

**NOTE**

# ***Enterospora* sp., an intranuclear microsporidian infection of hermit crab *Eupagurus bernhardus***

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**ABSTRACT:** Recent work at our laboratory has led to the discovery of a new genus of microsporidian parasite residing in the family Enterocytozoonidae. The type species of this new genus, *Enterospora canceri*, is an intranuclear parasite infecting the hepatopancreatocytes of the decapod crustacean *Cancer pagurus*. Here we provide the second description of a parasite within the genus *Enterospora*, this time infecting the hermit crab *Eupagurus bernhardus* from UK waters. The pathological manifestation and ultrastructural features of the hermit crab parasite are very similar to those described for *E. canceri*. Further taxonomic comparisons based upon ultrastructural and molecular affinities of *Enterospora* are now required to define firmer links between this new genus within the Enterocytozoonidae and all other microsporidian families. The opportunistic nature of the discovery of a second intranuclear microsporidian within the Crustacea suggests that their presence may be more common than in higher animal groups.

**KEY WORDS:** *Eupagurus bernhardus* · *Enterocytozoon* · Enterocytozoonidae · *Enterospora* · Epithelium · Hermit crab · Hepatopancreas · *Nucleospora* · Intranuclear · Nucleus · Crustacea

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## **INTRODUCTION**

Until the discovery of *Enterospora canceri* in European edible crab *Cancer pagurus* by Stentiford et al. (2007, this issue), microsporidians with features of the family Enterocytozoonidae had only been described from 2 genera: one (*Enterocytozoon*) cytoplasmic within mammalian hosts, the other (*Nucleospora*) intranuclear within finfish hosts. While *E. canceri* possesses features of both existing genera and has developmental stages typical of the family, its invertebrate hosting coupled with its infection location and specific development stages suggested that *Enterospora* is a separate genus. As such, the discovery provides an interesting link between existing members of the family and their apparently disparate neighbour families within the Microsporidia. Here we describe a second example of *Enterospora* sp., this time infecting the hermit crab *Pagurus bernhardus* (Decapoda: Anomura) captured from the Irish Sea, United Kingdom. As such, it is a second case of an intranuclear

microsporidian in the Crustacea. Whether the parasite from *E. bernhardus* is the same as that reported from European edible crab *Cancer pagurus* captured from the English Channel, or whether this marks the second species of the genus *Enterospora* remains to be shown.

## **MATERIALS AND METHODS**

Thirty hermit crabs *Eupagurus bernhardus* were captured as a by-catch during the UK National Marine Monitoring Programme (NMMP) annual survey in June 2003 from a site in Liverpool Bay, Irish Sea (53° 28.66' N, 3° 41.79' W). Hermit crabs were placed into running seawater for up to 1 h before preparation for histology and electron microscopy. Crabs were anaesthetized by chilling on ice for 30 min before removal of heart, gonad, gill, muscle and hepatopancreas. Dissected organs were fixed for 24 h in 10% Davidson's seawater fixative (Hopwood 1996) before transfer

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to 70% industrial methylated spirit (IMS). Fixed samples were processed to wax in a vacuum infiltration processor using standard protocols. Sections were cut at 3 to 5 µm on a rotary microtome and resulting tissue sections were mounted onto glass slides before staining with haematoxylin and eosin (H&E). Stained sections were analysed by light microscopy (Eclipse E800, Nikon) and digital images were obtained using the Lucia™ Screen Measurement System (Nikon). Hepatopancreas samples prepared for histology were also preserved for electron microscopy. Small blocks of tissue (2 mm<sup>3</sup>) were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and 1.75% sodium chloride for 2 h at room temperature. Fixed tissue samples were rinsed in 0.1 M sodium cacodylate buffer with 1.75% sodium chloride (pH 7.4) and post-fixed for 1 h at 4°C in 1% osmium tetroxide, reduced with 1.75% potassium ferrocyanide in 0.1 M sodium cacodylate buffer. Specimens were washed in 3 changes of 0.1 M sodium cacodylate buffer and stained en bloc in 0.5% aqueous uranyl acetate for 1 h. Specimens were embedded in epoxy resin 812 (Agar Scientific pre-mix kit 812) following dehydration through a graded acetone series. Thick sections were stained with Toluidine Blue for viewing with a light microscope to identify suitable target areas. Ultra thin sections (70 to 90 nm) of these areas, mounted on uncoated copper grids and stained with uranyl acetate and Reynolds' lead citrate (Reynolds 1963), were examined using a JEOL 1210 transmission electron microscope. Dimensions were assessed using the on-board measurement systems associated with this microscope.

## RESULTS

Hermit crabs displayed no external signs of disease. One specimen from the 30 assessed showed a severe degeneration of the hepatopancreas typical for that described previously in *Cancer pagurus* infected with the intranuclear microsporidian *Enterospora canceri* (Stentiford et al. 2007). The normal tubular structure of the hepatopancreas was almost entirely destroyed, with detachment of epithelial cells from the tubule basement membrane, a liberation of presumably free parasite spores to the tubule lumen and a pronounced haemocytic encapsulation response to affected tubules. Although the disease state was advanced, it was still possible to appreciate the intranuclear nature of the infection since hypertrophic, eosinophilic and granular nuclei were frequent amongst liberated spores and cellular debris within the bounds of the existing tubule basement membrane. In addition to the principle infection, colonies of Gram-negative bacteria were also apparent within the remnant tubules (Fig. 1).

Different life cycle stages of a microsporidian parasite were observed within the affected nuclei of epithelial cells of the hepatopancreatic tubules. In this example of presumably advanced infection, all tubular epithelial cells and their nuclei were implicated. Uninucleate meronts were not detected within infected nuclei, but presumptive subsequent stages were observed as plasmodia containing between 4 and >20 parasite nuclei in section. Larger plasmodia appeared to be lobed and occupied the majority of the hypertrophic host nucleus, with margination of the host chromatin. Typical of the genus *Enterospora*, early stage plasmodia contained a granular cytoplasm, were contained by a simple electron-dense membrane, developed small spherical membrane-bound vesicles and later, larger electron-lucent vacuoles. At this stage, paired parasite nuclei were closely opposed (Fig. 2A–D). Next, multiple copies of apparatus resembling the polar filament and anchoring disk of mature microsporidian spores formed within the plasmodial cytoplasm and became closely associated with the large electron-lucent vacuoles (Fig. 2E,F). Although sporoblasts were not observed budding from the sporogonal plasmodia, liberated sporoblasts were observed in direct contact with the host nucleoplasm. At this stage, the early polaroplast and developing polar filament and anchoring disks were present within a relatively electron-lucent and granular sporoblast cytoplasm. Occasionally small bleb-like protrusions emerged from the sporoblast outer membrane (Fig. 3A). Sporoblast stages matured to electron-dense spore stages that also were not contained within any sporophorous vesicle but, instead, lay in direct contact with the host nucleoplasm (Fig. 3B). In many cases, large numbers of spores (several tens to >100) were observed within a given section of host nucleus, suggesting that at least several hundred can be present within the nucleus as a whole (Fig. 3C). Spores contained 4 to 5 turns of the polar filament and measured  $1.4 \pm 19.7 \times 0.7 \pm 11.5$  µm (mean n = 30) and were contained within a trilaminate wall consisting of a cell membrane (7 ± 0.4 nm), endospore (30 ± 0.9 nm) and exospore (15 ± 1.4 nm). Gram-negative bacterial colonies were observed amongst cellular debris and remnants of the degenerated tubules (Fig. 3D, Gram stain not shown). Infected host nuclei frequently contained several development stages of the microsporidian, suggesting asynchrony between infections, even within the same host cell and nucleus (Fig. 4).

## DISCUSSION

The parasite described herein is the second reported example of an intranuclear microsporidian in an inver-

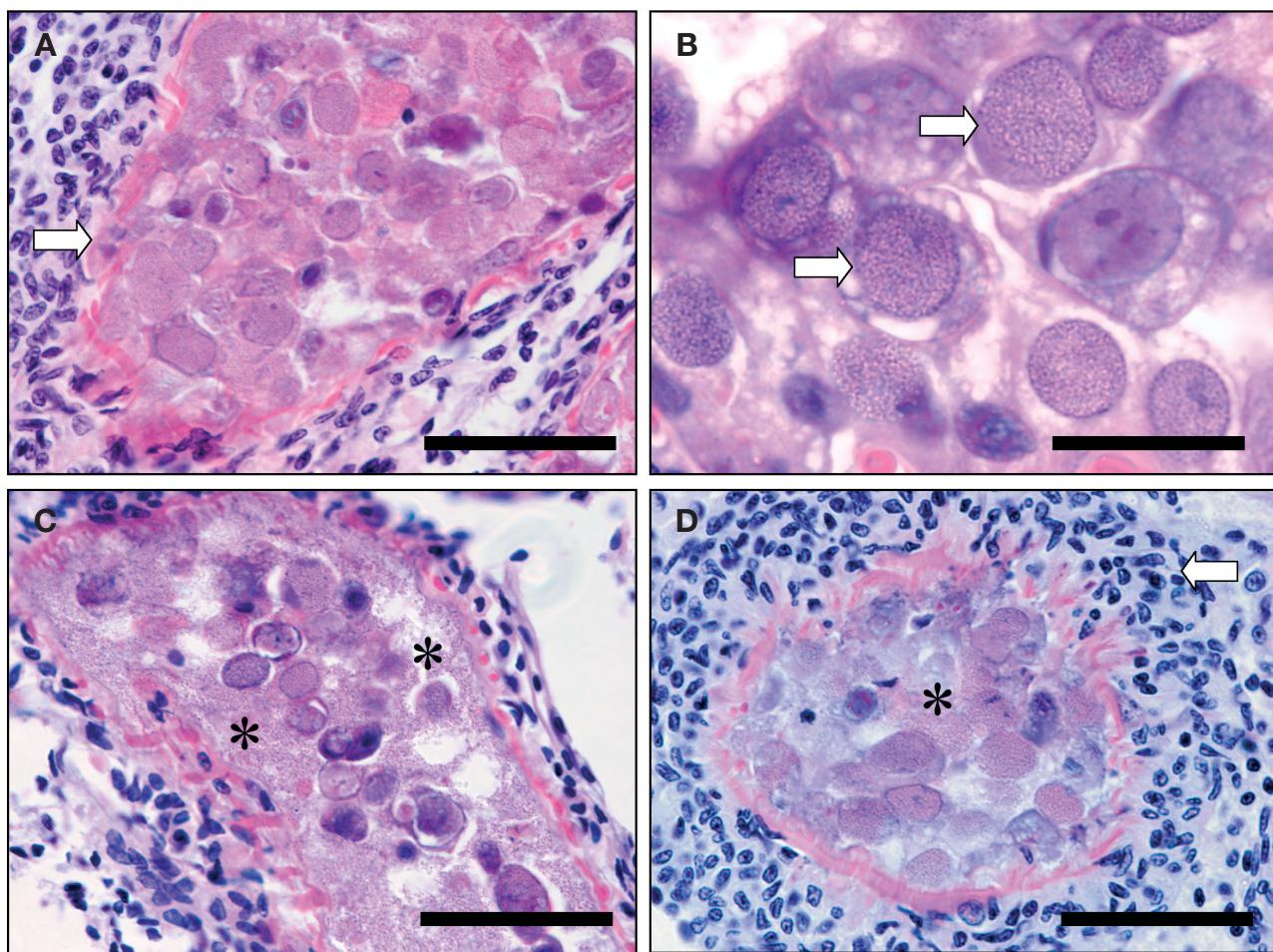


Fig. 1. *Enterospora* sp. infecting hepatopancreatic epithelia of *Eupagurus bernhardus*. Histopathology (A) heavily infected tubule showing dissociation of degenerate epithelial cells from existing basement membrane (arrows); sloughed infected cells fill tubule lumen. (B) Sloughed epithelial cells with hypertrophic, eosinophilic and granular nuclei (arrows); host chromatin is restricted to outer margins of infected nuclei. (C) Heavily infected tubule with sloughed epithelial cells and foci of bacterial microcolonies (asterisks). (D) Heavily infected tubule with disassociated epithelial cells, bacterial microcolonies (asterisk) and pronounced infiltration by host haemocytes (arrow). All images H&E. Scale bars = (A,C,D) 100 µm, (B) 20 µm

tebrate and follows a description by our laboratory of a new genus of intranuclear microsporidians infecting decapod crustaceans (Stentiford et al. 2007). The parasite from hermit crabs was discovered during routine monitoring of commercial and sentinel species during the UK NMMP in June 2003. As such, we can make no inference as to the prevalence of infection in hermit crabs at different times of the year nor at different locations.

A key feature of microsporidians within the family Enterocytozoonidae is the formation of spore organelles (extrusion apparatus such as the polar filament and anchoring disk) prior to fission of the sporogonic plasmodium into uninucleate sporoblasts and spores. The parasite described herein exhibits this key developmental feature and, as such, resides within this fam-

ily. Furthermore, due to the close similarity of this parasite with *Enterospora canceri* from *Cancer pagurus* (crustacean host, hepatopancreatic habit, intranuclear, organelles formed within plasmodium, 4 to 5 turns of polar filament, similar spore size, high spore production) it is appropriate that it is also placed within the same genus. Due to the low apparent prevalence of infection in the population sampled and the lack of opportunity to collect additional material from this population, it has not been possible to classify the parasite from the hermit crab to the species level. While key developmental stages clearly define the parasites from hermit crab and edible crab as genus relatives, the hermit crab parasite exhibited an intriguing feature that was not seen in the edible crab parasite (Stentiford et al. 2007). This feature involved the asso-

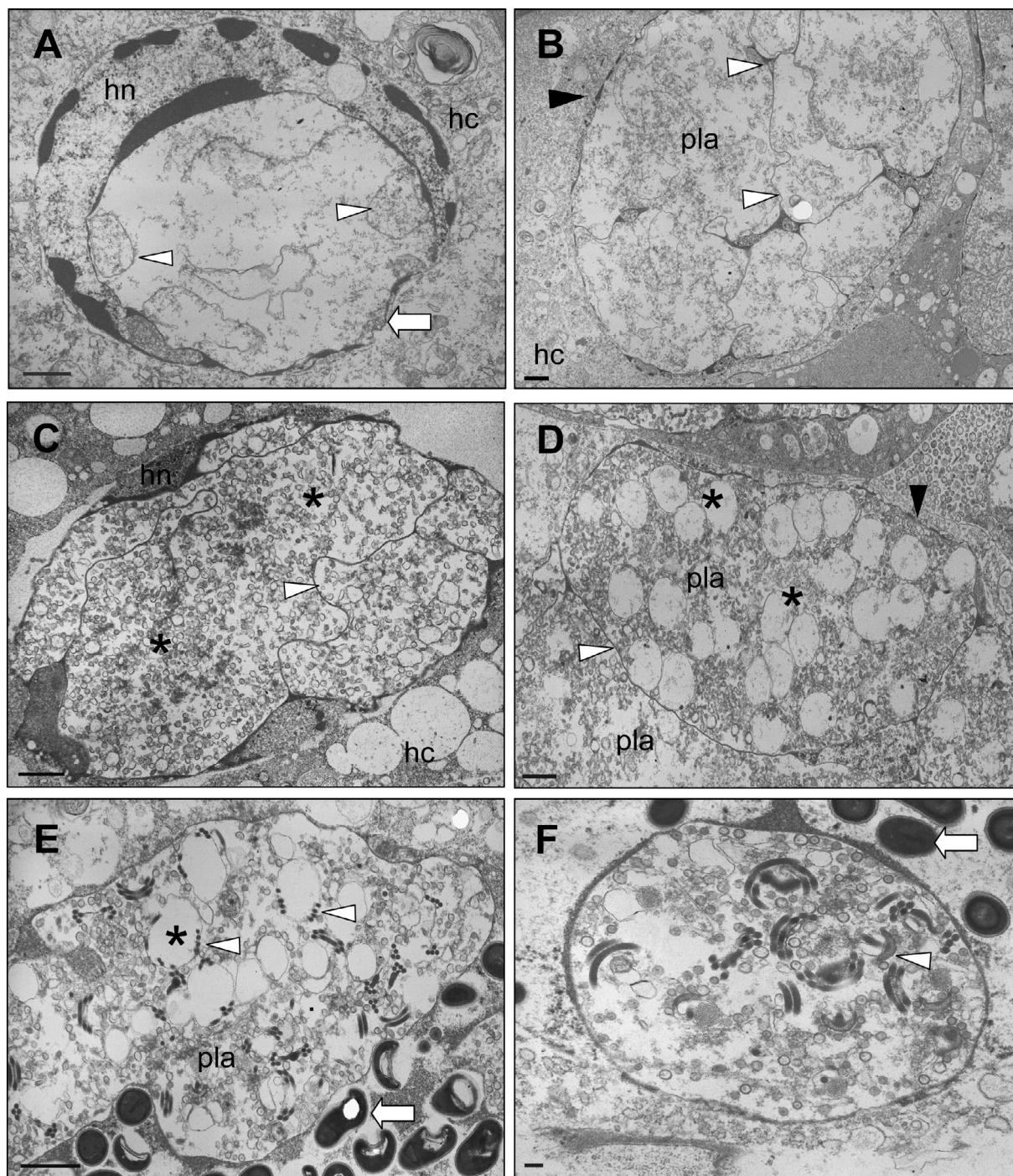


Fig. 2. *Enterospora* sp. infecting hepatopancreatic epithelia of *Eupagurus bernhardus*. Ultrastructure of early life stages. (A) Early meront stage (arrow) within nucleoplasm of host cell nucleus (hn); arrowheads: parasite nuclei; hc: host cell cytoplasm. (B) Early plasmodial stage filling nucleoplasm of host cell (black arrowhead); plasmodium (pla) contains internal septa (white arrowheads); hc: host cell cytoplasm. (C) Early plasmodial stage with internal septa (arrowhead) and small membrane-bound vesicles (asterisks); hn: host cell nuclei; hc: cytoplasm. (D) Early plasmodium (pla) with paired nuclei (asterisks) and internal septa (white arrowhead); different regions of plasmodium appear to be at different developmental stages; black arrowhead: host cell nuclear border. (E) Developing sporogonial plasmodium (pla) with large electron-lucent vacuoles (asterisk) and associated precursors of spore extrusion apparatus (white arrowheads); mature spores are visible outside plasmodium and in direct contact with host nucleoplasm (arrow). (F) Section of sporogonial plasmodium showing developing polar filaments with 4 to 5 coils and terminating in dome-shaped anchoring disk (arrowhead); mature spores are visible outside plasmodium in direct contact with host nucleoplasm (arrow). Scale bars = (A to E) 1  $\mu$ m, (F) 200 nm

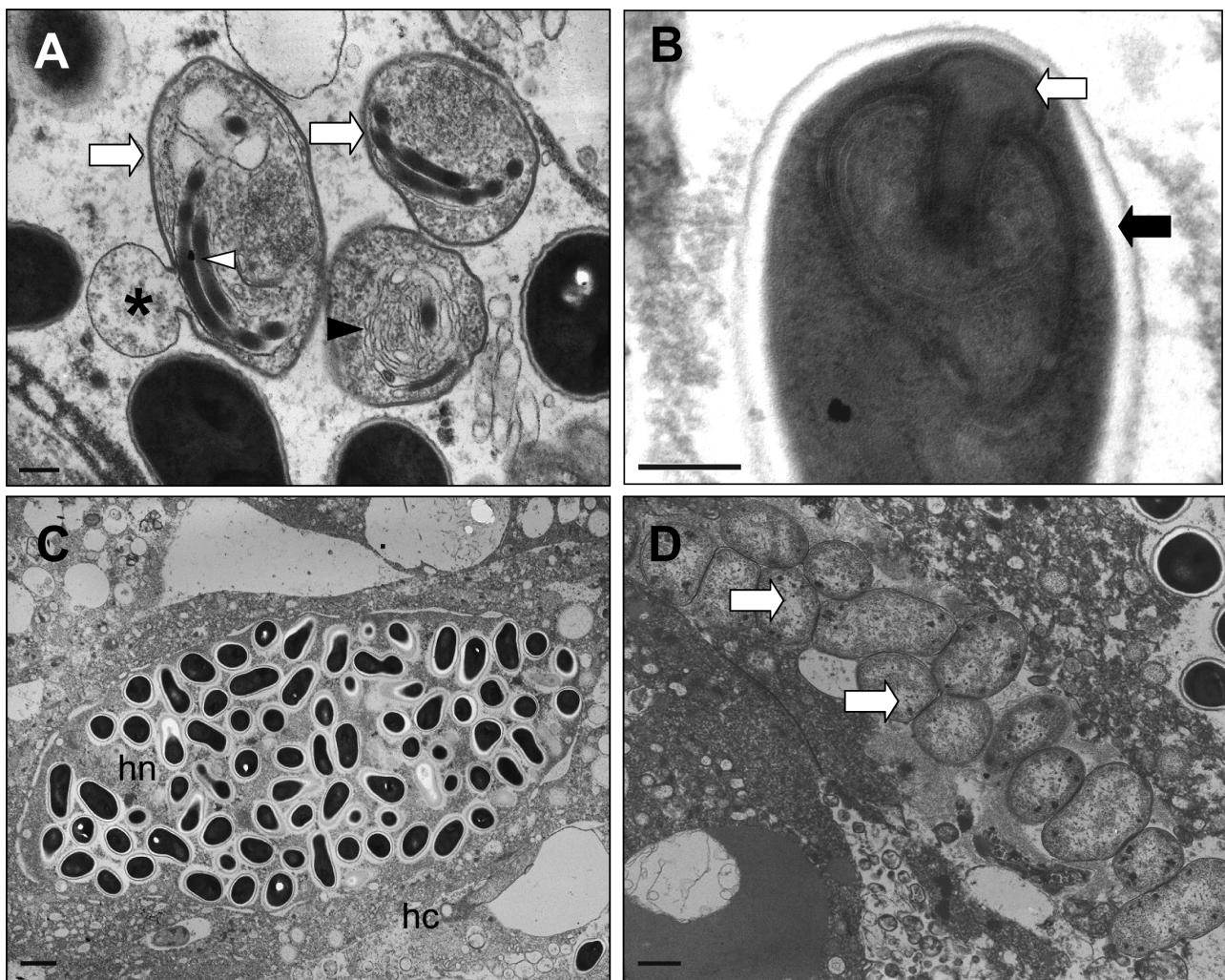


Fig. 3. *Enterospora* sp. infecting hepatopancreatic epithelia of *Eupagurus bernhardus*. Ultrastructure of spore development. (A) Sporoblast stages (arrows) with polar filament precursors (white arrowhead) and polaroplast (black arrowhead); small protrusions were occasionally observed in sporoblast wall (asterisk). (B) Maturing spore showing anchoring disk (white arrow) and trilaminar wall (black arrow). (C) Host nuclei during advanced infection; nucleoplasm (hn) is filled with mature spores; host cytoplasm (hc) is unaffected at this stage. (D) Late infection with microcolonies of bacteria (arrows) amongst liberated microsporidian spores and cell debris within tubule lumen. Scale bars = (A,B) 200 nm, (C,D) 1  $\mu$ m

ciation of polar filament precursors with the large electron lucent vacuoles present within the plasmodial cytoplasm (Fig. 2E). Whether this feature is common amongst parasites of the genus *Enterospora* or perhaps a unique feature of the parasite from hermit crabs will require analysis of further specimens.

Recent studies of microsporidian taxonomy based upon analysis of regions of the parasite genome have placed members of the Enterocytozoonidae as basal members within the microsporidia (Lom & Nielsen 2003). Since the crustacean parasites within the newly formed *Enterospora* appear to reside within the same family, it is feasible that a taxonomic comparison based upon genomic and ultrastructural features may now

allow firmer links to be drawn between the disparate Enterocytozoonidae and all other microsporidian families. A recent molecular phylogenetic study of a microsporidian from crustacean sea lice revealed similarity with the Enterocytozoonidae (particularly the *Nucleospora*) (Freeman et al. 2003) and Refardt et al. (2002) report that the daphnid parasite *Microsporidium* sp. also appears closely related to the *Enterocytozoon*. To this end, further work should gather appropriate *Enterospora*-infected material from *Cancer pagurus* and *Eupagurus bernhardus* and attempts made to compare these and other hepatopancreatic microsporidians from crustaceans with those found within higher animals.

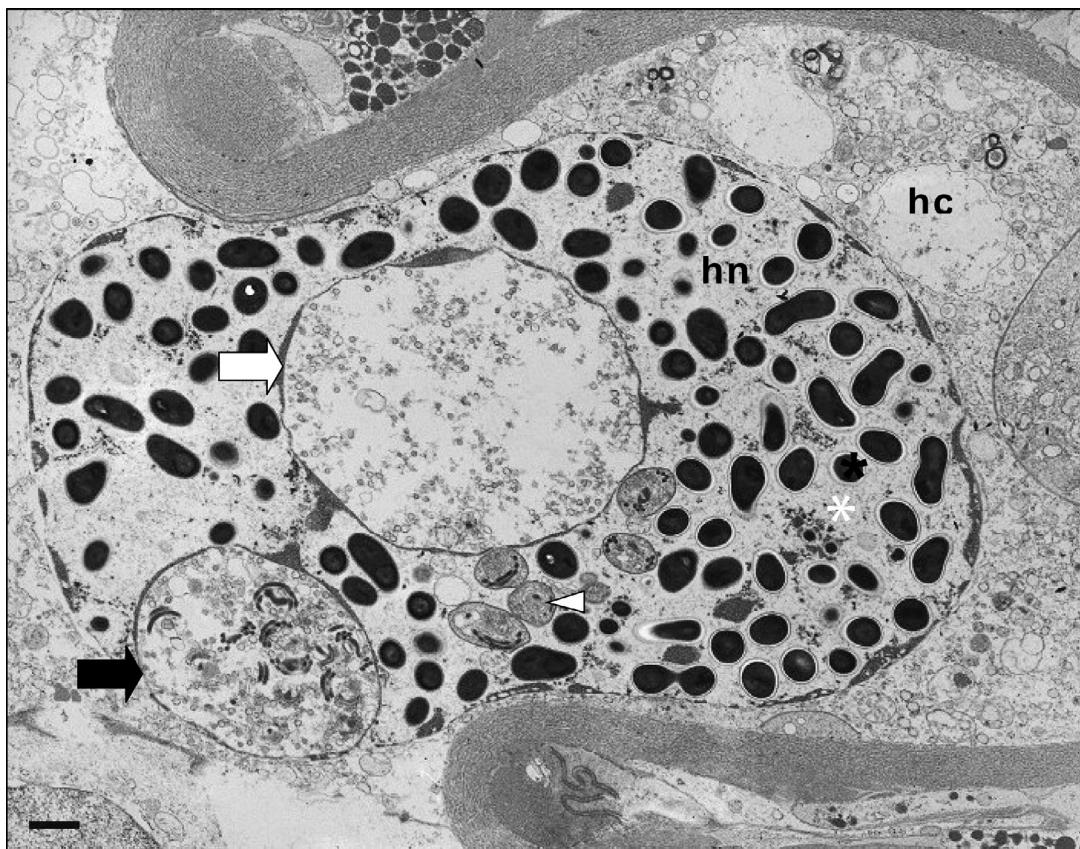


Fig. 4. *Enterospora* sp. infecting *Eupagurus bernhardus*. Ultrastructure of asynchronous development of *Enterospora* sp. within a single host nucleus (hn). Early meront (white arrow), sporogonial plasmodium (black arrow), liberated sporoblast (arrowhead) and mature spores (asterisk) are present. Host cytoplasm (hc) is unaffected at this stage. Scale bar = 1  $\mu$ m

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