged and subsequently explained to show a developmental sequence of the microsporidium. The fine structure and life cycle stages observed were consistent with the literature describing development in *Nosema* spp. (Larsson, 1986; Vavra, 1976). All stages were lacking a sporophorous vesicle.

Cytology of the Presporal Stages

The meront stage (Fig. 2) of the microsporidium was rounded ($2.5-3 \mu\text{m}$ in diameter) with a large central diplokaryon of approximately the same size as a mature spore ($2.1 \times 1 \mu\text{m}$ and $2.4 \times 1.2 \mu\text{m}$, respectively). The cytoplasm contained a poorly developed endoplasmic reticulum with numerous free ribosomes, and the unit plasma membrane adjacent to the host cell was surrounded by numerous host cell ribosomes. Thus the ultrastructure of the meront cytoplasm was similar to that reported by Canning and Sinden (1973) for *Nosema algerae* in *Anopheles stephensi*. Characteristically for microsporidia, no mitochondria were present at any stage.

In contrast to the meront stage, the larger sporonts (measuring $4.5-6.5 \times 2.2 \mu\text{m}$) possessed a better developed rough endoplasmic reticulum (RER) which was arranged concentrically around the diplokaryon. Each

sporont was separated from the host cell by an electron dense layer surrounding the cell membrane. The host's ribosomes were noticeably less ordered in relation to the sporont being no longer attached to the parasites outer surface.

Throughout development of the parasite, each of the two closely opposed nuclei making up the diplokaryon had a structure typical of nuclei from eukaryotic cells and was surrounded by a double membrane (Figs. 3, 8, and 16). Consequently, the area of contact between the two nuclei appeared as a thickened electron-dense plate and was occasionally the only part that was distinct (Fig. 6). Evidence of prophase during closed intranuclear pleuromitosis was found only in diplokarya (Fig. 5). The spindle plaque was seen as a thickened, electron-dense region of membrane and served as an attachment point for the microtubules which constitute the intranuclear spindle apparatus. The other ends of the microtubules were attached to the kinetochores of the chromosomes (Figs. 4 and 5). The plaque was about 230 nm wide (Fig. 5) and a number of cytoplasmic vesicles were seen around the spindle plaques where these were depressed into the nucleus (Fig. 5). The stage shown in Fig. 4 is probably a late anaphase stage of mitosis. This was seen more infrequently than the prophase stage of mitosis. Microtubules are shown extending between plaques on opposite sides of what appears to be a dividing nucleus. It is suggested that the chromosomes are subsequently pulled toward the poles and the nuclear envelope pinches in two.

Sporogony was disporoblastic. Two separate diplokarya are shown in Fig. 11, whereas Fig. 7 indicates a similar structure apparently undergoing transverse fission into two similar sporoblasts with cytoplasmic filaments linking them. Elsewhere individual sporonts containing diplokarya were common (Fig. 3). Disporoblastic sporonts measured about $8.5 \times 1.5-2.5 \mu\text{m}$.

Sporoblasts were recognizable due to the presence of finger-like projections extending from their external surfaces into the spaces which typically surrounded each cell (Fig. 8). However, the possibility that such spaces represent fixation artifacts cannot be discounted. The Golgi body was found in the posterior half of the sporoblast (Fig. 8). In the younger stages this appeared as a collection of membrane-bound vesicles which subsequently became filled with electron-dense

FIG. 1. Transverse section of the midgut showing numerous spores (S) in columnar (C) and goblet cells (G) and in mucous droplets (M) in the lumen (L). MV, microvilli. Light microscopy ($\times 550$).

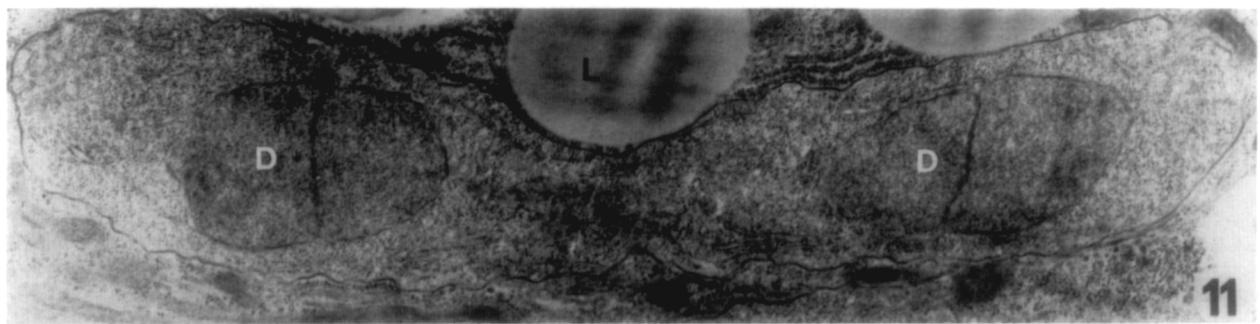
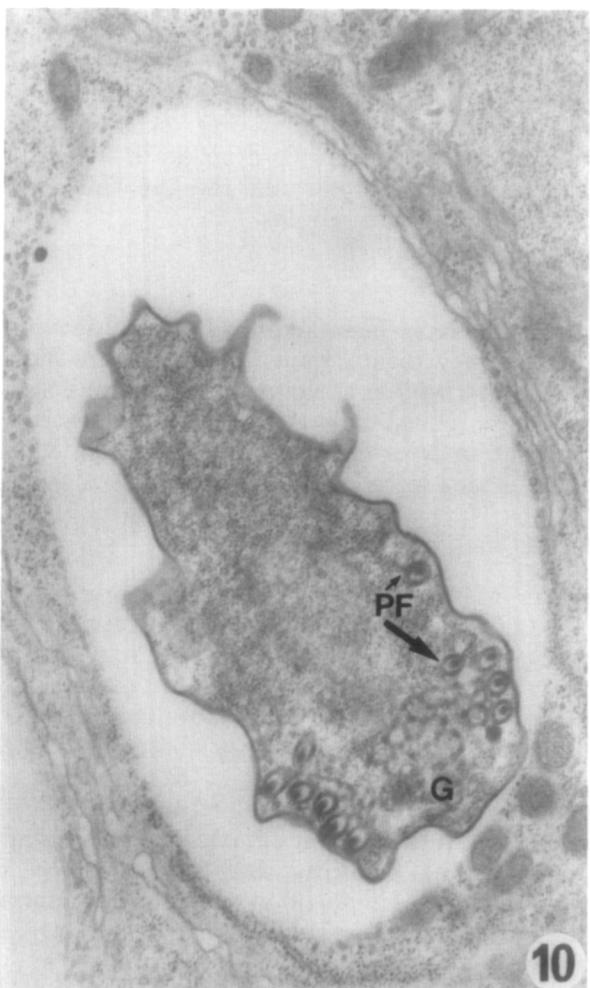
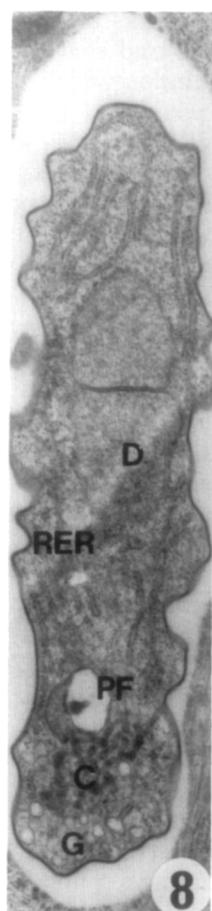
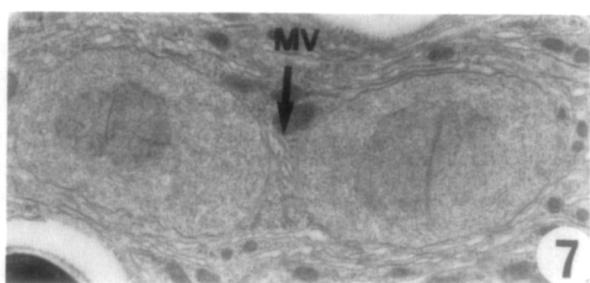
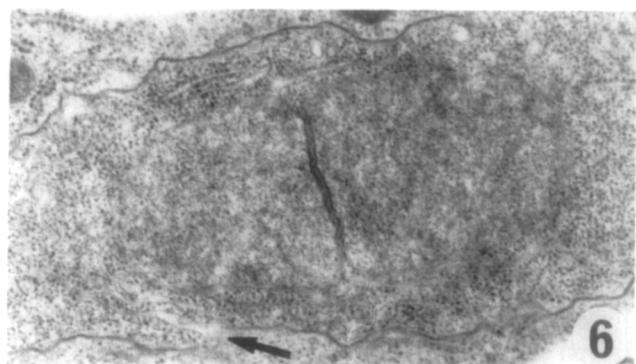
FIGS. 2-5. Meront and sporont stages of *Nosema* sp. found in the columnar cells of larval midgut of *H. armigera* (electron microscope).

FIG. 2. Meront with diplokaryon. D, diplokaryon; PM, plasma membrane; R, ribosomes of host cell surrounding parasite PM; L, lipid droplet ($\times 23,800$).

FIG. 3. Sporont showing formation of the wall (W) and the presence of a well-developed rough endoplasmic reticulum (RER) ($\times 13,100$).

FIG. 4. Sporont showing late anaphase stage of mitosis. K, kinetochores; SP, spindle plaque; spindle microtubules (arrow).

FIG. 5. Sporont showing prophase stage of mitosis. PV, polar vesicles; K, kinetochores; SP, spindle plaque; L, lipid droplet ($\times 17,500$).



material. These coalescences were most obvious initially in the region of the Golgi body nearest the nuclei.

Figures 8–10 and 13–15 show the likely developmental sequence of the polar filament as it extends from its site of production in the posterior region (Fig. 13) toward the anterior end where it terminated in the anchoring disc. The latter was closely moulded to the inner contour of the spore apex in the mature spore (Fig. 15).

The Spore

The average dimensions of mature spores were $2.43 \pm 0.12 \mu\text{m} \times 1.22 \pm 0.06 \mu\text{m}$ ($n = 13$) (mean \pm standard error) in the material prepared for electron microscopy. No macropores were found. Longitudinal sections through the spore revealed the presence of a peripherally situated coiled polar filament which was restricted to the posterior two-thirds of the cell.

The cytoplasm had numerous free ribosomes and areas of well-developed RER, the latter being situated within the helically coiled polar filament (Fig. 12). The diplokaryon was seen in the middle of the spore surrounded by the endoplasmic reticulum and the coiled polar filament (Fig. 16).

The polar filament develops from a simple tube filled with electron-dense material (Fig. 10) into a layered structure (Fig. 17 and 18) and up to nine layers can be seen in some sections. The majority of the coiled filament was isofilar. In the mature spore the coils of the filament were arranged in a single layer, but irregular arrangements were seen in immature spores (Fig. 17). The polar filament was approximately 70 nm in diameter, appeared elliptical or circular, depending on the plane of sectioning (Figs. 12 and 16) and formed a 12–13 coiled helix in the posterior two-thirds of the spore (Fig. 12). The uncoiled portion was somewhat wider, being about 90 nm in diameter. The angle of tilt of the coil with respect to the long axis of the spore was 65° (anterior coil) and 54° (posterior coil). In some sections, the diameter of the filament closest to the posterior body was smaller (50 nm) (Fig. 12).

The posterior body (posterosome) in *Nosema* sp. infecting *H. armigera* was approximately oval and tilted like the polar filament; the angle being approximately 48° with respect to the longitudinal axis of the spore (Figs. 12 and 16). The posterior body was about one-fifth of the total spore length (excluding the spore wall,

Fig. 12), and was limited by numerous layers of membranes (Fig. 16).

The exospore of the mature spore was 35 nm thick and followed the corrugated surface of the underlying electron-lucid endospore. No tubuli were seen projecting from the exospore as reported in *Pleistophora debaisieuxi* (Vavra, 1976). The endospore was present only in the mature spore and measured about 50–60 nm except at the anterior cap (Fig. 15) where it was considerably thinner (<10 nm).

Production of the polaroplast starts after the polar filament has formed a cap in the sporoblast or immature spore (Fig. 13). Subsequently, the anterior region of the polaroplast was seen surrounding the uncoiled part of the filament as stacks of numerous flattened lamellae (Fig. 14) and the more posterior vesicular region developed later (Figs. 15 and 19).

DISCUSSION

The microsporidian parasite from *H. armigera* has a number of ultrastructural features that are characteristic of the genus *Nosema* (Larsson, 1988; Toguebaye *et al.*, 1988). Table 1 shows a comparison between the ultrastructural features of various species of the genus *Nosema* from lepidoptera and the microsporidium found in *H. armigera*. It can be seen that the *Nosema* sp. infecting *H. armigera* has ultrastructural and morphological features similar to those of several other described species including *N. carposphae* (Malone and Wigley, 1981); *N. maniera* (Toguebaye and Bouix, 1983); and *N. transitella* (Kellen *et al.*, 1977). However, there are differences between these species and the *Nosema* sp. infecting *H. armigera*, e.g., no evidence of stages similar to the quadrinucleate meronts found in *N. maniera* were found and fixed spores from *H. armigera* were smaller than other *Nosema* spp. described (see Table 1). A complete identification of the current species must await further information concerning distribution, host specificity, and the extent to which environmental conditions effect variability.

In the *Nosema* sp. infecting *H. armigera*, the polar filament consisted of several concentric layers. Indeed, layering is thought to be similar in all microsporidian polar filaments with 11 separate layers variously described by Vavra (1976) and Larsson (1986). Using the nomenclature devised by the latter author, no subdivision of the central layer (Layer I) was distinguishable in the current study.

FIGS. 6–11. Electron micrographs showing sporont and sporoblast stages of *Nosema* sp.

FIG. 6. Immature sporont with developing wall (arrow) and indistinct nuclear envelope ($\times 28,000$).

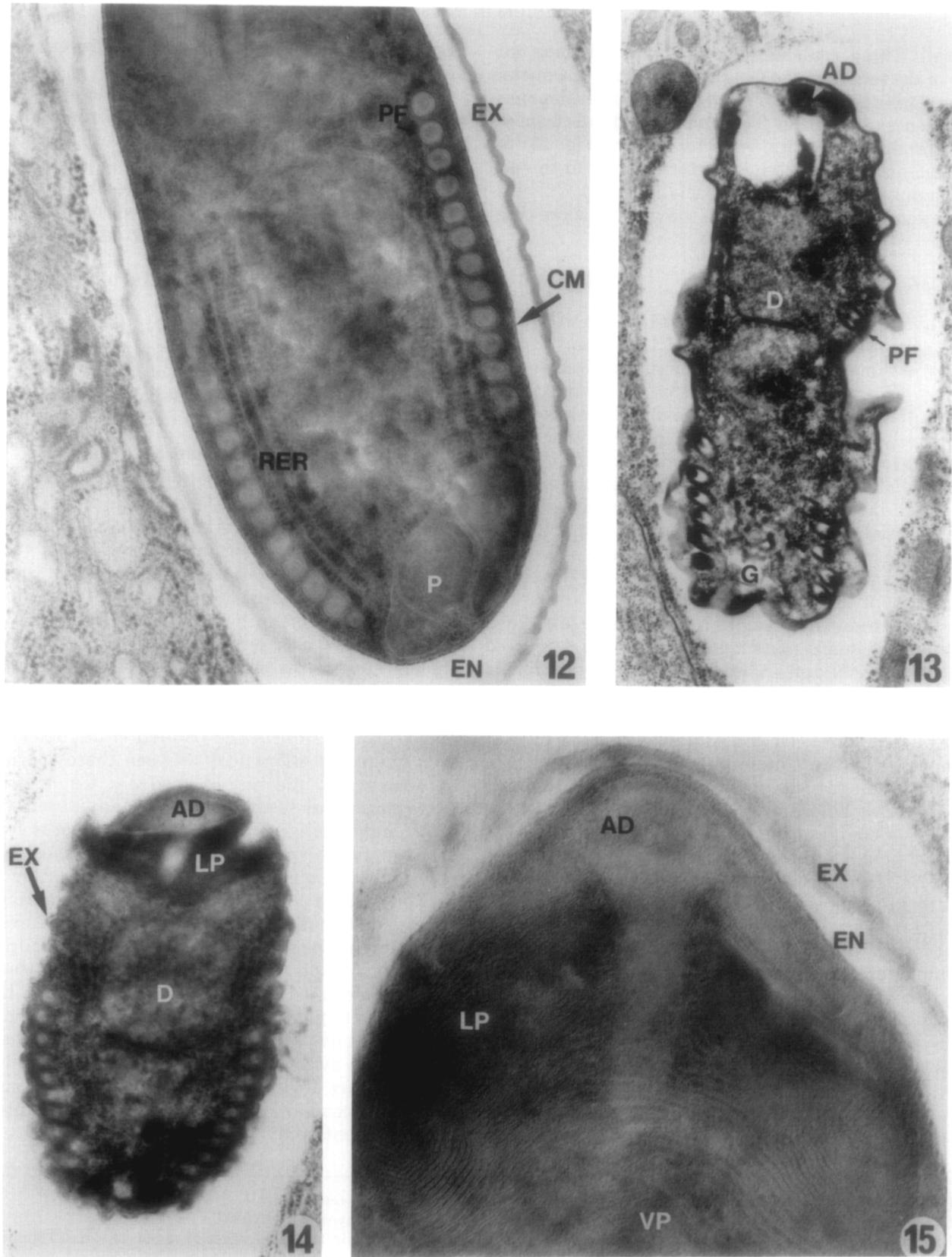
FIG. 7. Two sporoblasts with interdigitating microvillar projections (MV) found in the basal (serosal) region of a host columnar cell. ($\times 12,300$).

FIG. 8. Early sporoblast. Golgi (G); coalescences (C); anterior polar filament (PF); rough endoplasmic reticulum (RER); diplokaryon (D) ($\times 24,000$).

FIG. 9. Sporoblast showing further development of the polar filament (PF); Golgi body (G); coalescences (C) ($\times 26,000$).

FIG. 10. Sporoblast showing sections through the irregularly coiled polar filament (PF); Golgi (G) ($\times 26,300$).

FIG. 11. Sporont with two diplokarya (D); host lipid droplet (L) ($\times 20,400$).



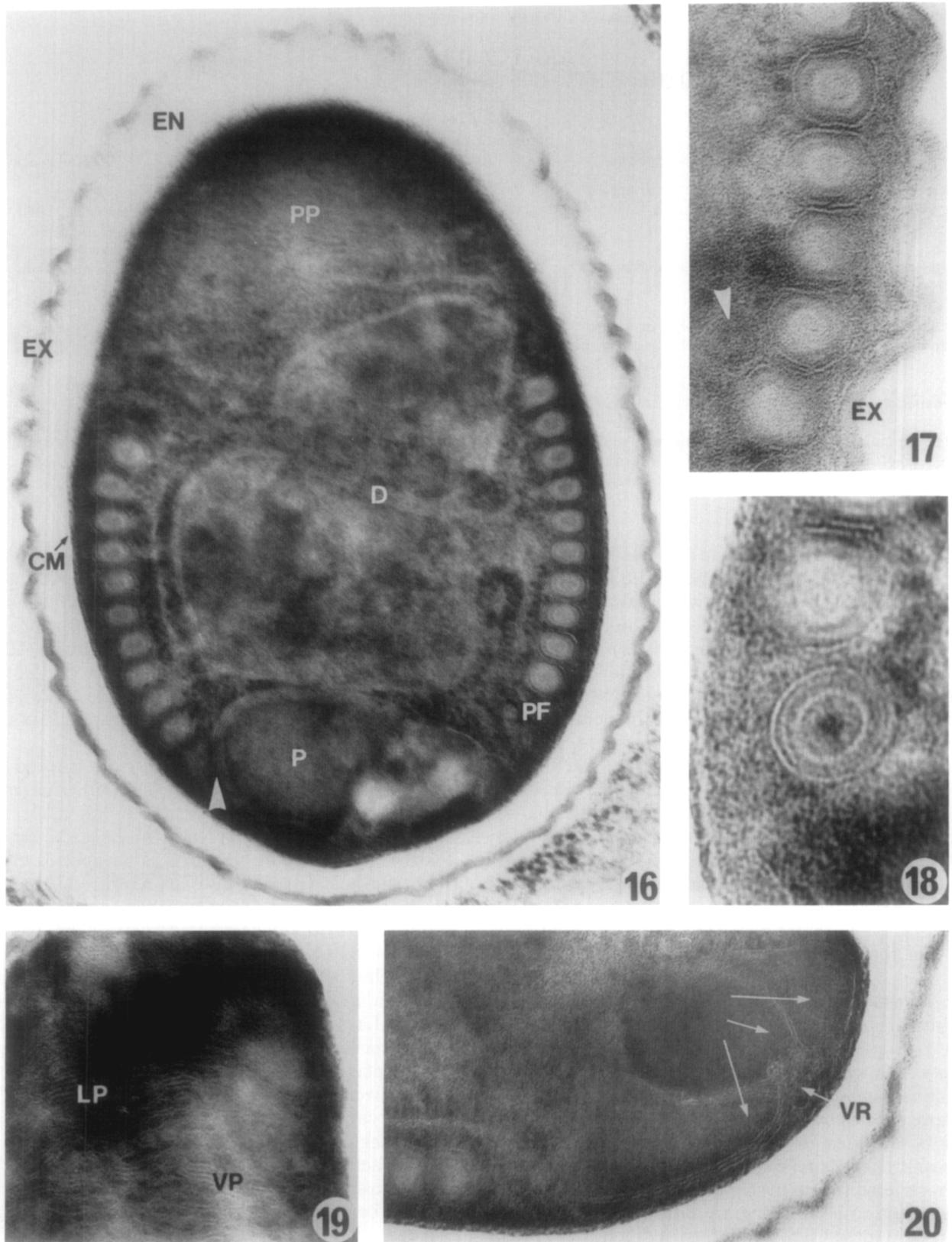
FIGS. 12-15. Electron micrographs of sporoblasts and spores of *Nosema* sp.

FIG. 12. Posterior region of a spore showing 12-13 coils of the polar filament (PF); exospore (EX); endospore (EN); and cytoplasmic membrane (CM); rough endoplasmic reticulum (RER); and posterior body (P) ($\times 58,000$).

FIG. 13. Sporoblast with anterior anchoring disc (AD); polar filament (PF); diplokaryon (D); Golgi body (G) ($\times 27,200$).

FIG. 14. Immature spore. Anchoring disc (AD); lamellar polaroplast (LP); exospore (EX); diplokaryon (D) ($\times 33,700$).

FIG. 15. Anterior region of mature spore showing exospore (EX); endospore (EN); anchoring disc (AD); lamellar polaroplast (LP); and the vesicular polaroplast (VP) ($\times 100,000$).



FIGS. 16-20. Electron micrographs showing spore structure.

FIG. 16. Posterior body (P); diplokaryon (D); polar filament (PF); polaroplast (PP); cytoplasmic membrane (CM); endospore (EN); exospore (EX); continuation of polar filament membrane around posterior body (arrowhead) ($\times 64,300$).

FIG. 17. Immature spore filament structure. Irregular coil (arrowhead); exospore (EX) ($\times 160,000$).

FIG. 18. Mature spore filament structure showing concentric rings of posterior-most filament ($\times 270,000$).

FIG. 19. Polaroplast of mature spore showing lamellar (LP) and vesicular portions (VP) ($\times 100,000$).

FIG. 20. Fine structure of posterior body showing three subdivisions (arrows) and vesicular region (VR) ($\times 100,000$).

TABLE 1
Ultrastructural Data for Various Species of *Nosema*

Species	Host species	Spore length × width (μm)	1	2	3	References
<i>Nosema</i> sp. in <i>H. armigera</i>	<i>Helicoverpa armigera</i>	2.43 × 1.22	70	12–13	54/65	Present work
<i>N. algerae</i>	<i>Heliothis zea</i>	3.2 × 1.8		11*		Street et al., 1980
<i>N. bombycis</i>	<i>Bombyx mori</i>	2.7* × 1.6*	65*	12.21	49/–	Sato et al., 1982
			80	13–14		Takizawa et al., 1975
<i>N. carpocapsae</i>	<i>Cydia pomonella</i>	3.13 × 1.88		9–13	53/–	Malone and Wigley, 1981
sp. M11	<i>Bombyx mori</i>	2.7* × 1.0*	80*	11.23	39/–	Sato et al., 1982
sp. M12		3.7* × 1.5*	90*	14.71	43/–	Sato et al., 1982
<i>N. maniera</i>	<i>Helicoverpa armigera</i>	3.6* × 1.25*		10–12	55*/52*	Toguebaye and Bouix, 1983
<i>N. pyrausta</i>	<i>Macrocentrus grandii</i> in <i>Ostrinia nubilalis</i>	4.2 × 1.75	100	10–12		Andreadis, 1980
<i>N.S.C.</i>	<i>Sceliodes cordialis</i>	4.11 × 2.05		7–14		Mercer and Wigley, 1987
<i>N. transitellae</i>	<i>Paramyelois transitella</i>	3.62 × 2.38		12.4	55/58	Kellen et al., 1977

Note. The numerically labeled columns refer to the following ultrastructural data from mature spores. (1) coiled filament width (nm); (2) number of polar filament coils; (3) angle of tilt of anterior coils/posterior coils to spore long axis (degrees).

* Indicates that the information has been measured from published micrographs and not quoted by the original authors.

The polar filament may form a specialized part of the posterior region of the spore called the posterior body. The location of this structure was identical to that occupied by the Golgi body earlier in the life cycle (Fig. 8), suggesting that the posterior body is also elaborated by the Golgi body (Vavra, 1976). This view is supported by the apparent continuity between the membrane surrounding the posterior region of the polar filament, as seen in longitudinal sections of the spore, and those associated with the posterior body (Fig. 16). Furthermore, it is reported that the posterior body spins vigorously during polar filament extrusion and is absent from the spore following filament discharge (Vavra, 1976). Examination of Fig. 20 shows the posterior body as being made up of a number of membrane-bound substructures which may play a part in water uptake, thus providing the pressure needed for polar filament extrusion and the germination of the sporoplasm (Undeen, 1990).

The exospore and polaroplast can be classified using the nomenclature of Larsson (1986) as Type 1B and Type 1, respectively. However, the corrugations of the spore wall may be real or a consequence of fixation and subsequent processing.

Many species of *Nosema* have been identified on the basis of the host in which they were first described. However, there is no evidence that *Nosema* spp. are in fact restricted to one host. For example, *N. algerae* has been shown to be infective in a variety of different insect orders including lepidoptera and noninsect arthropods (Fournie et al., 1990) whilst *N. bombycis* infects some 20 species of lepidopteran and other insects (Kashkarova, 1981).

Environmental conditions are known to affect protozoan spore development. *H. armigera* has a high mid-gut luminal pH like other lepidopteran larvae and has

been measured at pH 9.6 (unpublished result). This would suggest that the current microsporidian spores germinate effectively under alkaline conditions. Alkaline pH is known to act as a stimulus for spore germination in *N. locustae* (Whitlock and Johnson, 1990), *N. fumiferanae*, and *N. algerae*, whereas *N. heliothidis* germinated best at about pH 7.0 (Undeen, 1978). Thus it would seem unlikely that the parasite investigated here is *N. heliothidis*.

A number of researchers have attempted to use small differences in spore size as an indication of genus (Gassouma, 1972). However, the small differences in spore size should not be used alone as a taxonomic feature because spore size for a given species may vary with the host (Brooks and Cranford, 1972) and with geographic location (Brooks, 1968), and is affected by temperature (Maddox and Luckmann, 1966), age of the host, the host tissue infected, and also the medium in which they are measured (Mercer and Wigley, 1987). Fixation and staining may affect the size and shape of spores (Larsson, 1989). Furthermore, significantly different spore sizes have been reported for the same parasitic species in different individual members of the same species of host (Mercer and Wigley, 1987) and normal variations in spore size are to be expected as in any other population study (Lom et al., 1989).

The factors discussed above make accurate identification to species level extremely difficult and therefore on this basis the *Nosema* sp. infecting *H. armigera* is not readily distinguishable from some species infecting other Lepidoptera. Nevertheless, given the potent insecticidal action noted in laboratory cultures of the lepidopteran pest, *H. armigera*, further studies on this nosematid are clearly warranted to determine host specificity and assess its potential as a control agent under field conditions.

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