

Light and Electron Microscope Study of a New Species of *Thelohania* (Microsporida) in the Shrimp *Pandalus jordani*¹

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Thelohania butleri n. sp. was found in cells of skeletal muscles of the shrimp *Pandalus jordani*, from Queen Charlotte Sound, British Columbia, Canada. Sporulation stages were studied with the light and the electron microscope. Earliest stages were small and apparently uninucleate. Next were small diplokaryotic cells that possibly arose by fusion of the former. These enlarged and underwent sporogony. Sporogony was a series of three binary divisions, each producing unikaryotic cells. There was no sporogonial plasmodium. The spore was ovoid, 4.8 × 3.1 μm (stained), with a large crescentic nucleus and rounded posterior vacuole. The polar filament was isofilar, doubly coiled, with about 10 turns. This species closely resembles the type *T. giardi* Henneguy. It is concluded that sporogony by means of three binary divisions and lack of a sporogonial plasmodium may be essential characters of the genus *Thelohania* Henneguy, 1982.

KEY WORDS: Microsporida in shrimp, *Pandalus jordani*; electron microscopy; *Thelohania butleri* n. sp.; sporogony; genus *Thelohania* Henneguy, characters of.

INTRODUCTION

Recently, Vernick et al. (1977) made a brief electron microscope study of *Thelohania* sp. in the shrimp *Pandalus jordani*. That study was essentially limited to observations on the Golgi complex. Now we are describing the parasite more fully and proposing a name for it. Our study deals entirely with stages in the sporulatin sequence.

MATERIALS AND METHODS

In 1973 and 1974 Dr. T. H. Butler of the Fisheries Research Board of Canada, Nanaimo, British Columbia, Canada, sent

to one of us (V.S.) several lots of preserved material, either pieces of muscle or entire abdomens, from five species (already identified) of *Pandalus*. Most of the specimens were infected with microsporidia, of which there were four or five species. Only one of these, *Thelohania* sp. Vernick et al., 1977, in *Pandalus jordani*, is considered now, although two others were found in the same host species.

The infected *P. jordani* material used in this study consisted of the following specimens: five abdomens of shrimp caught off Tofino, West Coast of Vancouver Island, British Columbia, August 1, 1973, and fixed either in Bouin's solution or formalin-alcohol-acetic acid mixture (FAA); four pieces of abdominal muscle from shrimp caught at an unspecified locality on April 17, 1974, numbered 74-S-4, and fixed in glutaraldehyde; two pieces numbered 74-S-60 and one piece numbered 74-S-81, also fixed in glutaraldehyde but date and locality not specified; four abdomens of shrimp

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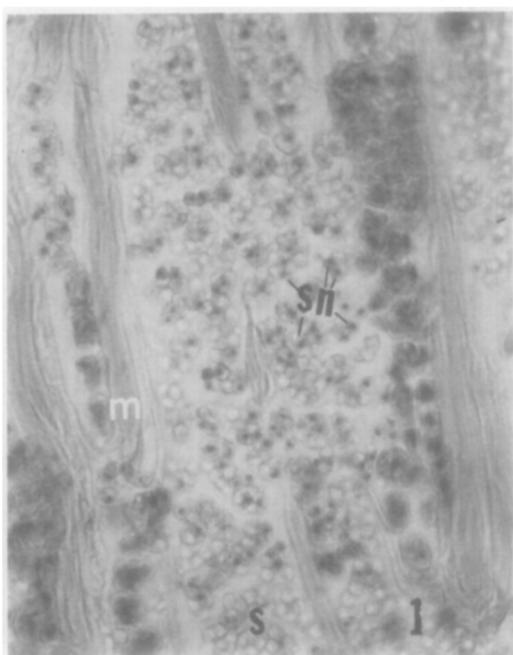


FIG. 1. Longitudinal section of rather heavily infected muscle, late stage. s, Spores that did not stain; sn, stained nuclei of spores; m, muscle. FAA-Heidenhain; $\times 520$.

caught in Queen Charlotte Sound on April 17, 1974, numbered 74-S-2, and fixed in FAA; two abdomens of shrimp caught on April 17, 1974, from an unspecified locality, numbered 74-S-4, and fixed in Bouin's solution.

All specimens were examined microscopically in wet mounts of fragments of the preserved muscles. Spore measurements were made from these preparations, using photographs of the spores and of a stage micrometer scale. Most specimens were sectioned and stained by one or more of the following methods: Gomori trichrome as modified by Alger (1966), Heidenhain's iron hematoxylin, periodic acid-Schiff (PAS), or PAS followed by iron hematoxylin. The material fixed in glutaraldehyde or FAA and stained with iron hematoxylin was especially useful for stages in sporogony. Gomori's trichrome was especially good for nuclei, which stained red against a green background.

The methods used for electron micro-

copy have already been given by Vernick et al. (1977).

RESULTS

Light Microscopy

In cross sections of the abdomen the parasites were most numerous in the peripheral portion and less numerous or absent in the central portion. When the infection was heavy, most microscopical fields consisted of enormous numbers of parasites (mainly spores); although some fields showed also fragments of muscle cells (Fig. 1). In light infections, the section consisted of mostly intact muscle tissue with scattered patches of the parasites (mainly early sporulation stages) in the muscle cells of the periphery of the section. The patches of developing parasites, when seen in longitudinal sections, were typically wedge-shaped with earliest

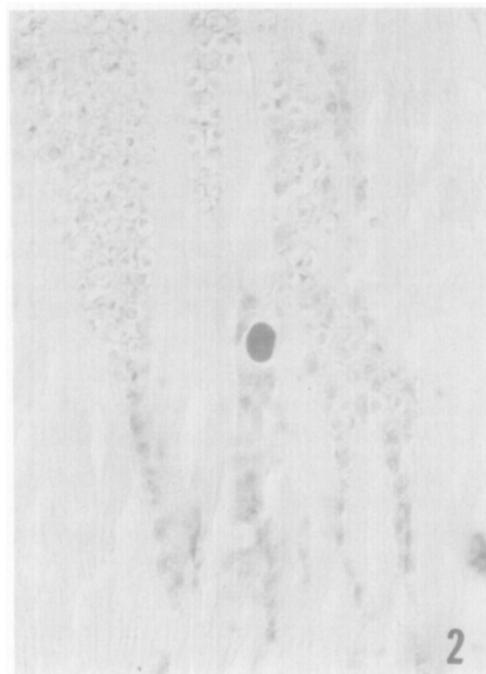


FIG. 2. Longitudinal section of muscle with early infection. Shown is a sequence of stages in sporogony with earliest stages at the bottom. Glutaraldehyde-Heidenhain; $\times 520$.

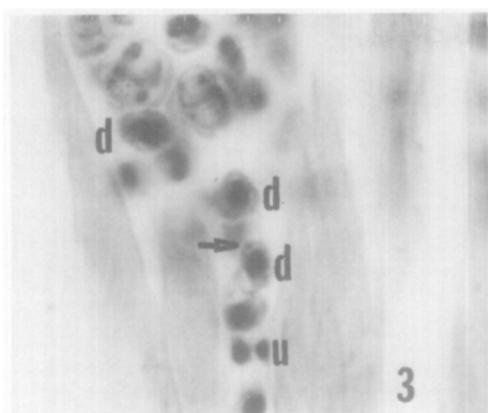


FIG. 3. Longitudinal section of muscle showing parasites in initial stages of sporogony. u, Uninucleate cells; d, small diplokaryotic cells of early sporonts (arising by fusion of uninucleate cells?); arrow, chromophilic granule. FAA-Heidenhain; $\times 1300$.

stages lying in single file at the pointed end of the wedge, latest stages irregularly massed in the broad part, and a more or less regular progression of developmental stages between (Fig. 2).

All the stages recognized were in the sporulation sequence, with the possible exception of the earliest ones. The earliest were small, irregularly shaped cells with very little cytoplasm. They were roughly 3 μm in diameter with, apparently, a single nucleus about 2 μm in diameter (Fig. 3, u). Most frequently these cells were compressed between the host muscle fibers and were quite elongated (Fig. 2). Slightly larger cells (3–4 μm in diameter) were clearly diplokaryotic (Fig. 3, d) and two black granules (about 0.5 μm in diameter) (Fig. 3, arrow) lay on opposite sides of the diplokaryon. We regard these cells (fusion products?) as early "sporonts," since they were destined to develop into large cells that underwent sporogony. The most conspicuous feature of the development was an increase in their size (Figs. 4, 5). The increase appeared to be essentially an expansion of the plasmalemma (formerly invisible) accompanied by formation of a vesicle (about 9–12 μm in diameter) containing the diplokaryon and the aforementioned granules (Figs. 5, 7). The cytoplasm became highly vacuolated as the cell

enlarged. Most of it was concentrated around the nuclei, giving them a very rough appearance. From some of the cytoplasmic protuberances on the nucleus tenuous strands of cytoplasm extended to the plasmalemma.

When the sporont reached its full size it was still diplokaryotic (Fig. 5). Many of these large cells appeared to be uninucleate, but a diplokaryon with one of its members covering the other would be indistinguishable from a single nucleus.

The sporont underwent sporongony to produce eight sporoblasts within a sporophorous vesicle (pansporoblastic membrane). Sporogony was clearly a series of three binary divisions (one cell \rightarrow two cells \rightarrow four cells \rightarrow eight cells, Figs. 5–8), each resulting in uninucleate cells (Figs. 6–8). The eight cells (sporoblasts) transformed into uninucleate spores. There was never a sporogonial plasmodium. The sporoblasts (Fig. 8, sb) had a somewhat polygonal shape because they were flattened in areas of contact with one another.

Spores in hematoxylin preparations often did not stain (Fig. 1, s). Many others showed only a large crescentic nucleus (Fig. 1, sn) while a few (probably young ones) showed clearly much of the internal structure (Fig. 8). In the latter the polaroplast and posterior vacuole appeared as two large light areas. The polar cap was centrally located in the anterior end. From it the polar filament extended diagonally backward and coiled around the posterior vacuole. The coil (Fig. 8, arrow), in optical section, was seen as a conspicuous dark band along the posteriolateral margin of the spore. The nucleus, not sharply differentiated when cytoplasmic components also stained with hematoxylin, lay posteriolaterally to the polaroplast. These stained spores were about $4.8 \times 3.1 \mu\text{m}$ ($4.5-5.0 \times 3.0-3.5 \mu\text{m}$; $n = 10$). In Gomori preparations the nucleus was red, sharply defined, large (about $2.5 \times 1.0 \mu\text{m}$), and crescentic.

Spores preserved in glutaraldehyde (Fig. 9) and unstained were ovoid, with rounded ends, with a thick wall, without ornamentation, with an anterior clear area (the

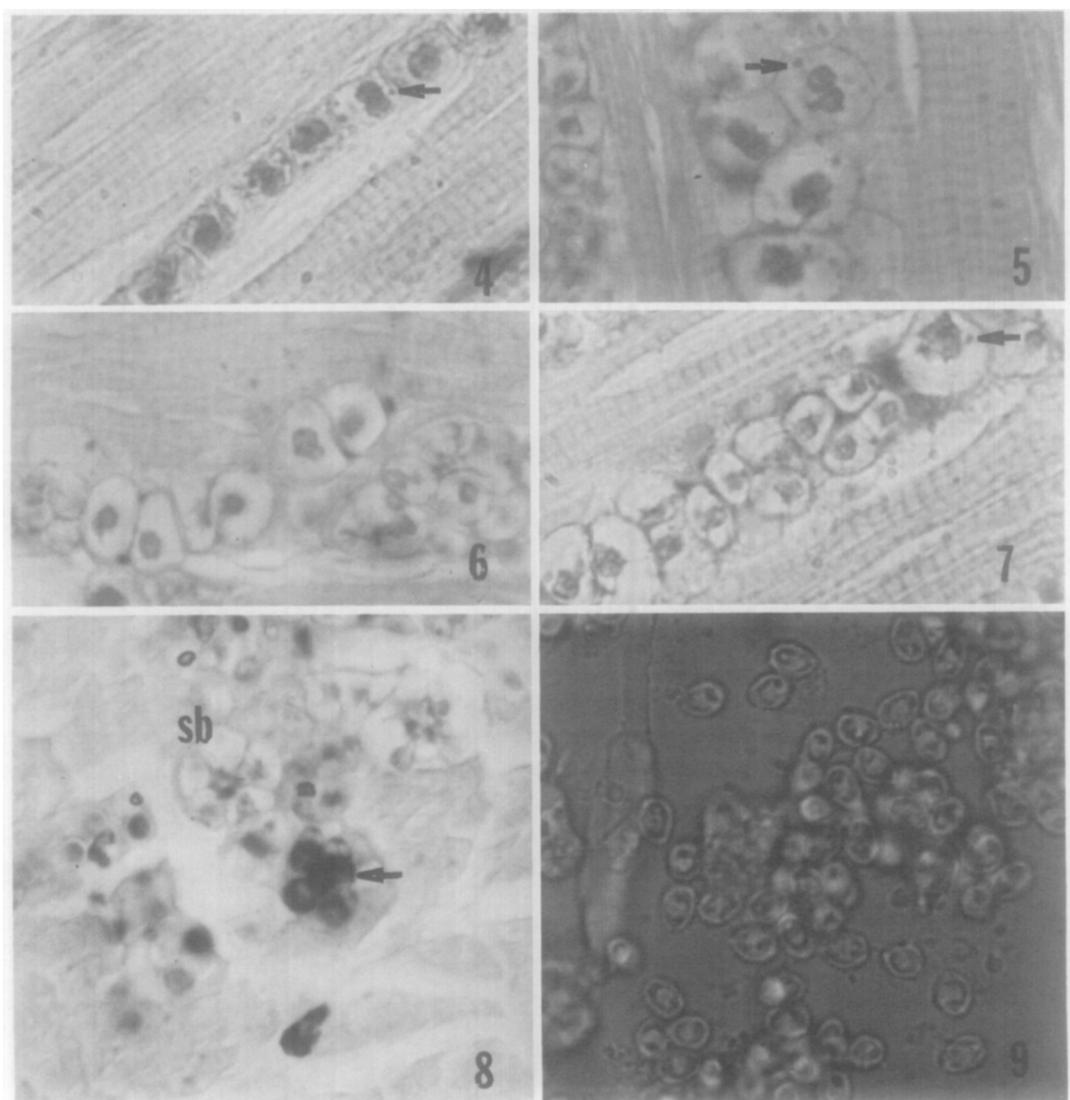


FIG. 4. Diplokaryotic cells (sporonts) in process of enlarging. Arrow indicates chromophilic granule. Glutaraldehyde-Heidenhain; $\times 1300$.

FIG. 5. Diplokaryotic cells (sporonts) of maximum size. Glutaraldehyde-Heidenhain. $\times 1300$.

FIG. 6. Two-cell stage, uninucleate cells resulting from first sporogenic division. Glutaraldehyde-Heidenhain; $\times 1300$.

FIG. 7. Mostly four-cell stages, consisting of uninucleate cells resulting from second sporogonic division. Glutaraldehyde-Heidenhain; $\times 1300$.

FIG. 8. Eight-cell stage (sporoblasts, sb) and spores. Arrow indicates coils of polar filament. Glutaraldehyde-Heidenhain; $\times 1300$.

FIG. 9. Spores fixed in glutaraldehyde and preserved in alcohol. $\times 1300$.

polaroplast), and with a rounded posterior vacuole containing an inclusion body ("secretion granule," according to Vernick et al., 1977). They were $4.7 \times 3.3 \mu\text{m}$ ($4.5-5.0 \times 3.2-3.5 \mu\text{m}$; $n = 10$).

Electron Microscopy

A few diplokaryotic cells (presumably sporonts) were seen. One of these has already been illustrated by Vernick et al.

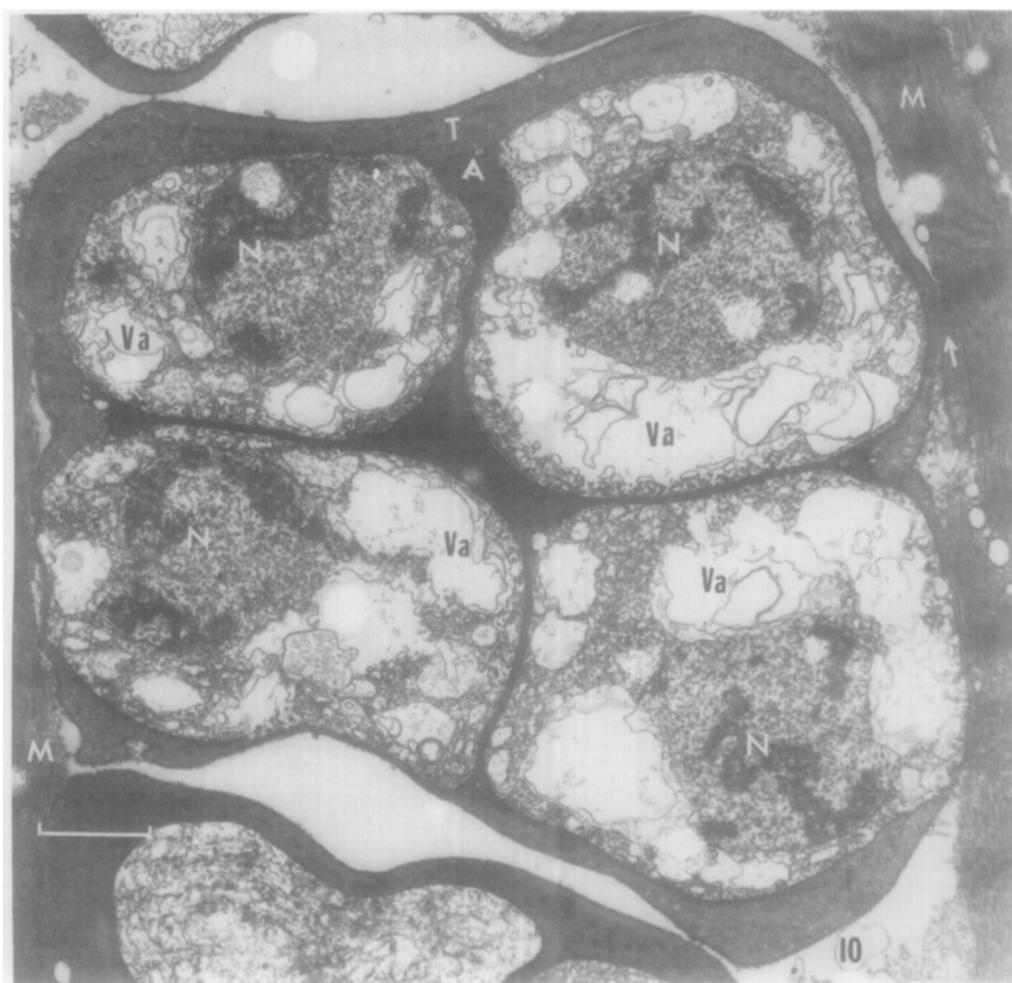


FIG. 10-17. Electron micrographs from glutaraldehyde-fixed material. The scale represents 1 μm unless otherwise indicated.

FIG. 10. Four-cell stage in sporogony. N, Nucleus; Va, cytoplasmic vacuole; M, muscle; V, vesicles derived from muscle; T, tubular (?) elements in pansporoblast; A, amorphous intercellular material.

(1977). It is noteworthy that the cells contained dense aggregates of vesicles which those authors identified as the "primitive Golgi" of Vavra (1965).

Cells produced by sporogonic division were unikaryotic, progressively less vacuolated and reduced in size. These cells were enclosed within a membrane, usually identified as the pansporoblastic membrane. The two-cell stage was not seen. During the four-cell stage (Fig. 10), the zone between the pansporoblastic membrane and the cells was mostly filled with a dense fibrillar (tubular?) material. Between the cells them-

selves was an amorphous, dense material. The fibrous or tubular zone (T) and the amorphous zone (A) were sharply separated, apparently by a membrane. The host muscle fibrils near the parasite were being lysed (Fig. 10, M). In some areas the contact between parasite and host muscle was very intimate, with confusion of their boundaries or actual interruption of them (Fig. 10, arrow). In such areas there appeared to be a direct continuity of host muscle and the fibrillar zone of the parasite. This zone appeared to be stratified, having as many as three or four layers. The

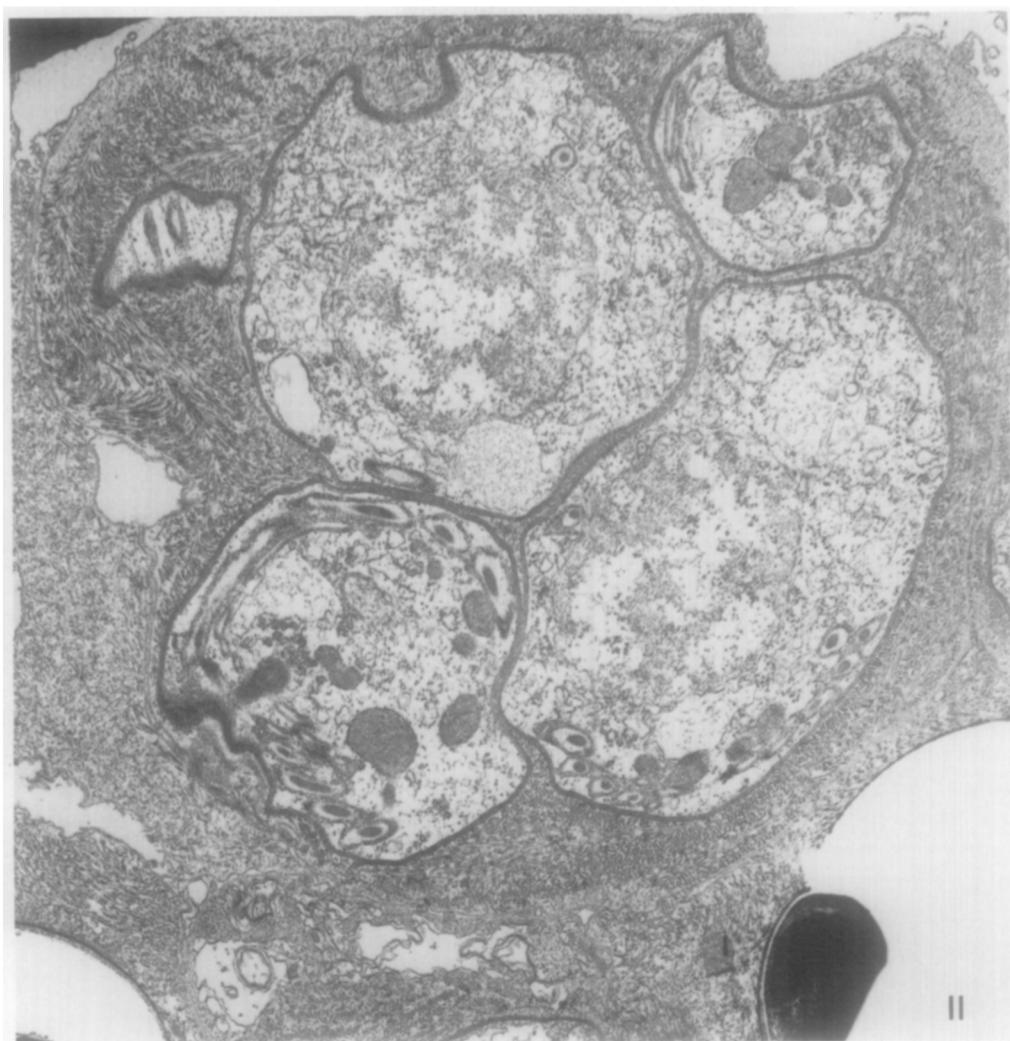


FIG. 11. Sporoblasts or eight-cell stage in sporogony. Only five cells are evident.

bounding membrane appeared to be composed of vesicles that resembled vesiculate elements formed in the lysed portion of the adjacent host muscle (Fig. 10, V). In some areas the parasite and the host muscle tissue seemed to be directly connected by means of such vesicles (Fig. 10, V).

In the eight-cell stage (Fig. 11) the intercellular amorphous material, mentioned above, had been replaced by the fibrous material. The latter still had a stratified appearance, with fibers of different layers often running in different directions. Each of the cells (early sporoblasts) was bounded by a membrane which appeared to be

trilaminar at first glance but seemed to be further divided (five layers?) when examined closely. The extracellular fibrous elements immediately adjacent to the sporoblasts showed a strong tendency to be perpendicular to the cell surface and were probably connected to it. Inside the sporoblast conspicuous changes were noted; the cytoplasm had become more dense, a polar filament was developing, and secretion granules (see Vernick et al., 1977) had appeared.

In a slightly later stage the limiting membrane of the sporoblast clearly was at least a five-layered structure, composed

of three quite dense and two less dense layers (Fig. 12, arrow). One layer, next to the cell contents, was extremely dense and possibly further laminated. At this stage the internal organelles were rather far advanced in their development and had largely assumed their final relative positions. It would be repetitious to describe in detail the morphology and development of these organelles because numerous publications have already dealt with this subject (Vavra, 1976, 1976a).

In the very late sporoblast stage (Figs. 13–15) the intercellular elements that formerly appeared filamentous now appeared definitely tubular in cross section (Fig. 14, T). Among the tubules were a few coarse granules (CG) of various sizes, some of them bounded by membranes. The limiting membranes of the sporoblasts consisted of two sets (Fig. 13). One set completely enclosed the sporoblast contents; it was a thin, dense (trilaminate?) membrane or plasmalemma, presumably identical with "the cytoplasmic membrane of the future spore content" (Vavra, 1976a). A portion of this membrane was in direct contact with the pansporoblastic membrane. The other set of membranes covered only the anterior half of the sporoblast; it was thick and complex, composed of several poorly defined layers constituting the exospore. The outermost layer of this exospore complex was very dense, frequently interrupted, and connected directly with the extracellular tubules. The innermost layer was covered by a deposition of coarse granules. Between the granular layer and the cytoplasmic membrane of the sporoblast contents was a wide space, apparently an artifact resulting from shrinkage of the sporoblast contents. The situation of this space in relation to membranes just described indicates that it was formed in the location of the future chitinous endospore. Some of the internal organelles, all of which were in an advanced state of development, were more clearly revealed in the late sporoblast (Figs. 12–14) than in the spore. Particularly clear were the secretion

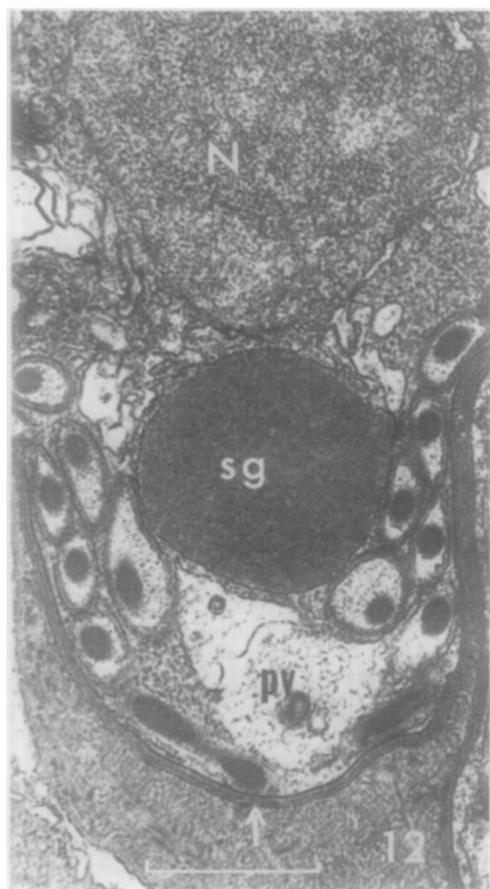


FIG. 12. Portion of young sporoblast (somewhat laterally compressed). N, Nucleus; sg, secretion granule; pv, posterior vacuole. Arrow points to membrane with five layers.

granules (sg), the lamellar and vacuolar portions of the polaroplast (PPL and PPV), the nucleus (N), the polar cap (PC) and posterior vacuole (pv). In or around the latter were some membranes but it is not clear that the vacuole was characteristically bounded by a membrane. A detailed description of the internal organelles is not made because it would contribute nothing new.

Micrographs of the mature spore (Fig. 16) revealed only a few noteworthy details. The polar cap was unusually thin and elongated, extending backward for almost one-third the length of the spore. The polar filament was slightly enlarged at the manubroid base but uniform in diameter throughout the rest of its length. It was

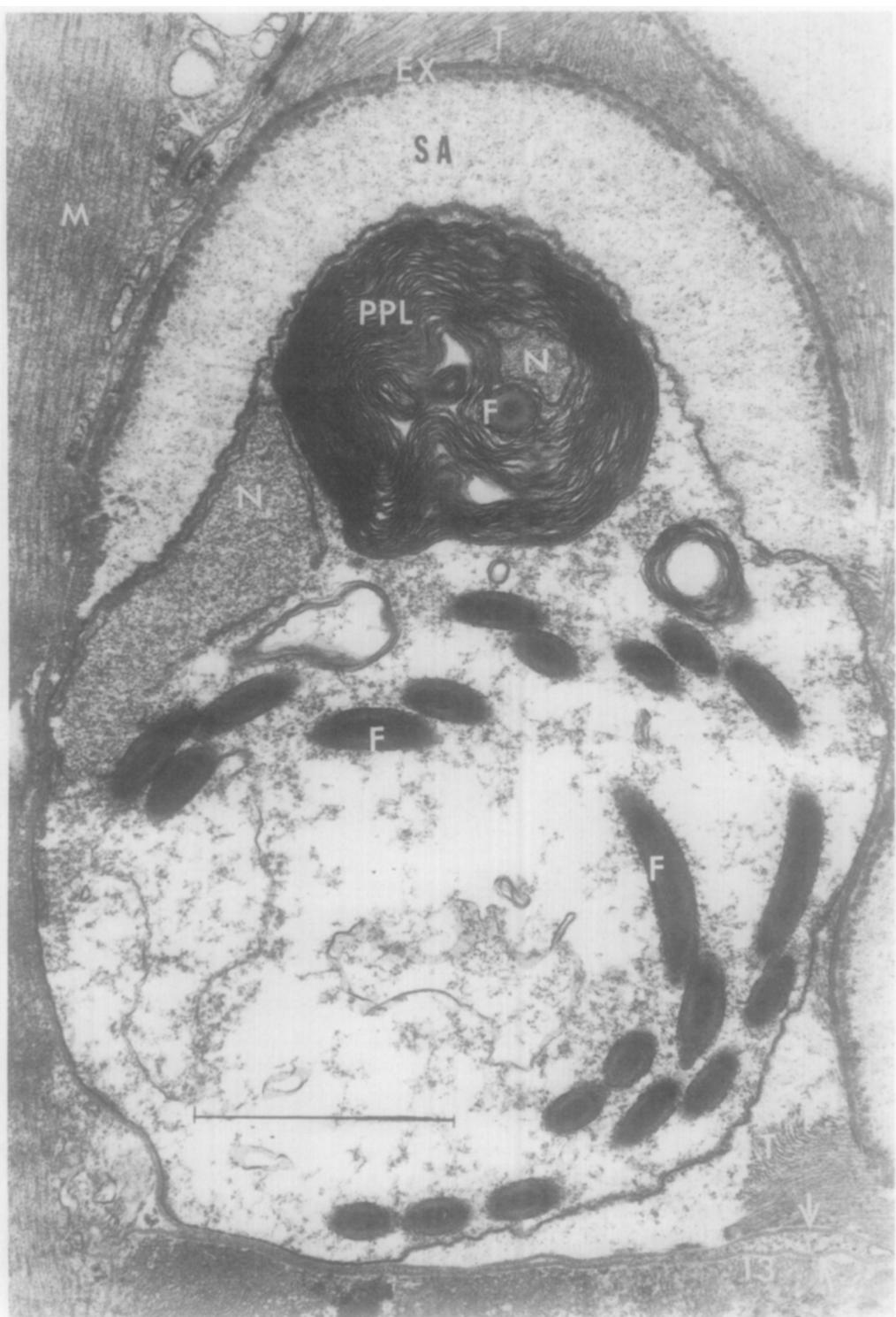


FIG. 13. Late sporoblast. SA, Shrinkage artifact in area of future endospore; EX, exposure membranes; N, nucleus; PPL, laminated part of polaroplast; F, polar filament; T, tubules; M, muscle. Arrows indicate pansporoblastic membranes.

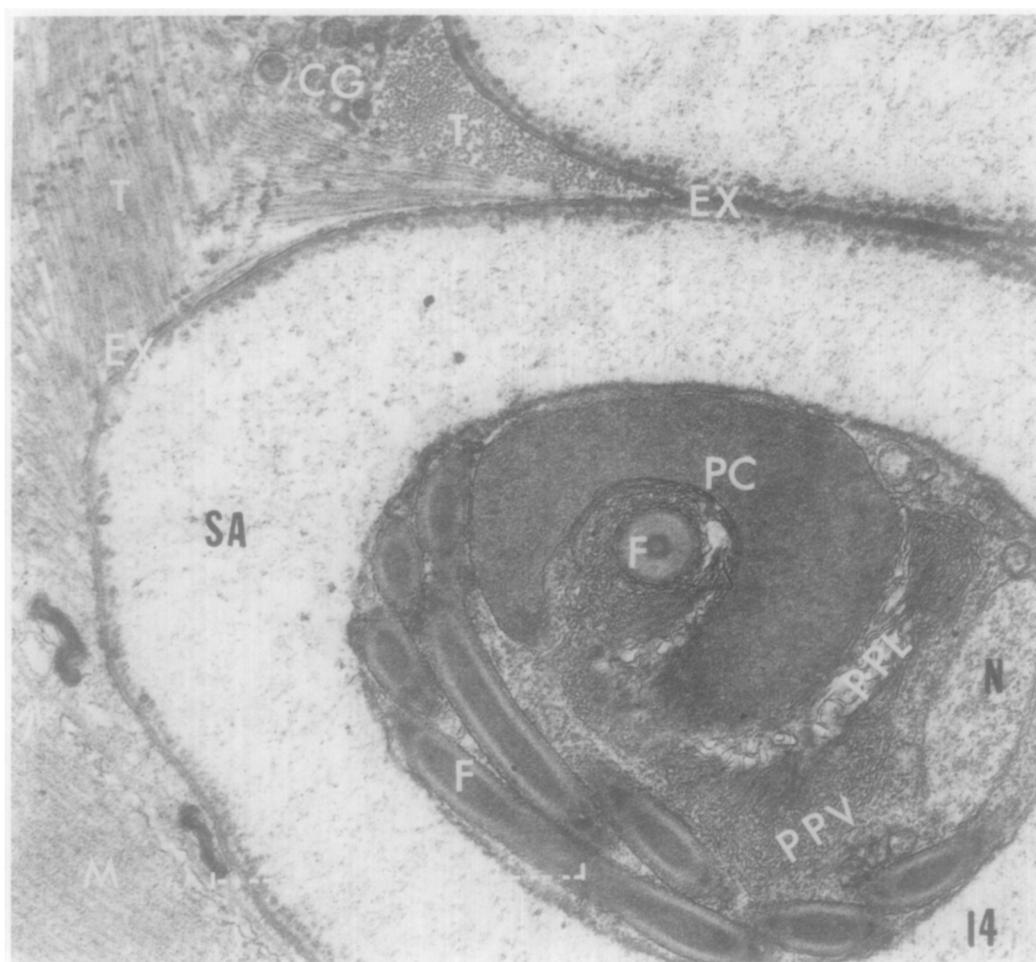


FIG. 14. Tangential section through anterior half of sporoblast. CG, Coarse granules; T, tubules, cs and ls; M, muscle; EX, exospore; SA, shrinkage artifact in region of future endospore; PC, polar cap; F, polar filament; N, nucleus; PPL, lamellate part of polaroplast; PPV, vesiculate part of polaroplast. Arrow indicates pansporoblastic membrane.

doubly coiled, with a total of about 10 or 11 turns. The polaroplast was composed of the usual two parts, an anterior lamellar one and a posterior vacuolar one, although the former was obscured by a staining artifact. The nucleus (N) was indistinct. The posterior vacuole was not evident in the few spores observed. The endospore (EN) was uniformly thick (about $0.4 \mu\text{m}$) except for a thin area at the anterior end. The exposure (EX) resembled that already described in the sporoblast but was thinner than formerly and the granules on the inner surface were finer. The spores were embedded in a matrix of fine and closely

packed granules (FG) and vesicles presumably derived from the former tubules, now almost completely disintegrated. The pansporoblastic membrane was a delicate structure composed mainly of small vesicles. The contiguous host muscle (Fig. 17, DM) was disintegrated and closely resembled the material within the pansporoblastic membrane, being composed of granules, vesicles, and a few tubules.

DISCUSSION

Mercier (1909), who described the life cycle of the type species *The洛hania giardi*

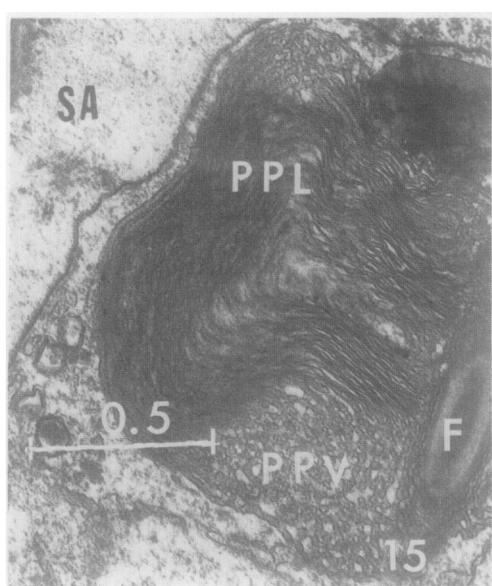


FIG. 15. Tangential section through polaroplast of sporoblast. PPL and PPV, Lamellate and vesiculate portions of polaroplast; F, polar filament (manubroid part); SA, shrinkage artifact.

Henneguy, 1892, saw essentially the same sporulation sequence that we have described. He believed that small uninucleate cells like our "earliest" stage (Fig. 3, u) were isogametes which underwent first cytoplasmic and then nuclear fusion ("copulation isogamique"), producing a cell with a single nucleus, the sporont. Our observation of apparently uninucleate cells followed by diplokaryotic cells is consistent with Mercier's view that there was cytoplasmic fusion of two uninucleate cells, although we saw no actual fusion. Unlike Mercier, we found no evidence for karyogamy. On the contrary, we saw a diplokaryotic condition of long duration, ending, in some undetermined manner, with the first sporogonic division. More data are needed to elucidate this and other problems relating to sexuality in *Thelohania*.

The kind of sporogony occurring in this species of *Thelohania* (three binary divisions) was previously reported in the type species, a fact that has been generally forgotten or ignored. The original description of *Thelohania giardi* by Henneguy and Théohan (1892) and the later more

detailed descriptions by Mercier (1909) both clearly indicated, by word and by illustration, that sporogony is a series of three cell divisions, each complete before the next begins. Kudo (1924) misinterpreted the report of Henneguy and Théohan by saying that three nuclear divisions "form eight nuclei, around each of which the cytoplasm becomes condensed." He gave an ambiguous account of Mercier's results that renders them obscure. Sprague (1977) considered the details of the early accounts of sporogony in *T. giardi* to be unreliable and simply said, "The sporont produces 8 sporoblasts within a pansporoblastic membrane. . . ." We can no longer ignore the previously published views that sporogony in the type species *T. giardi* is a series of three binary divisions. This conclusion has taxonomic implications. Sporogony of this kind precludes the existence of a sporogonial plasmodium. Nevertheless, several recent authors, losing sight of published data on the type species and basing their ideas on electron microscope studies of other species, have stated or implied that an important character of the genus *Thelohania* Henneguy is the possession of a sporogonial plasmodium. Note-worthy among these authors are Tuzet et al. (1971), Hazard and Oldacre (1975), and Sprague (1977). The evidence now suggests that the definition of the genus *Thelohania* should be amended to include sporogony by a series of three binary divisions and absence of a sporogonial plasmodium. Pending confirmatory data, however, it may be premature to make a definite proposal regarding this matter.

The nature of the "black granules" seen in our hematoxylin preparation is obscure. They probably are identical with the "chromidies vegetatives" observed by Mercier (1909) in *T. giardi* but this vague and obsolete term does not identify them. We could not definitely recognize them in the electron micrographs. In size and location, on opposite sides of the nucleus, they seemed to correspond with nothing but the "dense aggregates of vesicles"

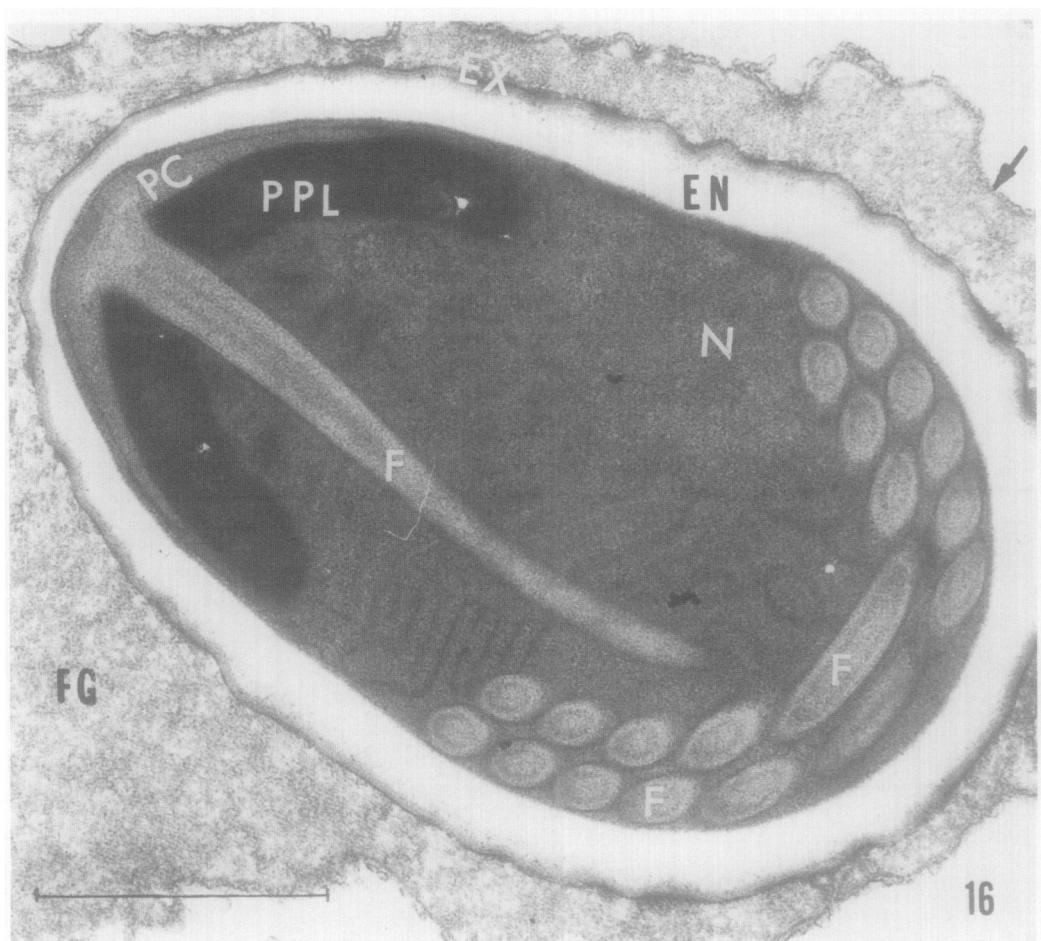


FIG. 16. Mature spore. EX, Exospore; EN, endospore, PC, polar cap; PPL and PPV, lamellate and vesiculate parts of the polaroplast; N, nucleus; F, polar filament; FG, fine granules, derived from former tubules (traces of tubules still present). Arrow indicates the pansporoblastic membrane.

which Vernick et al. (1977) interpreted as the "primitive Golgi" of Vavra (1965). If we concede the possibility that the granules seen with the light microscope and the aggregates of vesicles seen with the electron microscope are identical, this raises doubt that the latter were correctly identified because the intensely basophilic reaction of the former is not characteristic of Golgi vesicles. Thus, it seems that more information is needed to establish the identity of the "vesicles" as well as that of the "granules."

We noted that the "pansporoblast" of the parasite and the muscle tissue of the host were in very intimate contact (sometimes appearing to be continuous with one

another) while the former was being developed and the latter becoming disintegrated and diminished in quantity. This leads us to suspect that the pansporoblastic membrane and the tubules inside of it were being built up, at the expense of the host tissue, in some rather direct way. In other words, perhaps these structures are to be regarded as host material. If these are host material, perhaps the boundary separating the tubular zone (T) and the amorphous zone (A) in Fig. 10 is the "real" pansporoblastic membrane, which breaks down rather early and allows the tubules to penetrate between the sporoblasts. One can also speculate that none of the observed structures represents a pansporo-

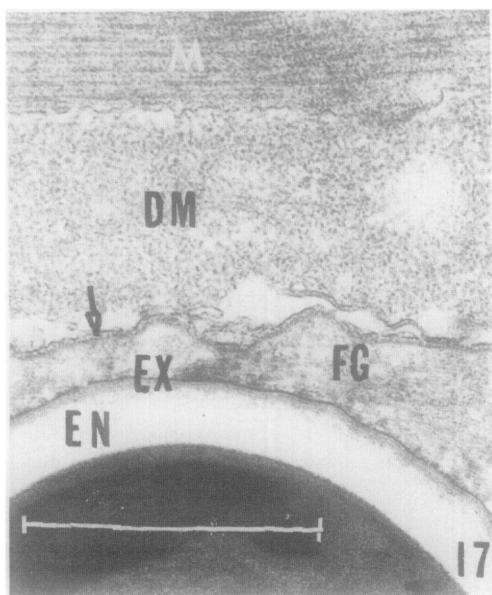


FIG. 17. Margin of a mature pansporoblast and the contiguous host muscle. M, muscle (intact); DM, disintegrated muscle; FG, fine granules and traces of tubules. Arrow indicates pansporoblastic membrane. Materials on the two sides of the pansporoblastic membrane show a remarkable resemblance.

blastic membrane but that the three binary fissions take place in a host vacuole. Apparently we have raised the whole complex issue of what is meant by "pansporoblast" and "pansporoblastic membrane," in this and other species, and whether the structures they signify are host or parasite material (assuming there is a sharp distinction). This set of problems is too complex and the present data are too meager to justify any detailed discussion or conclusion now. The problems, as they relate to *Thelohania*, may be partly resolved after data from electron microscope studies of the early sporogony stages become available.

Several species of microsporidia in shrimp were originally assigned to the genus *Thelohania*. We need to compare our *Thelohania* sp with only four named and one unnamed species because, according to Sprague (1977), the rest have been transferred to other genera. *Thelohania ceccaldi* Vivares, 1975, in *Processa edulis edulis* from the Mediterranean coast of France, is

clearly distinguished by having a fusiform pansporoblast. *Thelohania octospora* Henneguy, 1882, in *Palaemon rectirostris*, in France, has distinctly smaller spores ($3-4 \mu\text{m}$ long). *Thelohania duorara* Iverson and Manning, 1959, in *Penaeus duorara* from Florida, and *T. sp.* Thomas, 1971, in *Penaeus semisulcatus* from South India, both have spores about $5.4 \times 3.6 \mu\text{m}$ (probably not significantly larger than those of *Thelohania* sp. in *Pandalus*) but are distinguished by occurring in different host species, different infection sites, and different climates. Regarding infection sites, Kelly (1975) found that "*Thelohania duorara* is restricted to the periphery of muscle bundles" and "heavy infections . . . leave most of the muscle intact." One of us (V.S.) has observed *T. duorara* and *Thelohania* sp. Thomas in sections, noting in both the condition of which Kelly speaks. The appearance of the lesion is conspicuously different from that elicited by the present species; the parasite appears to be primarily in connective tissue between the muscle cell and only secondarily in the latter. *Thelohania giardi* in *Crangon vulgaris* was originally said to have spores 5 to $6 \mu\text{m}$ long, with sharply pointed anterior ends and with very fine longitudinal striations; its later description by Mercier (1909) gave no dimensions, did not mention striations, and illustrated spores with rounded ends. We can only reconcile these two descriptions by supposing that the pointedness was originally exaggerated and that the presence of striations is questionable, since the occurrence of neither of these morphological features has been confirmed for *T. giardi* or related microsporidian species. Thus, we think the spores of *T. giardi* are probably very similar in general appearance to those of *Thelohania* sp. in *Pandalus* and slightly larger ($5-6 \mu\text{m}$ long as compared with $4.5-5 \mu\text{m}$). Although these two species are very similar, we propose to treat them as distinct on the basis of difference in host and geographical location as well as slight differences in spore size.

Thelohania butleri n. sp.

Synonym: *Thelohania* sp. Vernick, Sprague, and Krause, 1977.

Host and site: (DECAPODA) *Pandalus jordani* Rathbun, 1902; cells of skeletal muscle.

Vegetative stages: No data.

Sporulation stages: Initial stage (early sporont) a small (4–5 μm), rather dense diplokaryotic cell with two or more basophilic granules in cytoplasm. This develops into a large (8–10 μm) diplokaryotic cell with highly vacuolated cytoplasm. Sporogony is a series of three binary fissions, all producing unikaryotic cells. During sporogony a dense mat of intercellular tubules arises. Tubules are transformed into fine granules during sporogenesis. Pansporoblast is fragile at maturity, permitting easy separation of spores.

Spore: Ovoid; 4.7 \times 3.3 μm preserved, 4.8 \times 3.1 μm stained; uninucleate, nucleus large and crescentic; polar filament iso-filar, doubly coiled, with about 10 turns; with rounded posterior vacuole enclosing secretion granule; with typical polaroplast, having anterior laminar and posterior vesicular portions; with thick (0.4 μm) exospore; with slightly undulated surface.

Locality: Queen Charlotte Sound, British Columbia, Canada.

Remarks: Dedicated to Dr. T. H. Butler, Fisheries Research Board of Canada, who furnished the material.

Deposition of type material (syntypes): Slide Nos. USNM 24529-VS74-14A and USNM 24530-VS74-18, in the International Protozoan Type Slide Collection, Smithsonian Institution, Washington, D.C.; slide Nos. 1978:5:16:1 and 1978:5:16:2 in the British Museum Protozoan Type Slide Collection; three slides in the collection of Dr. J. Weiser, Prague, Czechoslovakia; slide Nos. ROMP 133 and ROMP 134 in the Registry of Marine Pathology, National Marine Fisheries,

Oxford Laboratory, Oxford, Maryland; other syntypes in the collection of Victor Sprague (VS74-14A and VS74-18).

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