

# Cellular distribution of a feminizing microsporidian parasite: a strategy for transovarial transmission

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## SUMMARY

The cellular distribution of a vertically transmitted, feminizing microsporidian was followed in its host *Gammarus duebeni*. In adult females the parasite was restricted to gonadal tissue, in particular primary and secondary follicle cells. Spores were diplokaryotic with a thin spore wall and a short polar filament, characteristics typical of 'early' spores involved in autoinfection. The diplokaryotic life-cycle, absence of spore groupings and of a pansporoblast membrane typify the genus *Nosema*. However, the unusual globular polaroplast of the spore and restriction of this stage to host ovarian tissue have not previously been described in *Nosema*. Sporogony occurred only in follicle cells adjacent to developing oocytes and was in synchrony with the process of vitellogenesis. Oocytes were infected after formation of intracellular connections with follicle cells but harboured only vegetative stages of the parasite. Parasites were associated with the perinuclear cytoplasm and, in developing embryos, segregated to daughter cells along the axis of the spindle. In juvenile animals there was no evidence of pathology linked with feminization and the parasite was found at low density in cells under the cuticle. The parasite is highly adapted to transovarial transmission with an efficient mechanism of oocyte infection and no evidence of pathology.

**Key words:** microsporidia, transovarial transmission, *Gammarus duebeni*.

## INTRODUCTION

Transovarial transmission has been observed in a number of microsporidian parasites (Kellen *et al.* 1966; Nordin, 1975; Andreadis, 1983; Canning *et al.* 1985; Becnel *et al.* 1989; Raina *et al.* 1995). Among microsporidia infecting insect hosts, it is usually supplementary to the major horizontal route of transmission (Sweeney, Hazard & Graham, 1985; Avery & Undeen, 1990) and may involve a heterosporous life-cycle (Lord, Hall & Ellis, 1981; Becnel, Hazard & Fukuda, 1986; Sweeney, Graham & Hazard, 1988; Dickson & Barr, 1990). However, in the host *Gammarus duebeni* (Crustacea; Amphipoda) there have been several reports of microsporidia which have no obvious horizontal transmission (Bulnheim & Vavra, 1968; Bulnheim, 1971; Dunn, Adams & Smith, 1993) and may be transmitted exclusively by the transovarial route (Hatcher & Dunn, 1995). These parasites share several unusual characteristics: they are primarily located in gonadal tissue and they have no discernible pathogenic effects on the host other than their ability to feminize infected offspring.

The mechanisms of transovarial transmission and of host feminization are poorly understood (Smith & Dunn, 1991; Dunn *et al.* 1995). In order to approach these questions we have completed an ultrastructural study to map the cellular distribution of the parasite

at key points in the host life-cycle. Our main objective was to reveal the process of oocyte infection, by observation of oogenesis in adult gonadal tissue. In addition, we have followed the distribution of the parasite during early embryogenesis, and in juvenile animals at the time of sex determination (Bulnheim, 1978; Naylor, Adams & Greenwood, 1988). These latter experiments aim to provide information as to how the parasite is able to target gonadal tissue during host development and whether there is any obvious tissue-specific damage associated with feminization.

## MATERIALS AND METHODS

A random collection of approximately 200 *Gammarus duebeni* were collected from the Isle of Cumbrae (University Marine Biological Station, Millport). *G. duebeni* stocks were maintained at 12 °C in aerated brackish water with a specific gravity of 1005°, which corresponds to conditions in the field (Dunn & Hatcher, 1997). They were kept under long day conditions (16 h light:8 h dark) and fed rotted sycamore leaves and marine green algae, *Enteromorpha* spp.

To determine the infection status of the mother, embryos from 110 females were screened for microsporidian parasites and the females allocated to control or infected groups. *G. duebeni* adults form pre-copula pairs, with the male guarding the female, prior to eggs being laid into the brood pouch and fertilization. Pre-copula pairs were isolated and

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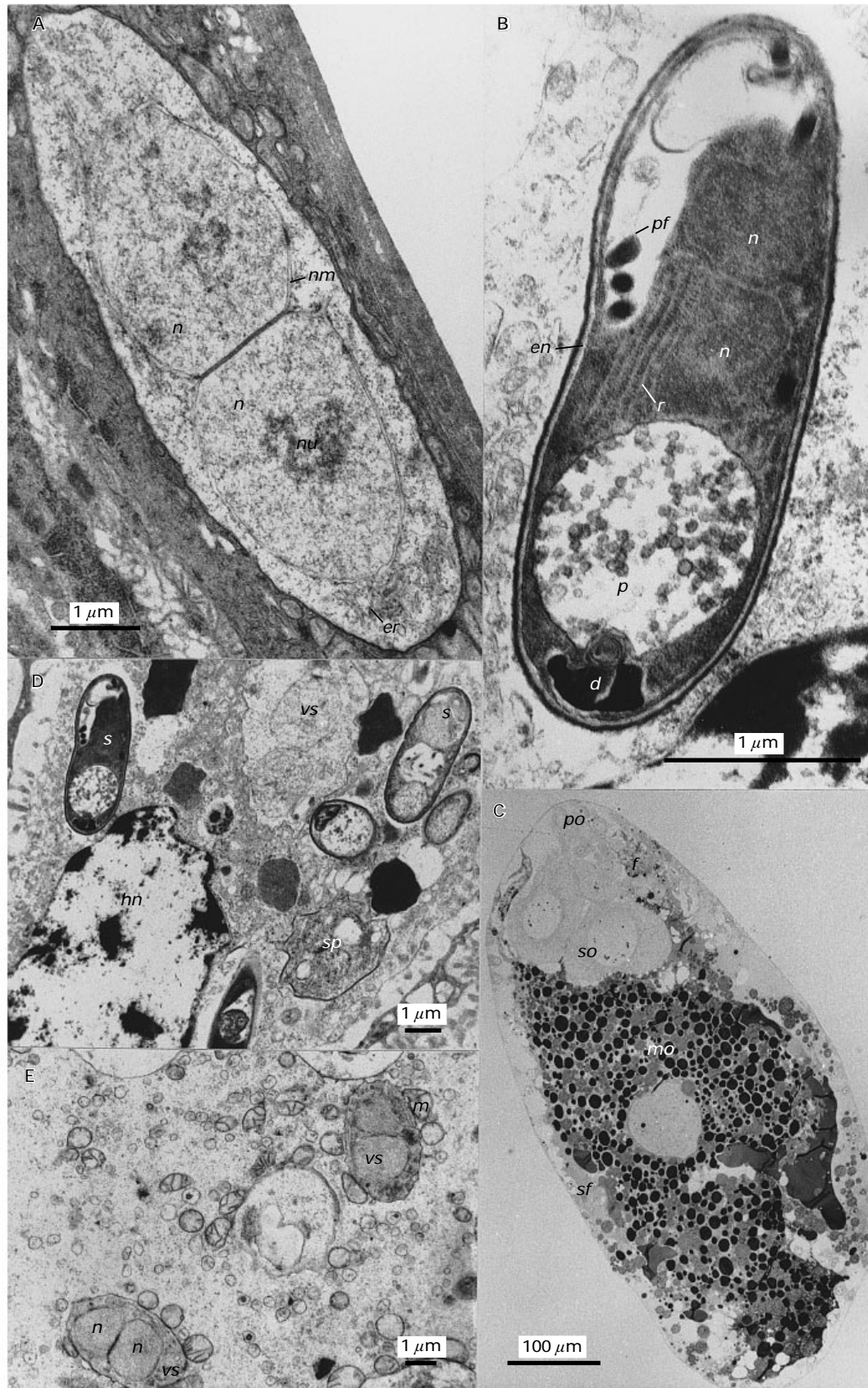


Fig. 1. Microsporidian parasites in the gonadal tissue of an adult female *Gammarus duebeni*. (A) Transmission electron micrograph (TEM) showing the vegetative stage of the parasite with diplokaryotic nucleus (*n*), double nuclear membrane (*nm*), nucleolus (*nu*) and endoplasmic reticulum (*er*). (B) TEM of microsporidian spore with a thin endospore (*en*) nucleus in diplokaryotic arrangement (*n*), polyribosomes (*r*), a polar filament (*pf*) with 3 coils and an

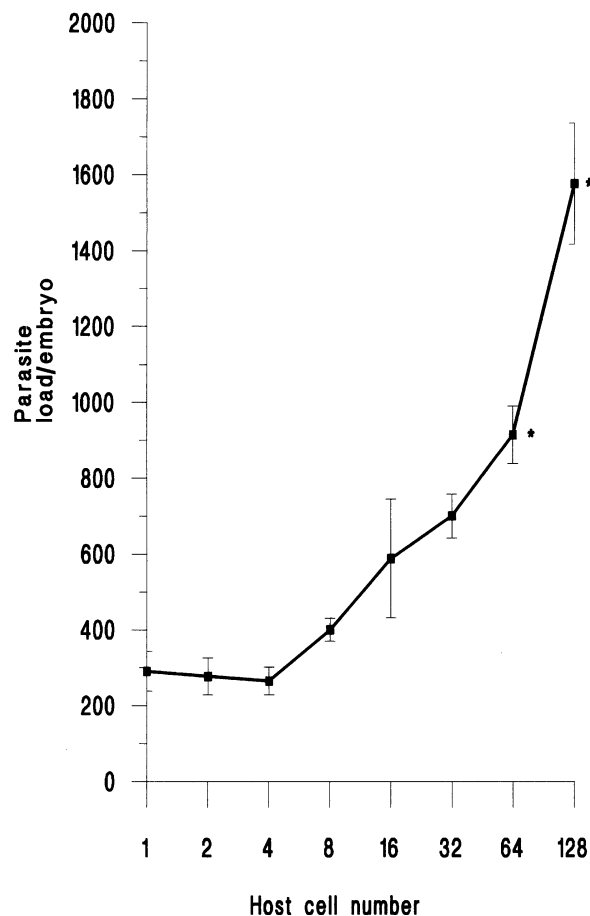


Fig. 2. Parasite growth rate in host embryos between 1 and 128 cell stages. Parasite numbers are constant over the first 2 host cell divisions. A doubling of parasites is seen over the 3rd and 4th host cell divisions and again over the 6th and 7th host cell divisions. Beyond the 32-cell stage it is difficult to visualize all cells within an embryo in DAPI-stained preparations. Therefore parasite numbers were estimated (\*) based on the number of host cells and parasites that were observed (Dunn *et al.* 1995).

examined daily for the presence of fertilized eggs. Embryos were removed from the brood pouches of anaesthetized females, permeated with 5 M HCl, rinsed in distilled water and fixed in acetone at  $-20^{\circ}\text{C}$ . Fixed embryos were squashed on a microscope slide and nuclei stained with DAPI (4,6-diamidino-2-phenyl-indole diluted 1/500 in 0.2 M  $\text{NaH}_2\text{PO}_4$  plus 8% glycerol, pH 7.5). Slides were screened for the presence of parasites using a Zeiss Axioplan fluorescent microscope.

Control and infected individuals were processed

for electron microscopy at 3 time-points in the host life-cycle. Ovaries were dissected from infected adult females 3–4 days prior to egg laying, embryos were removed from anaesthetized females during early development (1–100 cell stages) and juvenile animals were taken at the 4th moult (coinciding with the onset of sex determination, Bulnheim (1978)). Intact juveniles and ovaries dissected from adult females were fixed overnight in 3% (w/v) glutaraldehyde in Sorensen's phosphate buffer, pH 7.4 (SPB), rinsed in SPB, post-fixed with 1% osmium tetroxide overnight, dehydrated through a graded ethanol series and embedded in Araldite (Luft, 1961). Semi-thin and ultra-thin sections were cut through entire samples with a glass knife on a Reichert–Jung Ultracut microtome. Semi-thin sections were stained with Toluidine Blue (1% in borax) to assess the location and structure of tissue. Ultra-thin sections were taken every  $10\ \mu\text{m}$ , stained with uranyl acetate and lead citrate and screened with a Jeol JEM-1200EX electron microscope. Embryos were fixed in 2% paraformaldehyde and 0.05% glutaraldehyde (SPB) and then prepared as above.

## RESULTS

Examination of the ovaries from infected animals revealed the presence of microsporidian parasites which were absent from controls. All parasite stages observed lay directly in the host cell cytoplasm, with no evidence of a pansporoblast membrane or of regular groupings of parasites. Vegetative stages were elongate, ovoid and approximately  $8.1 \times 2.7\ \mu\text{m}$  (Fig. 1A) and were often closely associated with host cell mitochondria. They were characterized by the presence of a thin plasma membrane, diplokaryotic nuclei, endoplasmic reticulum and many ribosomes. Sporoblasts had a thickened plasma membrane, and developing spore organelles such as the polar filament and polaroplast could often be seen. Mature spores, observed in ultrathin sections, were cylindrical with a mean length of  $3.78\ \mu\text{m} + \text{s.e. } 0.15$  and width of  $1.22\ \mu\text{m} + \text{s.e. } 0.045$ ,  $n = 12$ . The exospore was densely stained and of normal width (approximately 30.5 nm) while the endospore, unusually, was of similar width to the endospore (approximately 34.4 nm). Spores were diplokaryotic with several rows of polyribosomes, a posterior vacuole, an anchoring disc and a polar filament which was short with only 3 coils (Fig. 1B). The polaroplast was

anchoring disc (*d*) and a polaroplast with unusual globular contents (*p*). (C) Light micrograph of a transverse section of ovary stained with toluidine blue. Primary oocytes (*po*) are located in a dorsal position; as they mature they move to a more ventral position, form links with primary follicle cells (*f*) and become secondary oocytes (*so*). As vitellogenesis commences the mature oocyte (*mo*) is surrounded by secondary follicle cells (*sf*). (D) TEM of a secondary follicle cell with an active microsporidian infection containing spores (*s*), a sporont (*sp*) and a vegetative stage (*vs*) near the host cell nucleus (*hn*). (E) TEM showing vegetative stages (*vs*) within a secondary oocyte. Again the parasite nuclei (*n*) are in diplokaryotic arrangement and the parasites are closely associated with host mitochondria (*m*).

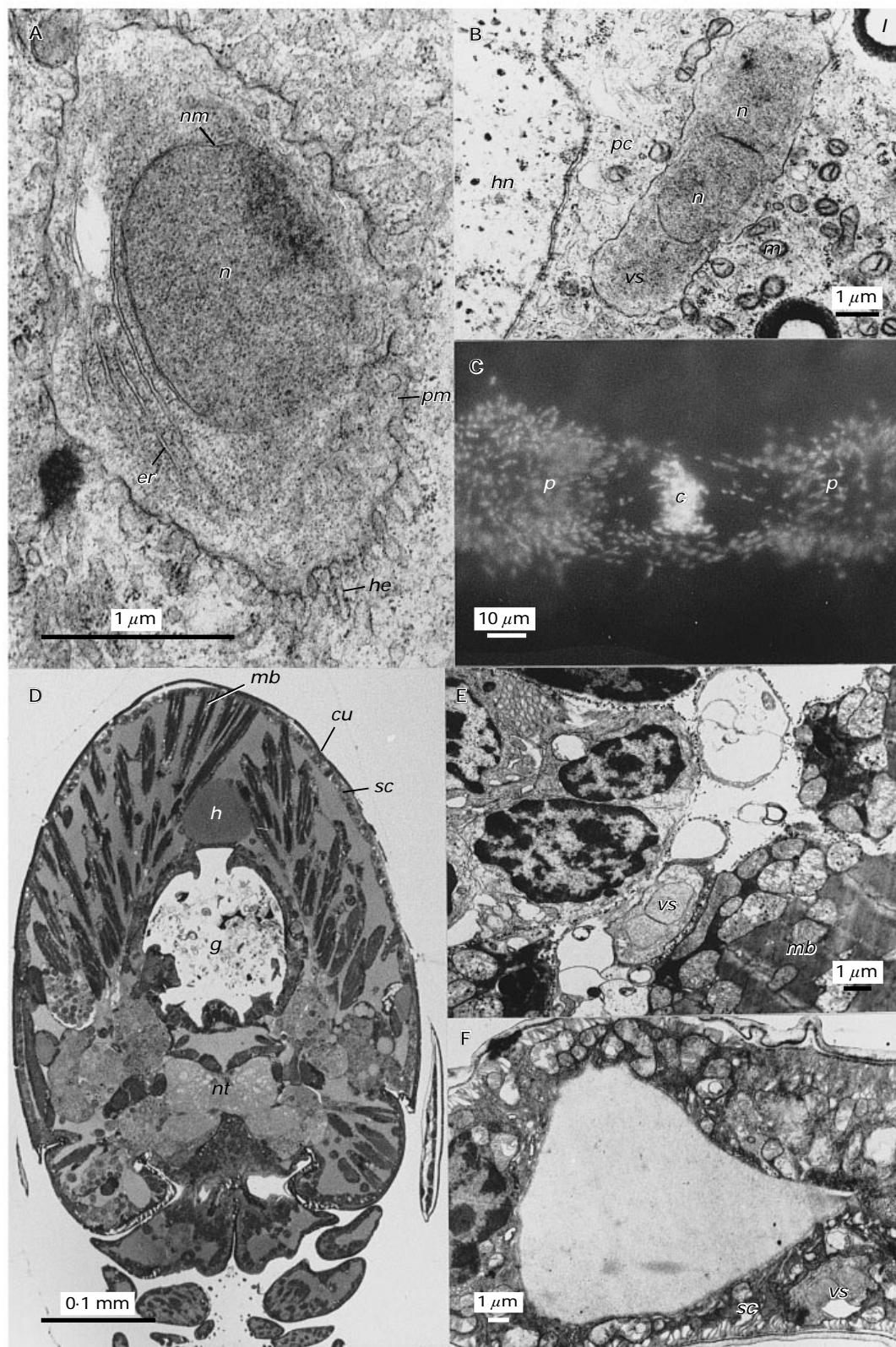


Fig. 3. Structure and distribution of microsporidian parasites in embryonic and juvenile *Gammarus duebeni*. (A) Transmission electron microscopy (TEM) showing a transverse section of a vegetative stage in a 16-cell embryo. Projections of the host ER (*he*) are closely associated with the parasite plasma membrane (*pm*). Also shown is the parasite nucleus (*n*), a nuclear membrane (*nm*) and endoplasmic reticulum (*er*). (B) TEM of an embryonic cell showing typical association between a vegetative stage (*vs*) and the host perinuclear cytoplasm (*pc*). Host cell nucleus (*hn*), host mitochondria (*m*), lipid globules (*l*) and parasite diplokaryotic nucleus (*n*) can also be seen. (C) Light micrograph (LM) showing a dividing nucleus of a host embryonic cell stained with DAPI. Parasites (*p*) are located at the opposite poles of the dividing nucleus in the region of the spindle poles. Host chromosomes (*c*) can be seen at

represented by a spherical, membrane-bound vesicle  $1.05 \mu\text{m} \pm \text{s.e. } 0.083$  in diameter containing abundant globules of up to  $73 \text{ nm}$  diameter in a clear matrix.

The *G. duebeni* ovary is a cylindrical tube with an oviduct on the ventral surface at a point approximately one third of the distance from the posterior end. Within the ovary there are 2 cell types: oocytes and follicle cells (Fig. 1 C). Examination of the ovary by light microscopy revealed that infection was not uniform and that parasites were concentrated in regions of developing oocytes. The ultrastructural study demonstrated that sporulation occurred only in follicle cells and that, as these cells matured, there was evidence of active infection with both an increase in the density of parasites and a shift in the proportion of infective stages. Spores were most commonly found in secondary follicle cells which surround the maturing oocyte (Fig. 1 D). Immature oocytes did not contain parasites, and vegetative stages (Fig. 1 E) of the microsporidian were first detected in oocytes which had formed connections with primary follicle cells. These vegetative stages were clustered around the nuclear envelope and under the plasma membrane but were only rarely found in the yolk.

Screening of DAPI-stained infected embryos revealed that the microsporidia increased in number during host development (Fig. 2). Parasite replication occurred at a slower rate than host cell division with only a 2-fold increase in parasite numbers over the first 6 cycles of host cell division (1–64 cells). The majority of cells within an embryo were infected with a mean parasite number of  $588 \pm \text{s.e. } 78.09$  in a 16-cell embryo. Intensity of infection varied between cells and ranged from 10 to 135, implying that differential segregation may occur at host cell division. Electron microscopy revealed vegetative stages of the parasite similar to those seen in the oocytes, except that the plasma membrane was irregular and undulating and was closely associated with finger-like projections of host endoplasmic reticulum (Fig. 3 A). Parasites were located adjacent to the nuclear envelope of interphase cells (Fig. 3 B) and at host cell division were seen to segregate along the axis of the spindle pole (Fig. 3 C) and thus infect both daughter cells.

Ultrastructural examination of juveniles revealed that, at this stage of development, the microsporidian was restricted to a small number of cell types and no evidence of pathology was seen. In sections through an entire juvenile (Fig. 3 D) only 33 parasites were seen: 27 in small cells lying immediately beneath the

cuticle (Fig. 3 F) and 6 distributed sporadically in other cells (Fig. 3 E), but never in muscle or nerve tissue or in the gut. Infected cells contained vegetative stages of the parasite and no evidence of sporulation was seen.

## DISCUSSION

The parasite described in the current study had the following structural characteristics: it was diplokaryotic throughout its life-cycle, it formed spores which were cylindrical with a thin endospore, it possessed an unusual polaroplast containing abundant globules and a short polar filament. Spores were detected only in the ovarian tissue of adult hosts and we found no evidence of regular groupings or of a pansporoblast membrane. The diplokaryotic life-cycle and absence of spore groupings and of a pansporoblast membrane are typical features of the genus *Nosema* (Sprague, 1978; Sprague, Becnel & Hazard, 1992). However, the unusual morphology of the polaroplast and its restriction to host ovarian tissue have not previously been described in *Nosema*. The genus is heterogeneous; however, it is clear that the majority of species belong to Lepidopteran or Coleopteran hosts. It has been suggested that the genus should be reviewed with probable re-assignment of species according to host type (Sprague, 1978). These questions can only be resolved by molecular phylogenetic analysis (Baker *et al.* 1994). We tentatively suggest that the organism described here represents a new microsporidian genus (E. U. Canning, personal communication).

The spores show strong structural homology with spores found in *Nosema* (FC spores, Iwano & Ishihara, 1991) and *Amblyospora* (Andreadis, 1983), which are involved in cell to cell transmission within the host (Lord *et al.* 1981; Iwano & Kurtti, 1995). These early spores are associated with vertical transmission (Andreadis, 1983; Han & Watanabe, 1988; Dickson & Barr, 1990). However, many of these species are heterosporous (Becnel *et al.* 1987; Iwano & Kurtti, 1995). In *Nosema* a second spore type, the MC spore (Iwano & Ishihara, 1991) is found late in infection causing pathology and mediating horizontal transmission (Han & Watanabe, 1988). There is no evidence either of a second spore type or of the typical host pathology associated with horizontal transmission in *G. duebeni*. This raises the possibility that the second spore type, MC spore, has been lost and the parasite relies solely on transovarial transmission.

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metaphase. (D) LM of a transverse section through a juvenile *G. duebeni* stained with toluidine blue. The soft tissue is enclosed in a hardened cuticle (*cu*); directly underneath this is the subcuticular tissue (*sc*). Muscle blocks (*mb*) are located predominantly in the dorsal regions and the gut (*g*) is found in the central region below the heart (*h*). The nervous tissue (*nt*) runs through the ventral region of the juvenile. (E) TEM showing a vegetative stage (*vs*) in a cell adjacent to a muscle block (*mb*). (F) TEM of a juvenile peripod with a vegetative stage (*vs*) within the subcuticular tissue (*sc*).

The location of the parasite is highly restricted in the adult host. Spores are found only in the follicle cells whilst vegetative stages are found in both follicle cells and oocytes. We propose that invasion of the oocytes may occur by spore germination. However, as we have not observed germination, it has not been possible to determine whether polar tube extrusion occurs across host cell membranes, as has been proposed for *Edhazardia aedis* and *Amblyospora* spp. in *Aedes* mosquitoes (Andreadis, 1983; Becnel *et al.* 1989), or whether the parasite utilizes intercellular connections between follicle cells and oocytes, as has been suggested for *Nosema pyrausta* in *Ostrinia nubilalis* (Sajap & Lewis, 1988). However, the absence of spore stages in the gametes, in contrast with other vertically transmitted *Nosema* spp. in insects (Canning & Hulls, 1970; Nordin, 1975) implies that spore germination may be essential for oocyte invasion.

Parasite development appears to be highly coordinated with the host reproductive cycle. Spores are found only in follicle cells which are adjacent to developing oocytes. Oocytes and follicle cells have a close association during development, with the follicle cell sustaining the developing oocyte (Zerbib, 1980; Charniaux-Cotton & Payen, 1988). Sporulation occurs in maturing follicle cells in parallel with the onset of vitellogenesis and transport. Similar relationships between the host reproductive cycle and sporulation have been observed in microsporidia of mosquitoes (Andreadis & Hall, 1979; Hall & Washino, 1986). We suggest that sporulation may be triggered, directly or indirectly, by the hormonal factors which induce maturation of the follicle cell.

Synchrony of sporulation with host oogenesis will maximize transmission of the parasite whilst minimizing the pathological effects on the host. There is evidence that a strategy of low pathology continues in the infected offspring. Although there are high numbers of parasites in early embryos the replication rate is low and juveniles contain very few parasites. Targeting of specific host tissues may be achieved through differential segregation at host cell division, as suggested by the uneven distribution of parasites between cells in early embryos in this system and in a previously described transovarially transmitted parasite of *G. duebeni* (Dunn *et al.* 1995). The mechanism of segregation may be linked to the association of the parasite with the perinuclear cytoplasm and its apparent segregation along the axis of the spindle (Callaini, Riparbelli & Dallai, 1994). Alternatively, invasion of target tissue may occur later in development (e.g. Andreadis, 1983; Sajap & Lewis, 1988; Becnel *et al.* 1989). At the onset of sexual differentiation gonadal tissue has not yet developed, the parasite is present in very low numbers and is restricted mainly to subcuticular tissue. However, infection in adults occurs only in follicle cells and oocytes. To determine the path of

transmission to the adult gonad, it is important to focus on the period of gonadal development. This in turn may shed light on the mechanism of feminization of the host.

There are several reports of vertically transmitted microsporidia in *G. duebeni*. Bulnheim & Vavra (1968) reported on a microsporidian which they referred to as *Octosporea effeminans* with a similar spore structure and location to the parasite studied here, but with frequent groupings of 8 spores. A second organism, *Thelohania hereditaria* (Bulnheim, 1971) had mononucleate spores within a pansporoblast membrane and infected both muscle and gonadal tissue. The only other EM description (Dunn *et al.* 1993) reports spores within a pansporoblast membrane with 7 polar filament coils and a thick spore wall. Despite their structural differences, these parasites all feminize their host (Bulnheim & Vavra, 1968; Bulnheim, 1971; Dunn *et al.* 1993; R. S. Terry unpublished observations). We suggest that this transmission strategy may be more common than previously expected and may have evolved a number of times.

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