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Enterocytozoon salmonis N. Sp.: An Intranuclear Microsporidium from Salmonid Fish

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ABSTRACT. The developmental stages of a recently described microsporidian from the nucleus of hematopoietic cells of salmonid fish were found to be unique among the Microsporida. All observed stages, including meronts, sporonts, and spores were in direct contact with the host cell nucleus (principally hematopoietic cells) of chinook salmon (*Oncorhynchus tshawytscha*). There is no parasitophorous vacuole and sporogony does not involve formation of a pansporoblastic membrane as with other members of the suborder Apansporoblastina. The extrusion apparatus differentiates prior to division of sporogonial plasmodia. The spores are ovoid (1 × 2 μm) and uninucleate, and possess a coiled polar tube with 8–12 turns. Developmental stages of the salmonid microsporidian are similar to those described for *Enterocytozoon bieneusi* as found in the intestinal mucosa of human AIDS patients. However, the intranuclear development, different cell types, and host infected clearly separate the salmonid and human parasites. Accordingly, the intranuclear parasite of salmonids is given the name *Enterocytozoon salmonis* n. sp. within the suborder Apansporoblastina.

Key words. *Enterocytozoon salmonis,* Microspora, salmonid anemia, salmonid microsporidium, taxonomy, ultrastructure.

MICROSPORIDIANS have been observed as parasites of numerous species of animals. Most however, are found in fish and insects [2]. Although all microsporidians are obligate intracellular parasites, only two have been reported to infect the host cell nucleus. Both of these intranuclear microsporidians were found in salmonid fish. Modin [8] first described *Microsporidium rhabdophilia* as it occurred in the nuclei of rodlet cells among several salmonid species in California, US. Although *M. rhabdophilia* was frequently encountered, it was not associated with any known disease signs. In contrast to the rodlet cell parasite, intranuclear microsporidian infections of hematopoietic cells resulted in anemia, lymphoblastosis, and a leukemic condition in chinook salmon (*Oncorhynchus tshawytscha*) from Washington [4, 9] and California [5]. Initially the cell type infected by this second microsporidian was thought to be an erythroid precursor [4], but later studies indicated the principal cell involved was of the lymphocyte lineage [5, 9]. The clinical signs observed in microsporidian-infected chinook salmon are similar to a plasmacytoid leukemia described from 2 to 3 year-old chinook salmon from seawater net pens in British Columbia, Canada [6, 7]. The two conditions do share similar clinical signs but the role of the microsporidian in the plasmacytoid leukemia remains unclear. It is neither seen in all cases of the leukemia nor evident when the disease is transmitted experimentally to chinook salmon [6].

Hedrick et al. [5] showed the unique ultrastructural characteristics of the spore of the salmonid microsporidian most closely resembled descriptions of *Enterocytozoon bieneusi* occurring in the intestinal mucosa, or enterocytes, of humans with AIDS [3]. The two parasites share similar developmental cycles that include differentiation of the extrusion apparatus during merogony (prior to division into sporoblasts). The following report describes details of the developmental stages of this salmonid parasite as it compares with those unique to *Enterocytozoon* and establishes its taxonomic position among the Microsporida.

METHODS

Fish. Juvenile chinook salmon (30–50 g) were obtained directly from the Darrah Springs Hatchery, California Department of Fish and Game.

Light and Electron Microscopy. Samples from the kidney of five infected fish were placed into 2.5% glutaraldehyde in 0.06 M cacodylate buffer (pH 7.4) and fixed for 24 h at 4° C. The tissues were rinsed twice in buffer and then post-fixed in 1% aqueous OsO_4, dehydrated through a graded ethanol series, infiltrated, and embedded in epoxy resin. Semi-thin sections (1 μm) were stained with toluidine blue and photographed with an Olympus BH-2 photomicroscope. Thin sections (10–20 nm) were stained with 4% uranyl acetate and lead citrate prior to examination with a Phillips EM 400 electron microscope at 80 kV.

RESULTS

Hematopoietic cells in the spleen, kidney, and blood leukocytes were the main cell types infected with the intranuclear microsporidian (Fig. 1). In the kidney, rodlet cells (Fig. 2), epithelium of the urinary tubules (Fig. 3), and mesangial cells of the glomerulus (Fig. 4) were also infected but at lower rates than the hematopoietic cells.

With the exception of the infectious sporoplasm, which is injected into the host cell cytoplasm by the spore, all intranuclear developmental stages (including meronts, sporonts, and mature spores) were observed in the life cycle of the salmonid microsporidian.

Meronts. The earliest stages observed were rounded or elongated uninucleate cells found in the host cell nucleus (Fig. 5, 6). These early meronts were characterized by a clear cytoplasm with few ribosomes, and flattened and smooth endoplasmic reticulum (ER). A single unit membrane separated the parasite cytoplasm from the host cell nucleoplasm (Fig. 5). Binucleated meronts possessed more ribosomes and a more developed ER (Fig. 6). Mitochondria and diplokaryons were never observed. The ER was organized into several contiguous folds that separated the two nuclei or nucleoplasms. In the vicinity of each nucleus was a cluster of ribosomes associated with thin tubular structures presumed to be precursors of the polar tube. Some parasites located in the core of the host cell nucleus displayed long expansions to remain in contact with the host cell cytoplasm (Fig. 6).

Sporonts. Sporonts were found both deeply embedded or on the periphery in an apparent budding position with respect to the host cell nucleus (Fig. 7). In each case the host cell nuclear membrane isolated the parasite from the host cell cytoplasm. More developed sporonts contained several nuclei with thin or inapparent nuclear membranes (Fig. 8). An abundant sporogonial material corresponding to the extrusion apparatus begins to appear and assemble prior to formation of the sporoblast nuclei (Fig. 7, 9). This sporogonial material is principally precursor of the polar tube. In transverse section this material appears as round to ovoid bodies delimited by a thick electron-dense layer surrounding a hollow core (Fig. 7, 8). The primor-

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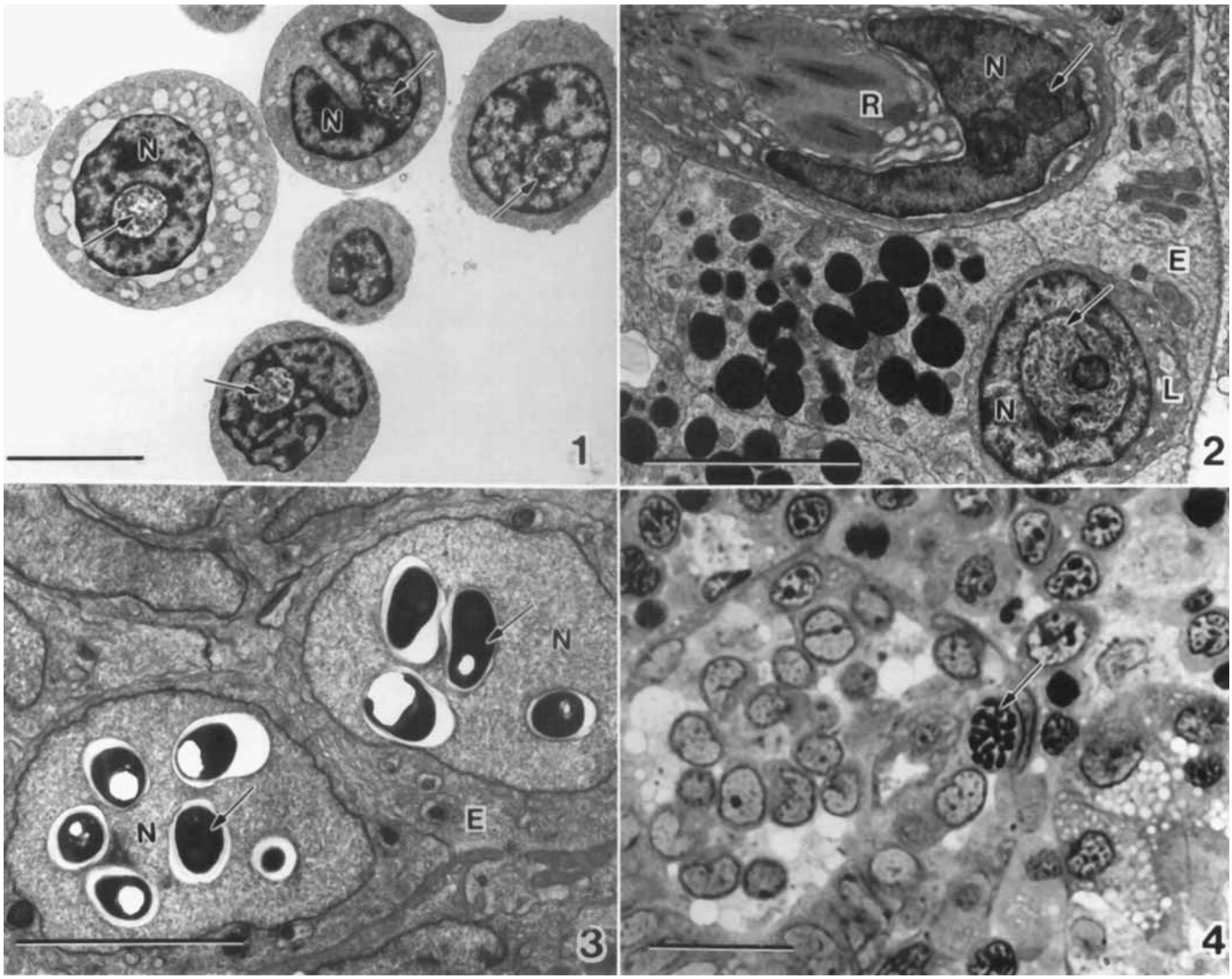
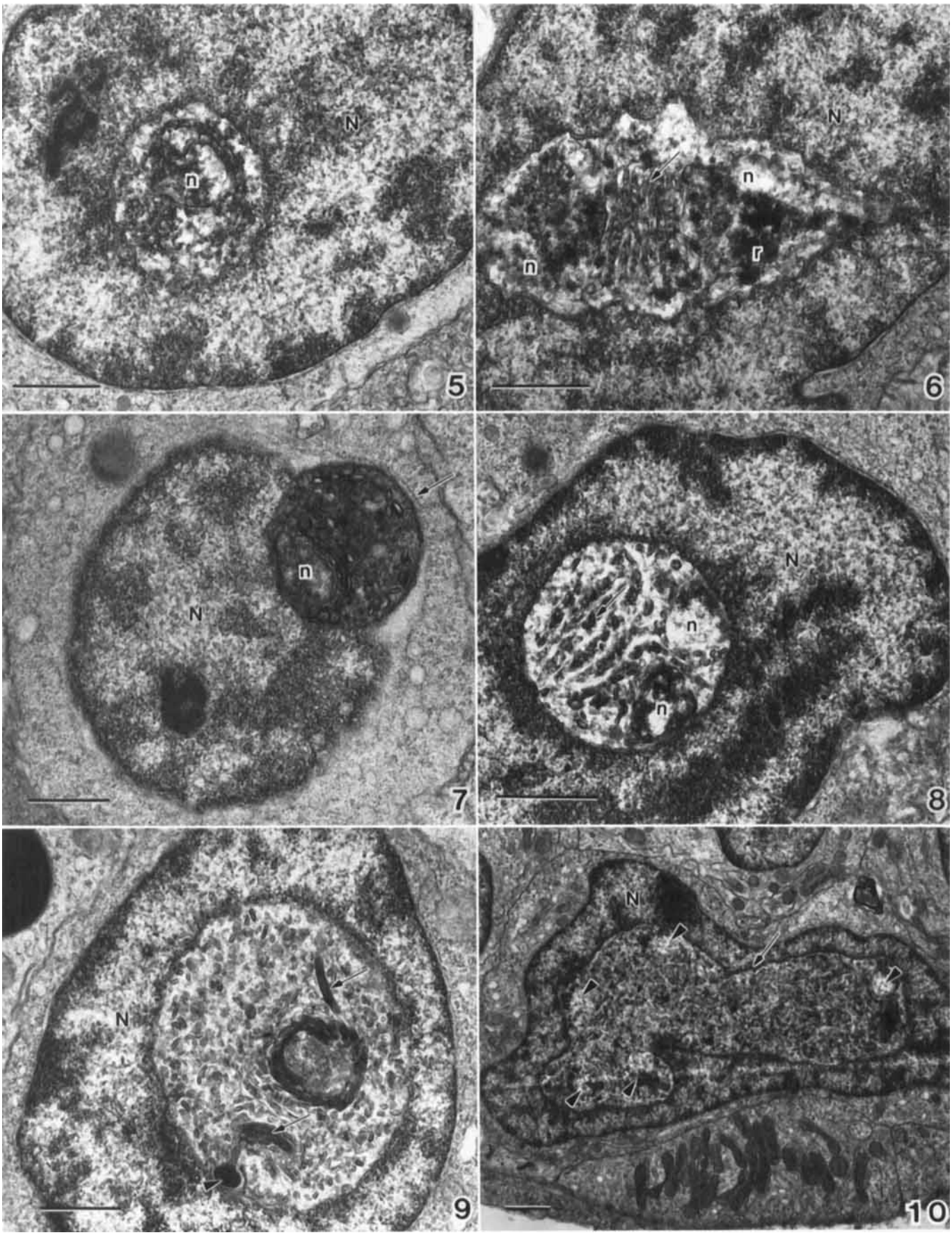


Fig. 1-4. *Enterocytozoon salmonis* n. sp. developmental stages and spores (arrows) in the nuclei (N) of cells from the kidney of chinook salmon (*Oncorhynchus tshawytscha*) cells. Electron micrographs. Bars = 5 μm. **1.** Leukocytes, **2.** A rodlet cell (R) and lymphocyte (L) infiltrating the urinary tubule epithelial cells (E). **3.** Spores within the epithelium of a urinary tubule. **4.** Light micrograph of a group of spores within the nucleus of a mesangial cell of the glomerulus. Toluidine blue stain. Bar = 25 μm.

dium of the anchoring disc connected to the anterior part of the polar tube becomes recognizable as do the laminate and vesiculate parts of the sporoplasm in more advanced sporonts (Fig. 9, 10).

Sporogonial Division. The number of sporoblasts dividing from each sporont is apparently dependent on the size of the host cell. Lymphoblasts contained fewer spores (1–8) compared with up to 18 in the nucleus of the large epithelial cells of the urinary tubules or mesangial cells of the glomerulus (Fig. 4). The first sign of sporogonial division was a thickening and invagination of the plasmalemma of the parasite (Fig. 11). The invaginating membrane separates several (depending on the size of the host cell) uninucleate sporoblasts within the host cell nuclei. Each sporoblast contains 4–5 coils of the polar tube, a single large vacuole, an anchoring system, and sporoplasm (Fig. 12).

Spores. Mature spores were ovoid and approximately 1 × 2 μm as measured from fixed tissues (Fig. 3, 13–15). Because of a retraction of the sporoblast cytoplasm the mature spores appeared to be surrounded by a peripheral host vacuole, which may be further enlarged by shrinkage of the host cell nucleoplasm during fixation (Fig. 3, 14, 15). The spores are coated by a thin membrane delimiting the spore contents and the two thicker layers of the spore wall: the inner electron-lucent endospore and the rough outer electron-dense exospore (Fig. 13). The anterior part of the polaroplast is characterized by a thickened outer laminate layer surrounding an inner reticulate component (Fig. 14, 15). The thick anchoring disc is composed of alternating electron dense and lucent layers. Its base forms an electron-lucent membrane covering the top of the laminar part of the polaroplast (Fig. 14, 15). The polar tube originating at the anchoring disc extends posteriorly through the vesicular polaroplast and turns repeatedly into 8–12 coils that can be arranged in single or double rows around the posterior vacuole in cross section (Fig. 14). Transverse sections of the coils show the multilamellar structure of the polar tube (Fig. 14, insert). The nucleus abuts the lamellar polaroplast occupying a mediolateral position (Fig. 14).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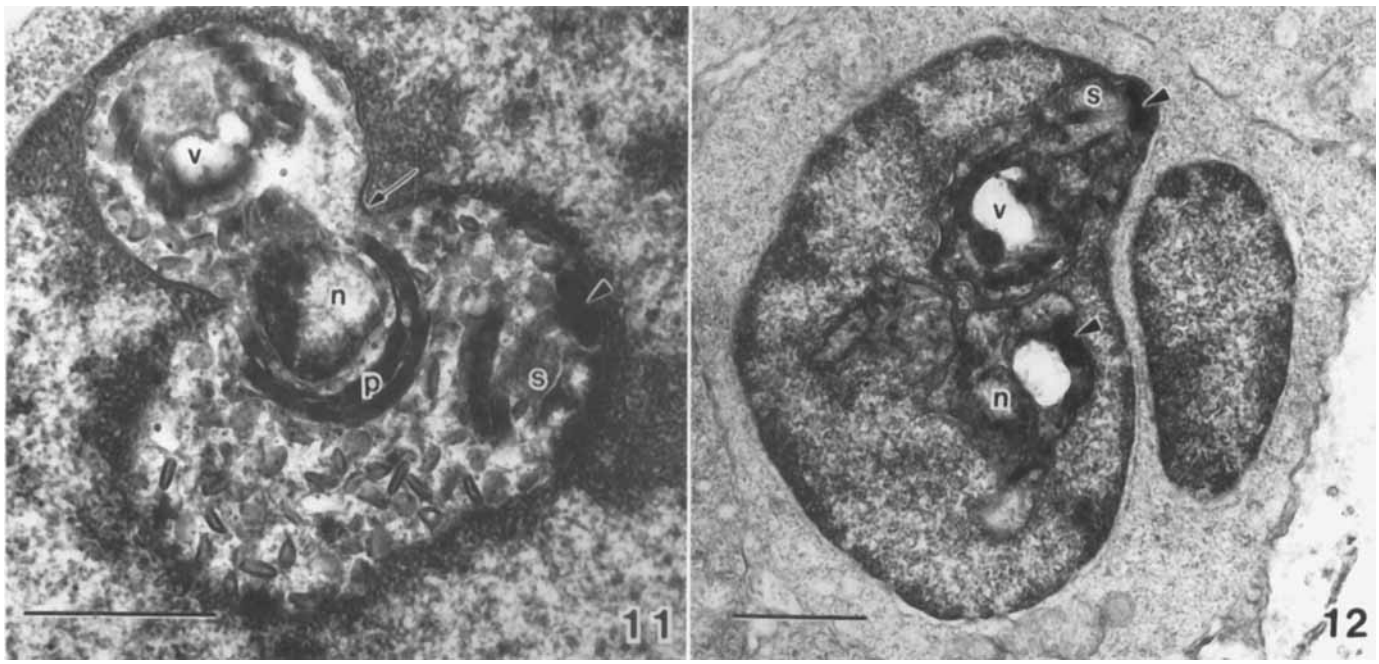

Fig. 11, 12. Sporogonial division of *Enterocytozoon salmonis* n. sp. showing nuclei (n), polar tube (p), vacuole (v), sporoplasm (s), and anchoring disc (arrow head). Bars = 1 μm. **11.** Early division with invaginating plasmalemma (arrow) separating two future sporoblasts. **12.** Sporogonial plasmodium showing two newly formed sporoblasts.

DISCUSSION

This study describes the developmental stages and spores of a unique intranuclear microsporidian occurring in chinook salmon in North America. Although previous observers [4, 5, 9] have recorded certain stages of the parasite, the present report describes a continuum of development beginning with the first recognizable and poorly differentiated meront to a spore with an elaborate extrusion apparatus and sporoplasm. The unique features of the development of this parasite clearly separates it from other Microsporida and therefore warrants its taxonomic placement as *Enterocytozoon salmonis* n. sp.

Geographic Range. The geographic range for *Enterocytozoon salmonis* was originally thought to be restricted to Washington and California states, but reports of similar intranuclear hematopoietic microsporidian infections by light microscopy in steelhead (*Oncorhynchus mykiss*) and rainbow trout (*O. mykiss*) from Idaho state (E. MacConnell, pers. comm.) and British Columbia, Canada (M. L. Kent, pers. comm.) indicate the parasite is found in several fish stocks in the western regions of North America. The same parasite has also been detected by one of the authors (S. C.) among rainbow trout in France (unpubl. data).

Affinities of *Enterocytozoon salmonis* to other Microsporida. Intranuclear microsporidians have only been reported from salmonid fish and all previous studies seem to describe a similar agent, perhaps with the exception of *M. rhabdophilia. Microsporidium rhabdophilia* is found in the nucleus of chinook salmon, coho salmon (*O. kisutch*), and steelhead trout in the same geographic region. It has small spores (1.5 × 1.5 μm in fixed preparations) of approximately the same size as *E. salmonis* (1 × 2 μm). However, it differs from the descriptions of *E. salmonis* in two important characteristics; its apparent specificity for rodlet cells and the absence of overt disease associated with parasitism [8]. The intranuclear hematopoietic microsporidian shows a preference for lymphoblasts; parasitism results in anemia and an accompanying leukemic-like condition [4, 5, 9]. Although host cell specificities might seem sufficient to distinguish the two parasites, this is confused somewhat by our finding of merogonic and sporogonic stages of what appears to be the hematopoietic microsporidian in the nuclei of rodlet cells, urinary tubule epithelium, and mesangial cells of the glomerulus (Fig. 2, 3, 4) of chinook salmon with clinical infections with *E. salmonis*. Although parasites were detected in these nonhematopoietic cells, they were found only on occasion compared with high rates of infection in hematopoietic cells. Since electron microscopic studies on salmonids with *M. rhabdophilia* (and no evidence of clinical disease) have not been conducted, directly comparing the merogonic and sporogonic stages with *E. salmonis* is not possible.

Developmental sequences of *E. salmonis* are very similar to those described for *E. bieneusi* [1, 3]. In both *E. salmonis* and *E. bieneusi,* the precursors of the extrusion apparatus are produced prior to the formation of individual sporoblasts. Sporoblasts are formed by a process of invagination of the plasmalemma—although this process is more accentuated in *E. bieneusi*

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Fig. 5, 6. Merogonic stages of *Enterocytozoon salmonis* n. sp. Host cell nucleus (N) and parasite nucleus (n). Bars = 1 μm. **5.** Early uninucleate stage. **6.** Proliferative binucleated form. The parasite cytoplasm contains a developed endoplasmic reticulum (arrow) and aggregations of ribosomes (r).

Fig. 7-10. Sporogonic stages of *Enterocytozoon salmonis* n. sp. Host cell nucleus (N) and parasite nucleus (n). Bars = 1 μm. **7.** Uninucleate sporont stage with abundant polar tube precursors. The host nuclear membrane surrounds the parasite. **8.** Binucleate sporont displaying precursors of the polar tube (arrow). **9.** Sporonts with development of the extrusion apparatus: anchoring disc (arrow head) and polar tube (arrow). **10.** Large sporont characterized by a thickened plasmalemma (arrow) surrounding at least five nuclei (arrow head).

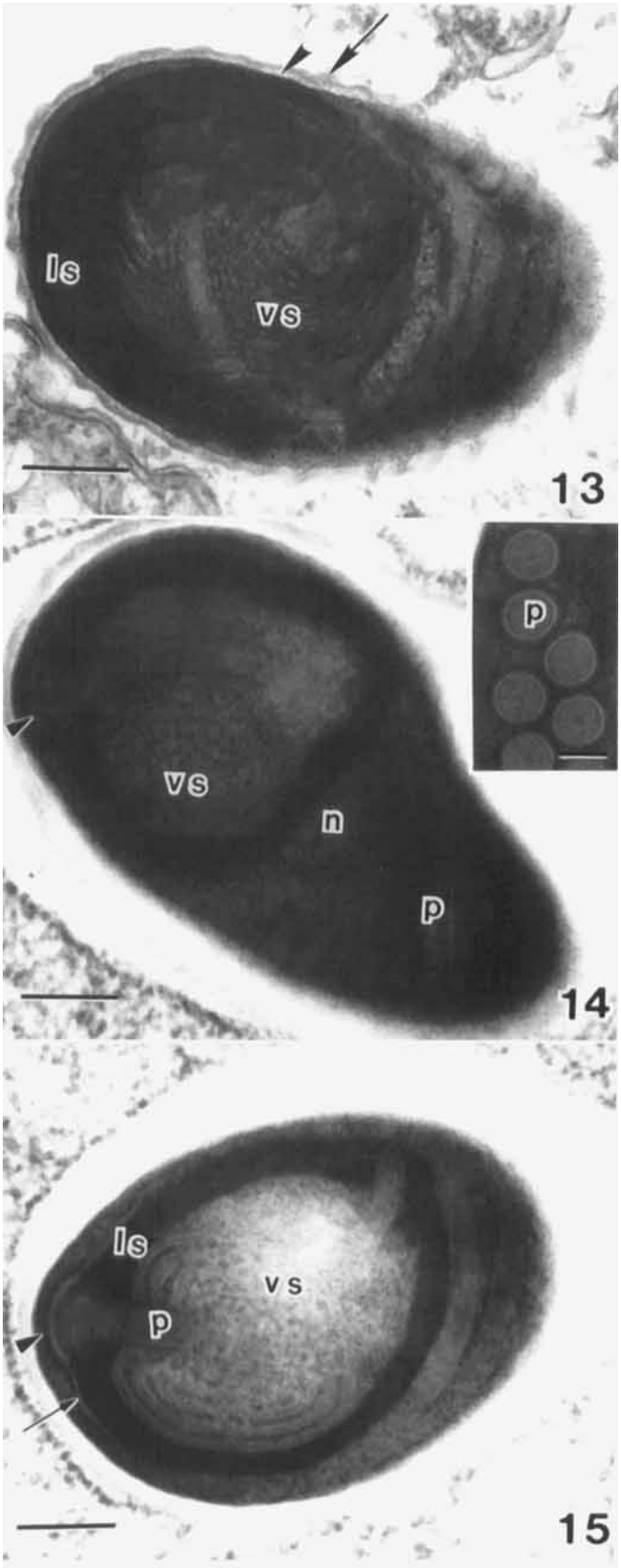

Fig. 13–15. Sections through mature spores of *Enterocytozoon salmonis* n. sp. Bars = 250 nm. **13.** Spores have an outer exospore (arrow) and an inner electron-lucent layer or endospore (arrow head). The vesiculate polaroplast (vs) is surrounded by a laminate part (ls). **14.** The main inner components are evident: anchoring disc (arrow head), nucleus (n), vesiculate polaroplast (vs), and polar tube coils (p). **15.** The complete extrusion apparatus from the anteriorly located anchoring disc (arrow head) connected to the polar tube (p) to the vesiculate polaroplast (vs) in the posterior. The laminate polaroplast (ls) is partly covered by anchoring disc extensions (arrows).
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[1]. Several features of *E. salmonis,* however, were never observed in *E. bieneusi* infections: (1) merogonic and sporogonic stages of *E. salmonis* were found only in the nucleus not in the cytoplasm as with *E. bieneusi*; (2) electron-lucent inclusions and electron-dense discs developing during the merogony and sporogony of *E. bieneusi* were not observed in our study; and (3) the spores of the human parasite display only 4–5 turns on the coiled polar tube [1, 3]. Although the presence or absence of diplokaryon during development of *E. bieneusi* is unsettled [1, 3] there was no evidence for this in *E. salmonis.*

Taxonomy. *Enterocytozoon salmonis* exhibits the main characteristics of the Microsporida. It does not develop within a parasitophorous vacuole and sporulation does not involve formation of a pansporoblastic membrane; both features of microsporida grouped in the suborder Apansporoblastina [2]. According to the unique developmental stages, *E. salmonis* is placed into the same family as *Enterocytozoon bieneusi.*

Characteristics of *Enterocytozoon salmonis* n. sp. The parasite is found among salmonid fish from the western US, Canada, and France. The nucleus of: hematopoietic cells (principal location), rodlet cells, epithelial cells of the urinary tubule, and mesangial cells of the glomerulus are sites for infection. All stages are in direct contact with the host cell nucleoplasm. The parasite displays no diplokaryon at any stage of development, the extrusion apparatus begins to differentiate into multinucleated meronts before forming the sporogonial plasmodia. Ovoid spores (1 × 2 μm) are uninucleate with 8–12 turns of the coiled polar tube.

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Gas Vacuoles and Flotation in the Testate Amoeba *Arcella discoides*

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ABSTRACT. The natural formation of gas vacuoles as a method of movement is described for the testate amoeba *Arcella discoides*. These vacuoles are used to float the organism from the substrate to the surface in an inverted position.

Key words. *Arcella discoides*, gas vacuoles, testate amoeba.

THE phenomenon of gas-vacuole formation in species of *Arcella* has been observed previously and is summarised by Bles [1]. In early experiments gas-vacuole formation was induced [1, 2] by inverting the specimen in small drops of water on a microscope slide. Such specimens righted themselves either by the extrusion of a long pseudopodium, which attached to the substrate and returned the shell to an upright position, or by a combination of pseudopodium and gas-vacuole formation. Gas vacuoles were formed only in those individuals that could not extend a large enough pseudopodia to reach the substrate. Bles [1] considered that gas-vacuole formation was a by-product of the animal's struggle to turn over. He did not observe vacuole formation at one side and the subsequent vertical tilting described by Engelmann [2]. The reaction times given [1] for vacuole formation in *Arcella discoides* of between one and fourteen minutes agree well with current results. Although this phenomenon has been known for some time, no evidence supports the theories of vacuole formation or its natural occurrence. Therefore, an investigation into the changes accompanying gas-vacuole expansion and contraction in cultures was undertaken, and the results are presented here.

MATERIALS AND METHODS

The strain used in this study was collected in a water sample taken from a small pond at High Halstow, Rochester, Kent, England during April, 1984. Clonal cultures were established by isolating single active animals; one of which subsequently has been maintained in small plastic containers [5]. Specimens with gas vacuoles were taken from the culture vessels, placed in a drop of water, and immediately examined by interference contrast microscopy.

RESULTS AND DISCUSSION

The shell of *Arcella* is composed of a rigid proteinaceous casing, usually circular, domed, and with an invaginated aperture. Natural gas-vacuole formation is observed in specimens of *Arcella discoides* two weeks after they are inoculated into new culture medium, when they migrate from the agar substrate to the surface of the covering medium. At this stage a bacterial film has often formed on the substrate. The upward movement is due to the formation of small gas bubbles or vacuoles within the cytoplasm at specific sites, which are evenly spaced around the periphery of the shell. The vacuoles materialize close to the plasmalemma in the posterior cytoplasm (i.e. the margin furthest away from the aperture). Initially, one or two vacuoles are seen at one side of the shell casing. Then, two or more start to appear almost equidistant from each other. They expand slowly to a more or less standard spherical size (approximately 20 μm in diameter), although some sausage-shaped vacuoles, fewer in number but twice as large, are occasionally seen.

About two minutes after the first vacuole sighting, one side of the casing is lifted free of the substrate. A minute later, we see the shell tilts into a vertical position when viewed from above and the pseudopodia remain extended and trail back toward the substrate. The vertical shell then begins to lift slowly from the substrate—in our culture vessels, with a covering of 2–3 mm of fluid medium, the shell reaches the surface in approximately 10 minutes. Here as the shell touches the meniscus, it gradually turns so that the aperture faces upward. Pseudopodia, which remained extended from the posterior margin throughout this process, now begin to radiate evenly beneath the medium surface. The gas vacuoles at this stage appear to be equal in volume and equally spaced.

The gas vacuoles deflate when floating shells are disturbed. Initially there is no apparent reaction, but after about two or three minutes the vacuoles become smaller (Fig. 1, 2). Four or five relatively equally spaced vacuoles can be reduced to three or four (Fig. 3, 4); after about five minutes they are usually reduced to three (Fig. 5). In the next minute these gradually reduce to either two, usually situated at opposite margins, or one (Fig. 6). One or two minutes later these disappear and the shell often descends to the substrate. Some shells retain their surface position held in the meniscus of the dish after deflation.

Gas-vacuole formation has been observed under similar conditions in a culture of *Arcella catinus*, although the times taken for inflation and deflation in the species were longer, about twenty minutes. A culture of *A. vulgaris* did not exhibit the same phenomenon.

It is assumed that flotation allows the individual to move from an unfavourable environment, in cultures possibly either low dissolved oxygen concentration or toxic exudates produced by the bacterial film that overlays the agar substrate, to one that is more acceptable. This would certainly agree with accounts of other protozoa migrating into the upper water levels in lakes and ponds [3, 4].