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Interrelationships of cytoplasmic structures in *Bonamia* sp. (Haplosporidia) infecting oysters *Tiostrea chilensis*: an interpretation

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ABSTRACT: Reticular structures, confronting cisternae (CC), including cylindrical confronting cisternae (CCC), an unusual surface projection, formation of haplosporosome-like bodies (H-LB) and haplosporogenesis are described from *Bonamia* sp. infecting oysters *Tiostrea chilensis* from New Zealand. Formation of H-LB began when balls of putative nuclear material derived from indentations in the nuclear surface were encircled by cisternae of Golgi bearing a layer of dense material on their inner, concave surface. Also, H-LB were formed when CC split longitudinally and putative nuclear material filled the lumen of the resultant tubular structure, which subsequently pinched off H-LB. Haplosporogenesis began with the formation of cytoplasmic multi-vesicular bodies (MVB), which filled with dark osmiophilic material derived from nearby Golgi. The characteristic membranes of haplosporosomes appeared to form within these MVBs. It is suggested that H-LB are unusual structures in haplosporidans and that their core is formed from nuclear material. The CC from which they may form are also unusual, and in mammals CC occur in rapidly dividing or diseased cells. As CCC can only be induced in virally infected, transformed or neoplastic mammalian cells, it is considered that in *Bonamia* sp. CCC may be overt signs of an underlying disease. The significance of this is discussed in relation to the sudden appearance and pathogenicity of *Bonamia* sp. and, possibly, *Haplosporidium nelsoni* (MSX).

INTRODUCTION

Haplosporidians are a group of obligate parasites among which some of the most pathogenic species infect oysters. *Haplosporidium nelsoni* (MSX), which infects *Crassostrea virginica*, first appeared in Delaware Bay (USA) in 1957 and Chesapeake Bay in 1959, where it causes mortalities throughout most of the year (Andrews 1982). Its distribution before 1957 is unknown. With it co-exists *Haplosporidium costalis*, which unlike MSX only kills *C. virginica* for a short time in May–June, and it is thought to be endemic on the east coast of the USA (Andrews 1982). Historically *Mikrocytos mackini* has caused mass mortalities in *Crassostrea gigas* at Denman Island on the Canadian west coast, and *Mikrocytos roughleyi* has similarly affected *Saccostrea commercialis* around the Australian east coast (Farley et al. 1988). The roles of *Mikrocytos* spp. as pathogens are currently being investigated.

Bonamia ostreae, which has caused widespread mortalities among *Ostrea edulis* around Europe, was probably introduced into France in live *O. edulis* from California in 1979 (Grizel et al. 1988). It has caused mortalities in *O. edulis* in California in the past (Katkansky et al. 1969) and more recently (Elston et al. 1986, Friedman et al. 1989). *Bonamia* sp., the subject of this study, is highly pathogenic in *Tiostrea chilensis* in Foveaux Strait on the south coast of the South Island of New Zealand (Dinamani et al. 1987), and since 1986 has destroyed ca 80 % of the oyster stocks. Infection at barely detectable levels elsewhere around New Zealand probably derive from oysters moved from Foveaux Strait before the current epizootic. Little is known of how or why these parasites are so pathogenic, despite their considerable economic impact on commercial fisheries and aquaculture. However, pioneering work by Perkins (1968, 1969, 1979) and cytochemical studies by Azevedo & Corral (1985, 1987) have started to reveal the development and composition of some cytoplasmic structures.

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This study reports on the interrelationships of some cytoplasmic structures in *Bonamia* sp. during its annual cycle (Hine 1991a), using the same material as in an earlier study (Hine 1991b). After comparison with other species, a hypothesis on the pathogenicity of *Bonamia* sp., and possibly *Haplosporidium nelsoni*, is presented.

MATERIALS AND METHODS

Techniques were as described in Hine (1991b). Samples of 50 oysters *Tiostrea chilensis* (syn. *T. lutaria*) were collected in January 1987 (1/87), and April (4/90), June (6/90), June–July (6–7/90), July (7/90) and August (8/90) 1990. Oysters were kept chilled on ice and opened one at a time; the flat valve was removed, the heart excised and cut in half, one half fixed for transmission electron microscopy (TEM), and an imprint made from the other half. For TEM 1/87 oysters and replicate tissue of 4/90 oysters were fixed in 2.5 % glutaraldehyde in cacodylate buffer (pH 7.2), and the other replicate 4/90 and subsequent samples were fixed in 2.5 % glutaraldehyde in filtered (0.22 µm) seawater. After 1 to 2 h, tissues were washed twice in buffer or seawater. Heart imprints were stained using the Merck Hemacolor system, and after identification of heavily infected oysters their TEM-fixed tissues were post-fixed in 1 % OsO_4 for 1 h, stained en bloc with 5 % uranyl acetate in 0.1 M sodium acetate buffer for 45 min, dehydrated in ascending (50 to 100 %) ethyl alcohol, embedded in Araldite, sectioned, stained with 5 % uranyl acetate for 10 min and 5 % lead citrate for 5 to 6 min, and examined on a Philips 420ST electron microscope.

RESULTS

Four groups of cytoplasmic structures are described: reticular structures, confronting cisternae, and structures involved in the formation of haplosporosome-like bodies, and in haplosporogenesis.

Reticular structures

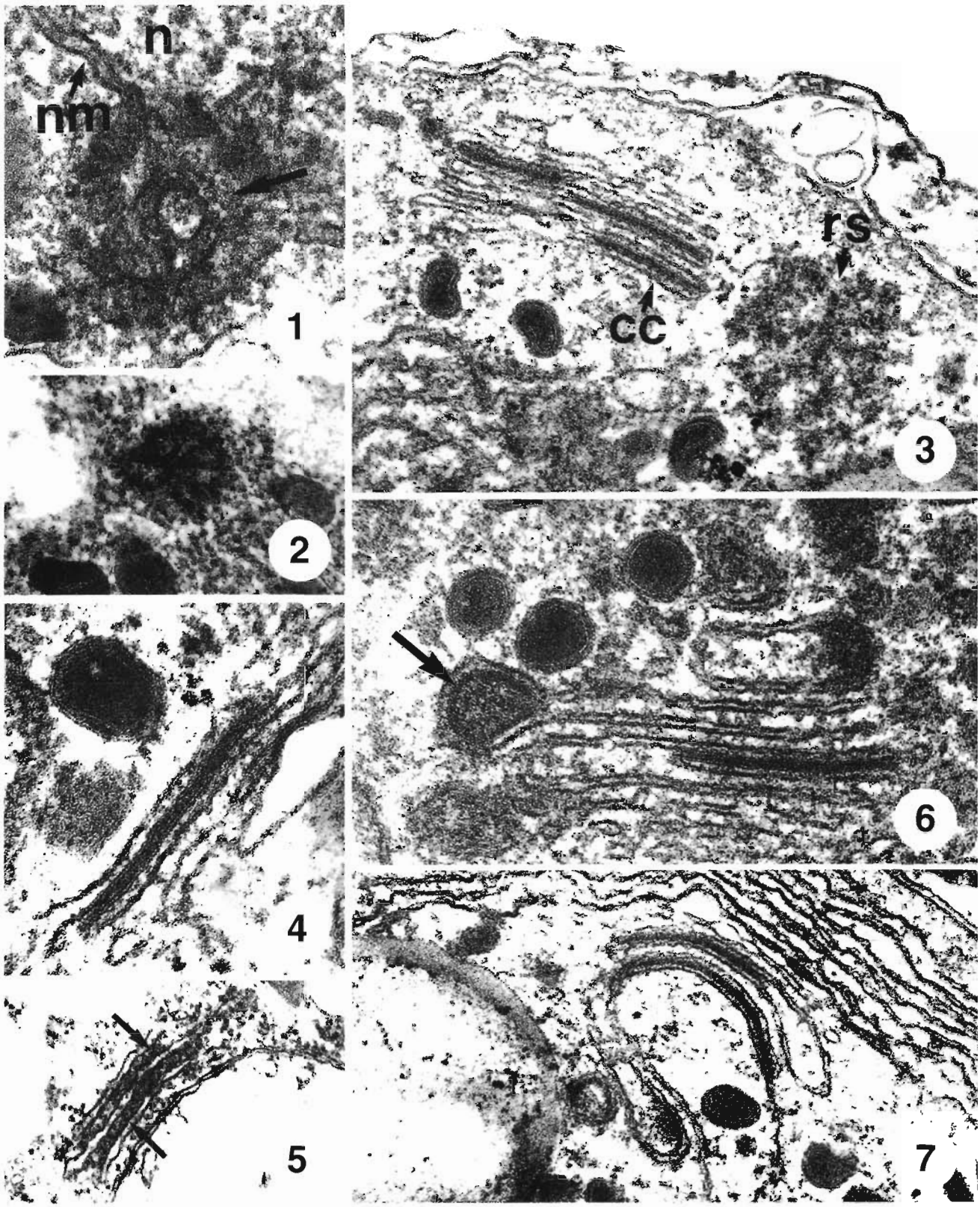
Reticular structures (RS) were observed in the peripheral cytoplasm at all stages of development, but were most common in dense forms of *Bonamia* in January (Table 1). They appeared to develop from material on the nuclear surface (Fig. 1), but RS usually appeared as a reticulated pattern of moderately to strongly electron-dense material 240 to 440 nm in diameter (Figs. 2 & 3). A few short disorganised 5 nm diameter tubular or membranous inclusions were occasionally seen in RS (Fig. 1). There was no delimiting membrane and no apparent association with other cytoplasmic structures.

Confronting cisternae

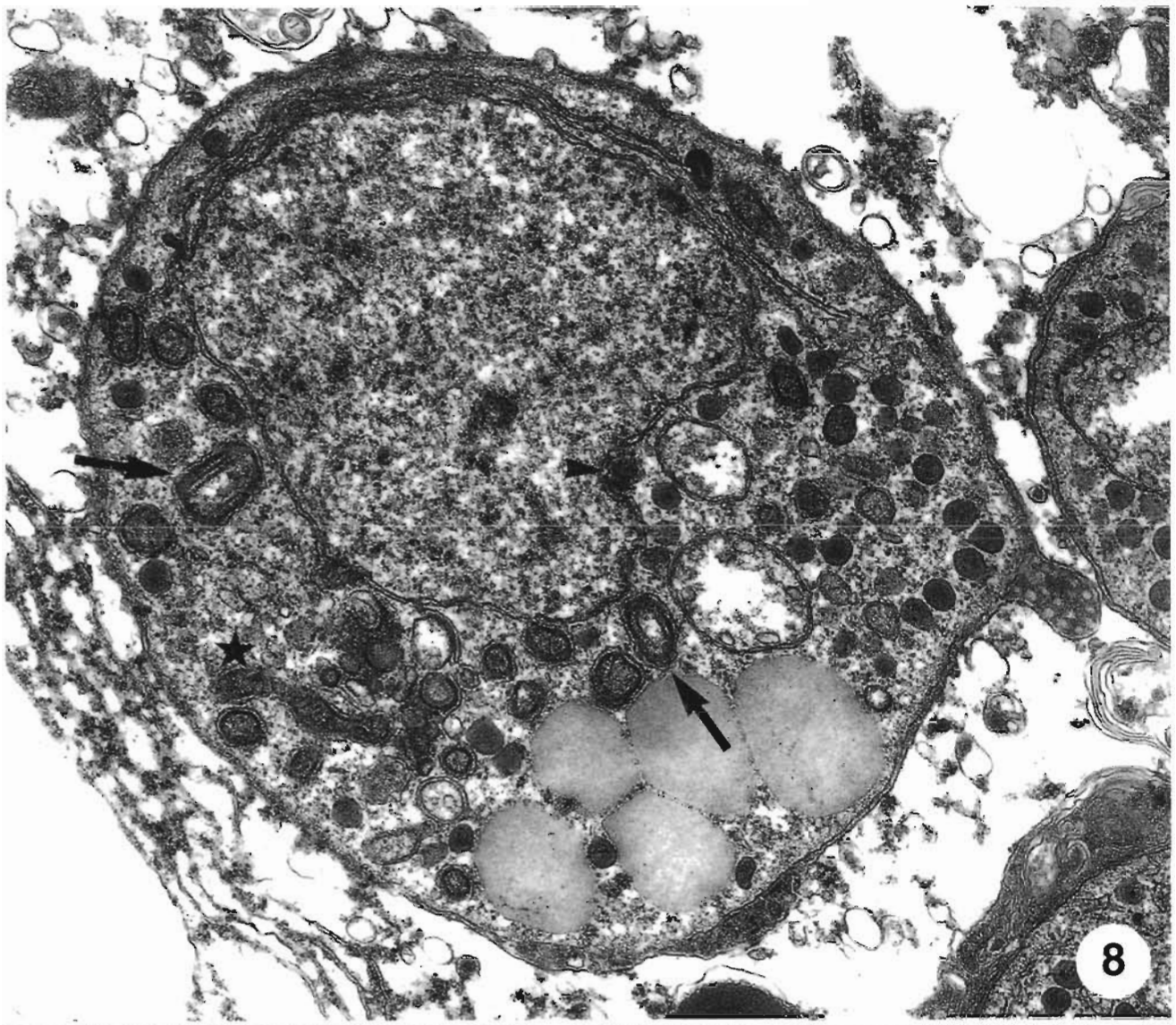
Parallel arrays of endoplasmic reticulum (ER) with sparse ribosomes on their outside surface (rER) were often observed with a dense lamina between them (Figs. 3 & 4), over the sampling period (Table 1). The dense lamina between these confronting cisternae (CC) was formed from the ribosomes trapped between the rER cisternae (Fig. 5). The dense lamina was 22 to 25 nm thick and the rER lumen was 12 to 15 nm in diameter with walls 5 nm wide. A dense lamina was also observed between Golgi cisternae near the surface of the nucleus (Fig. 6). In July and August pseudopodial, often diplokaryotic, forms contained meandering arrays of CC (Fig. 7), some of which showed round, ovoid or elongated circular or tubular profiles (Fig. 8, Table 1). These cylindrical confronting cisternae (CCC) were 320 nm in overall diameter, appeared as 2 concentric circles in cross section and frequently enclosed other cytoplasmic structures (Figs. 9 & 10). Occasionally CC or CCC formed with mitochondrial membranes (Fig. 11). One cell with CCC (Fig. 8) had a surface projection 430 × 266 nm, containing membrane-bound light ovoid particles 58 × 50 nm in diameter (Fig. 12).

Table 1. *Bonamia* sp. Prevalence (% occurrence) of cytoplasmic structures in relation to month sampled, and mean number of haplosporosomes. n: no. of observations; RS: reticular structures; CC: confronting cisternae; CCC: cylindrical confronting cisternae; MVB: multi-vesicular body; H-LB: haplosporosome-like body

Month/year	n	RS	CC	CCC	Extranuclear material	Nuclear indentations	Golgi:nuclear material	Golgi:MVB	H-LB	Haplosporosomes (mean no. ± SD)
1/87	71	17	10	0	14	6	32	0	20	15 ± 7
4/90	57	4	14	0	14	11	44	0	19	21 ± 9
6/90	26	8	19	0	54	50	58	0	35	32 ± 17
6–7/90	29	3	15	0	55	38	96	0	38	30 ± 13
7/90	49	2	2	8	51	26	92	2	49	44 ± 25
8/90	147	1	15	9	63	53	98	10	50	58 ± 24



Figs. 1 to 7. *Bonamia* sp. in *Tiostrea chilensis*. Fig. 1. Nuclear surface in a dense form showing the nucleus (n), nuclear membrane (nm), and an extranuclear cytoplasmic mass with a few 5 nm wide membranous inclusions (arrow); ×93 800 Fig. 2. Dense cytoplasmic reticular structures; ×69 000. Fig. 3. Reticular structures (rs) near a Golgi-like profile with dense laminae between confronting cisternae (CC); ×86 250. Fig. 4. CC showing the ER cisternae flanking the dense lamina between them, ×134 000. Fig. 5. Closely apposed rER cisternae showing ribosomes (arrows) between them, ×57 500. Fig. 6. Golgi with dense lamina of CC between cisternae, and with a cisterna bearing dense material on its inner concave surface (arrow) encircling granular material; ×107 200 Fig. 7. Meandering ER arrays forming CC; ×70 300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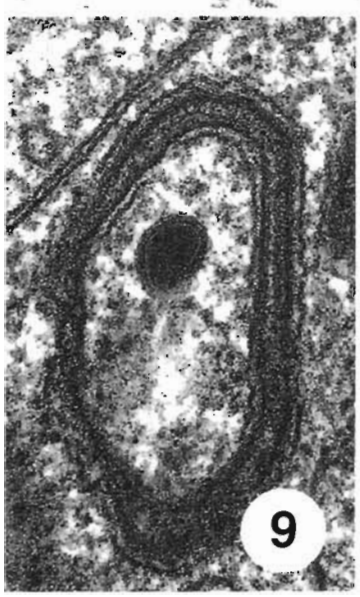
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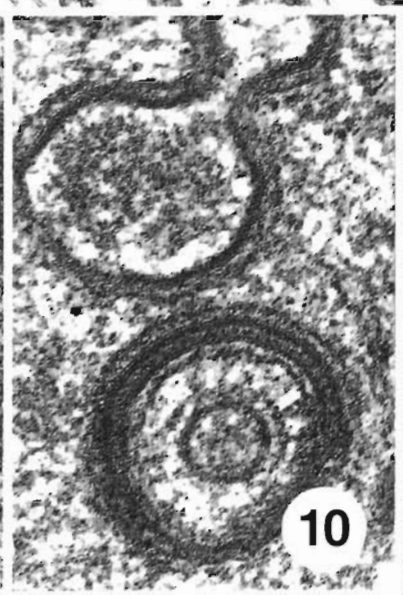
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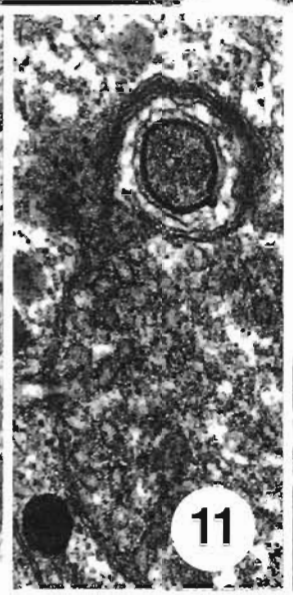
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Formation of haplosporosome-like bodies

Formation of haplosporosome-like bodies (H-LB) began at cup-like indentations in the nuclear surface, which contained amorphous fine granular or vesicular (Fig. 13) material. The granular material resembled dense material in the nucleus. Nearby, a flattened or slightly concave area of nuclear surface was overlaid with 2 to 3 beaded profiles of Golgi apparatus that derived from, and was attached to, the nuclear membrane (Fig. 14). Balls of cup material passed along the nuclear surface under the Golgi cisternae (Fig. 15), after apparent formation where Golgi cisternae were in contact with the surface of the material in the cup (Fig. 16). They were then encircled by a curved Golgi cisterna with dense material on the inner concave surface (Fig. 6), to form an H-LB.

Golgi cisternae were the same diameter as rER in CC and CCC, but the dense layer was 11 to 13 nm thick, half the thickness of the dense lamina in CC and CCC. This resemblance to a longitudinal half of a CC was more than coincidental. In many sections CC appeared to split longitudinally as putative nuclear material from the cup-like indentations filled the lumen formed by the split (Fig. 17). This formed an elongated tubular structure (Figs. 8 & 17), from which H-LB then pinched off. Granular or vesicular masses, some enclosed by Golgi profiles, also occurred in the cytoplasm away from the nucleus. In rare cases the nuclear surface became invaginated, with dense material forming on the enclosed inner surface (Fig. 18), to form an intranuclear H-LB (Fig. 19).

There was an increase in the percentage of *Bonamia* with H-LB and the number of H-LB per parasite (Fig. 20), from the earliest sample in January onwards (Table 1). The occurrence of cup-like indentations was greatest from June to August (Table 1), and the number of indentations per section increased until, in July and August, it was common to see 3 indentations in one section of a nucleus. Also in July and August samples, dense moribund or necrotic *Bonamia* predominated, and these were often packed with large irregular H-LB configurations (Fig. 21).

Haplosporogenesis

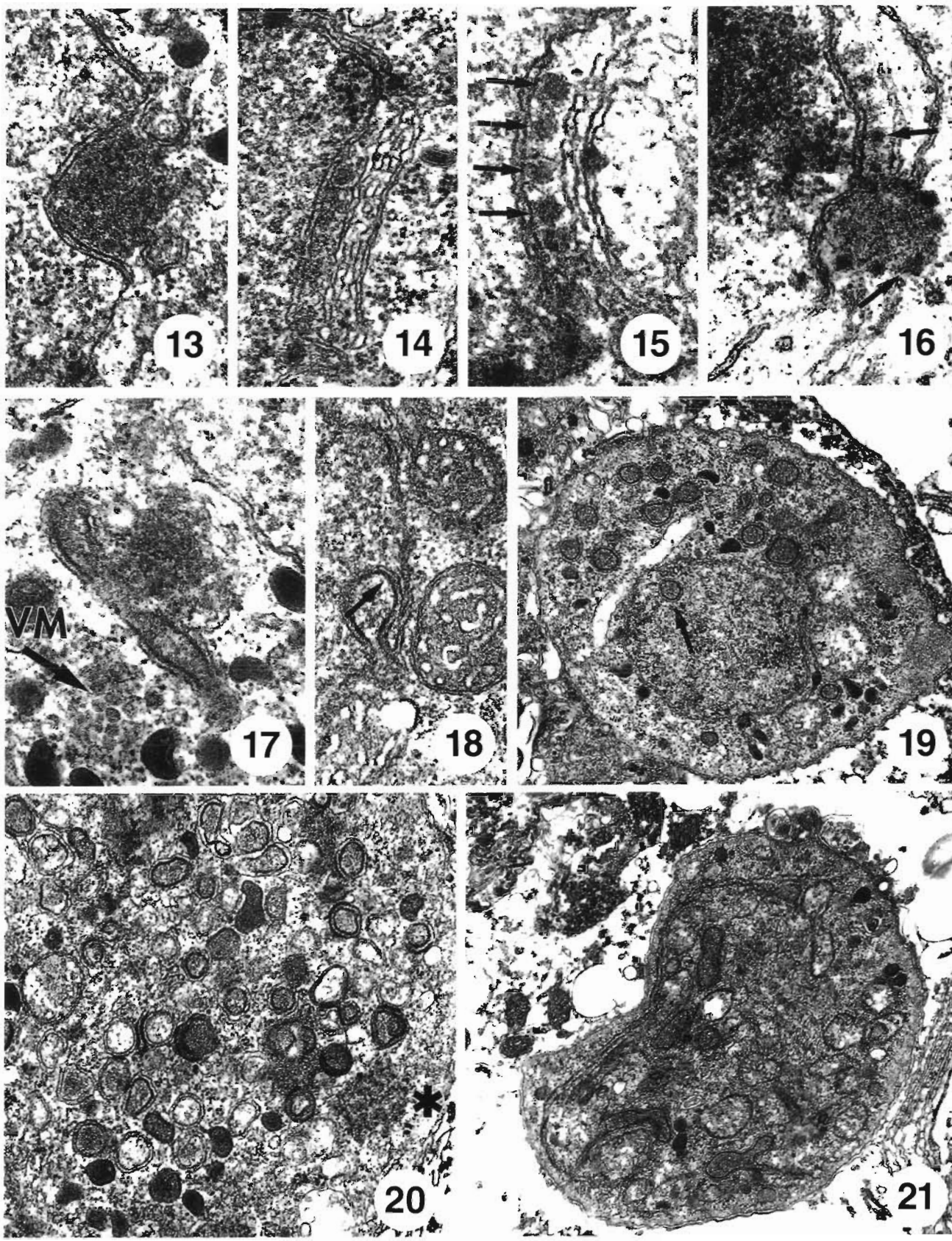
The apparent sequence of haplosporogenesis is shown in Figs. 22 to 25. Light, membrane-delimited, multivesicular bodies (MVB) <700 nm in diameter, containing pleomorphic vesicles 32 to 220 nm across, each with a bilaminar membrane 5 nm wide, formed in the cytoplasm (Fig. 22). Dense material from the cisternae of nearby Golgi (Fig. 23) appeared to enter the MVB, increasing its density (Fig. 24). Spherical or reniform haplosporosomes, 115 to 140 nm in largest dimension, formed at the periphery of these MVBs, but during haplosporosome formation an MVB-delimiting membrane was rarely observed (Fig. 25). Often, Golgi was seen forming H-LB near masses where haplosporogenesis occurred. In July and August some *Bonamia* contained numerous large MVB containing irregular, apparently malformed, haplosporosomes.

DISCUSSION

Structures resembling haplosporosomes are not confined to haplosporidians, but also occur in *Paramyxa paradoxa* (Desportes 1981), in the vegetative stages of myxozoans (Smith et al. 1984, Kent & Hedrick 1985, 1986, Lom et al. 1989), and in the primary cells of *Marteilia sydneyi* and its relatives (Perkins 1976, Perkins & Wolf 1976, Ginsburger-Vogel & Desportes 1979). The haplosporosomes of haplosporidians (Fig. 5 in Scro & Ford 1990) and myxozoans (Fig. 12 in Kent & Hedrick 1985) sometimes orientate along the plasma membrane in a remarkably similar manner. Haplosporosomes develop either directly from the Golgi (= spherule) in the spore (Perkins 1969, 1979, Desportes & Nashed 1983, Smith et al. 1984, Azevedo & Corral 1987, Larsson 1987, Lom et al. 1989), from dense or formative inclusions derived from the spherule (Perkins 1975, Marchand & Sprague 1979, Ball 1980, Ormières 1980, La Haye et al. 1984, Hillman et al. 1990), or from MVB (Perkins 1968, 1976, 1979, Perkins & Wolf 1976, Seagrave et al. 1980, Desportes 1981).

In this study, Golgi was involved in the development of MVB, and it therefore appears that haplosporosomes are indirectly Golgi-derived. They may also derive directly from Golgi, as their numbers increased over the

Figs. 8 to 12. *Bonamia* sp. in *Tiostrea chilensis*. Fig. 8. Cell containing CCC (arrows), H-LB including a tubular H-LB structure (★), dense material in a cup on the nuclear surface (arrowhead) and a beaded projection from the cell surface; ×33 400. Fig. 9. Early CCC in which the dense lamina is forming from the parallel ER cisternae; ×90 900. Fig. 10. CCC surrounding an H-LB, and lying next to an H-LB-like structure. Note the similarity of the wall of the H-LB-like structure to half the wall of the CCC; ×92 200. Fig. 11. H-LB surrounded by CC formed from ER and the mitochondrial membrane; ×57 500. Fig. 12. Enlargement of the surface projection from the cell in Fig. 8 showing the membrane-limited ovoid particles; ×112 900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months sampled (Table 1; Hine 1991b), but MVB were only observed in the last 2 months (July and August) of this study. In *Haplosporidium nelsoni* haplosporosomes form from Feulgen-positive MVB and acquire their outer membrane from the membrane delimiting the MVB (Perkins 1968, 1979). In *Bonamia* sp. an MVB-delimiting membrane was only seen in developing MVB, and haplosporosomes appeared to arise by membrane assembly in the dense mass, in a manner resembling some viruses (Fig. 2c in Comps 1988).

Haplosporosomes have a matrix of protein, an external membrane of glycoprotein and an internal membrane containing lipid (Azevedo & Corral 1985), but their function is unknown. There is evidence they are exocytosed (Perkins 1968, 1979, Smith et al. 1984, Azevedo & corral 1985, Scro & Ford 1990), and may cause host cell lysis (Perkins 1979, Scro & Ford 1990). Alternatively, it has also been suggested that they participate in spore wall formation (Newman et al. 1976), and Golgi-derived dense bodies in *Paramarteilia orchestiae* appear to fulfil this function (Ginsburger-Vogel & Desportes 1979). Haplosporosomes contain periodate-reactive complex carbohydrates (Perkins 1976) which may act as an energy reserve in sporulation (Azevedo & Corral 1985). If haplosporosomes are involved in sporulation, it would be more likely that they would form before sporulation and be absent from other stages, rather than be present at all stages except early sporulation. There is no evidence that *Bonamia* spp. (Pichot et al. 1979, Hine 1991a, b) or *Mikrocytos* spp. (Farley et al. 1988), and possibly other haplosporidians (Newman et al. 1976, Dyková & Lom 1988), produce spores, and yet all contain haplosporosomes.

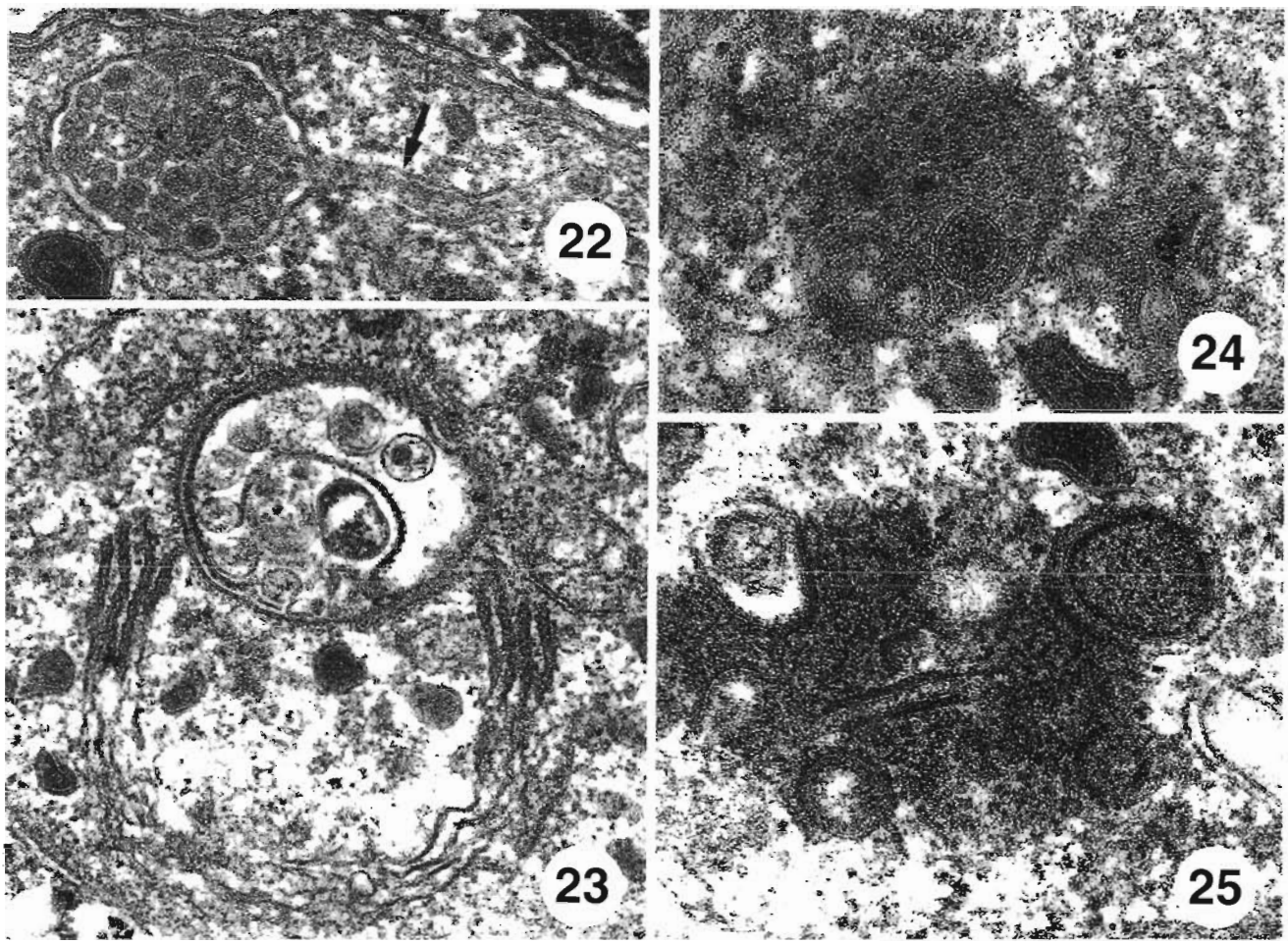
The formation of H-LB described here was similar to that reported from *Haplosporidium nelsoni* by Perkins (1979). However, few other haplosporidians contain H-LB [Pichot et al. 1979 (Fig. 13), Dyková & Lom 1988 (Fig. 11), Farley et al. 1988 (Fig. 20), Hillman et al. 1990 (Fig. 4)], and they cannot be regarded as a common or possibly even a normal feature of haplosporidians. In *Bonamia* sp. their formation appeared to involve movement of nuclear material into cups on the nuclear surface, and packaging of this material by Golgi cisternae bearing a layer of dense material on one surface. The presence of putative nuclear material in cup-like nuclear indentations is similar to observations on *Haplosporidium costalis* by Perkins (1969: Fig. 9), but H-LB were not reported from this species. The dense layer on the cisternae appeared to be the same as that of the dense lamina of CC, which, being formed from ribosomes of rER, is probably ribonucleoprotein.

Among haplosporidians CC have only previously been observed in *Bonamia ostreae* by Pichot et al. (1979: Fig. 13). In mammals CC are formed by apposition of rER cisternae, as observed here, and have been reported from many neoplastic and some rapidly dividing normal cells (Ghadially 1988). After consideration of known occurrence, Ghadially (1988) concluded '...they have been seen more often in neoplastic and virus infected cells than in normal cells. One may argue that this is at least an atypical if not an overtly pathological event because not all cells in mitosis develop confronting cisternae.' The common occurrence of CC in *Bonamia* sp. may therefore indicate an underlying disease. Also, the involvement of CC in H-LB formation may indicate H-LB are abnormal structures. This is also suggested by the increased number of H-LB in some cells when the *Bonamia* population rapidly declines in July and August (Hine 1991a, b), and the packing of large irregular H-LB in moribund and necrotic *Bonamia* at that time.

Further support for the suggestion that *Bonamia* has an underlying disease comes from the presence of CCC in July and August. In mammals CCC are 225 to 300 nm in diameter (Ghadially 1988), with rER cisternae 20 to 25 nm across (Luu et al. 1989) and a dense lamina 20 to 24 nm wide (Ghadially 1988, Luu et al. 1989). They occur in virus-infected, virally transformed and neoplastic cells and are induced by exposure of those cells to α and β interferon (Bockus et al. 1988, Luu et al. 1989). From their review of CCC Luu et al. (1989) concluded that the production of CCC in the presence of interferon may require the existence of a viral genome in the DNA of the CCC-positive cell.

It would be premature to assume that the trigger of CCC formation in mammalian cells is identical to that

Figs. 13 to 21. *Bonamia* sp. in *Tiostrea chilensis*. Fig. 13. Cup-like indentation in the nuclear surface containing vesicular material; ×51 700. Fig. 14. Golgi profiles overlying the nuclear surface; ×61 400. Fig. 15. Spherical bodies (arrows) between Golgi cisternae and the nuclear membrane; ×96 600. Fig. 16. Vesicular material above the nuclear surface, into which Golgi cisternae ramify. Note the spherical bodies on the surface of the material and on the nuclear surface (arrows); ×57 000. Fig. 17. Vesicular mass (VM) apparently entering the lumen of a tubular structure thought to be a longitudinally split CC. Note 32 to 38 nm diameter cytoplasmic vesicles (arrow) ×56 300. Fig. 18. Invagination of the nuclear membrane showing dense material on the concave invaginated surface (arrow); ×50 000. Fig. 19. *Bonamia* with several H-LB, one of which is intranuclear (arrow); ×22 800. Fig. 20. Cytoplasm of parasite in the 8/90 sample. Note the many irregular H-LB and the peripheral RS (*); ×35 000. Fig. 21. Necrotic parasite containing large irregular H-LB; 8/90 sample; ×19 900



Figs. 22 to 25. *Bonamia* sp. in *Tiostrea chilensis*. Fig. 22. Early development of an MVB. Note the tubular extension of the MVB (arrow); ×91 700. Fig. 23. MVB next to Golgi cisternae containing intracisternal dense material. Unlike Golgi in Fig 14, this apparatus is in the cell periphery; ×58 300. Fig. 24. Dense MVB with a thin delimiting membrane and containing vesicular membranes; ×108 200. Fig. 25. Haplosporosomes developing from an MVB. Note the lack of a delimiting membrane; ×96 000

observed here, for 3 reasons. Firstly, in mammals short-term exposure of cells to interferon results in the formation of other structures, called tubuloreticular structures (TRS), that may occur in continuity with CCC (Bockus et al. 1988). The RS reported here do not resemble TRS, as the latter are composed of a distinct network of 22 to 25 nm tubules, but RS only contained a few fragments of 5 nm membrane-like structures. Unlike CCC, TRS can be induced in non-virally infected healthy cells (Luu et al. 1989). Secondly, limited investigations on molluscs have not detected the presence of interferon (T. C. Cheng pers. comm.). Thirdly, if TRS and CCC formation is a basic cellular response of eukaryotic cells to integration of a viral genome, TRS and CCC would also be expected in non-mammalian animals. They may have gone unnoticed, however, as TRS and CCC only occur in a small proportion of infected cells in mammals, and it is only since the recognition of their occurrence in Human Immunodeficiency Virus (HIV) infected lymphocytes that they have received attention (Sidhu et al. 1985, Kostianovsky et al. 1987, Luu et al. 1989).

Despite the lack of TRS in *Bonamia*, the CCC observed here are similar to those reported from mammalian cells in the method of formation, the entrapment of other organelles within them (Kostianovsky et al. 1987) and their occasional formation with mitochondrial membranes (Sidhu et al. 1985). On the basis of these observations it is hypothesised that CCC are an overt sign of an underlying pathology in *Bonamia* sp. The report of a 300 nm diameter structure of 2 concentric circles in *Haplosporidium nelsoni* by Scro & Ford (1990), similar in size and appearance to CCC in this study, may indicate that *H. nelsoni* has a similar underlying disease. *H. nelsoni* is the only other haplosporidian in which H-LB formation appears to be a

common occurrence. Both *Bonamia* sp. and *H. nelsoni* are very pathogenic and both seem to have suddenly appeared, *H. nelsoni* in Delaware Bay in 1957 and Chesapeake Bay in 1959, and *Bonamia* sp. in Foveaux Strait in 1986. If the very pathogenic forms of these 2 parasites had pre-existed in those areas, they would have made an obvious impact on the oyster fisheries. The parasites were either brought into these areas, or they altered from a pre-existing non-pathogenic form.

In *Bonamia* sp. the presence of an underlying disease may also explain why the parasite population declines rapidly in July and August. It was previously thought that this decline was due to increased phagocytosis by host hemocytes in winter (Hine 1991a, b), but examination of parasites in July and August suggests many die without being phagocytosed. Moribund and necrotic *Bonamia* are often packed with many large irregular H-LB, and it appears the development of these bizarre forms of H-LB is rapidly followed by cell death. The collapse of the *Bonamia* population may therefore be due to endogenous rather than exogenous factors, with death occurring before sporulation takes place.

If *Bonamia* sp. is a virally infected, possibly transformed, cell, is there any evidence of viruses in these cells? Virus-like particles (V-LP) have been reported from *Bonamia* sp. by Hine (1991b), in a haplosporidian in *Ostrea edulis* by Bonami et al. (1985), and in *Mikrocytos mackini* by Farley et al. (1988), none of which is known to sporulate. The status of these V-LPs is unclear, but in the case of *Bonamia* sp. they may have been mis-identified vesicles similar to those observed in MVBs. Viruses contain nucleic acid and in *Haplosporidium nelsoni* several DNA masses and one RNA mass occur in the cytoplasm (Myhre 1969), and the MVB from which haplosporosomes form are Feulgen (DNA) positive (Perkins 1979). Haplosporosomes may also resemble viruses, such as those of *H. costalis* reported by Perkins (1979: Figs. 12 & 13) and rhabdoviruses, and if MVB do contain DNA, they are virus-like in composition. However, the most suspect particles are H-LB as they have a virus-like core of nuclear material and a ribonucleoprotein coat, they are common in *Bonamia* sp. and *H. nelsoni*, they are formed in CC in *Bonamia* sp., and they proliferate in moribund *Bonamia*. They are therefore virus-like particles in direct association with host cell pathogenesis.

In conclusion, *Bonamia* sp. contains CC and, more significantly, CCC, which in mammals can only be induced by interferon when there is a viral genome in the DNA of the host cell. These structures participate in the formation of H-LB which have a virus-like structure and are associated with moribund *Bonamia* in July and August. *Bonamia* sp. and *H. nelsoni* (MSX) both form H-LB, both may contain CCC, and both appeared suddenly to cause mortalities in oyster fisheries. Considering the economic impact of these 2 pathogens, further TEM studies need to be carried out on the relationship between cytoplasmic structures and nucleic acid distribution.

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