

Occurrence and characteristics of the haemocyte parasite *Bonamia* sp. in the New Zealand dredge oyster *Tiostrea lutaria*

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ABSTRACT: Bonamiasis, first detected in the New Zealand dredge oyster *Tiostrea lutaria* (Hutton) during the southern autumn of 1986 was investigated through 2 surveys of oyster beds carried out over spring (Sep 1986) and summer (Jan 1987). In spring, a 'lighter' form of the parasite occurred most frequently, confined largely to the sub-epithelial tissue. In summer, a 'dense' form, nearly identical to the 'classic' European and American *Bonamia ostreae*, was the dominant type; it occurred in sub-surface tissue around the digestive diverticula and gut, and invaded gonadal follicles and gill filaments. Other forms, such as a second type of the 'dense form' and what appeared to be intermediate stages between the 'lighter' and 'dense' types were also detected. The significance of these different types in the life cycle of the parasite is discussed. The New Zealand species shows distinct size and morphological differences from *B. ostreae*.

INTRODUCTION

About the end of autumn (May to July) 1986, oyster-men reported gaping, dead or moribund oysters and heavy mortalities (~63 %) in their catches of wild populations of the native dredge oyster *Tiostrea lutaria* (Hutton 1873) in Foveaux Strait, New Zealand, particularly from the western beds from April to June 1986. Microscopic examination revealed intense haemocyte infiltration of connective tissue around gut, digestive diverticula of and supra-branchial areas. Also present in many oysters were intracellular parasites, 3 to 5 µm in size, within granular haemocytes throughout the connective tissue. Fine structure studies of the parasite were therefore carried out, and these indicated that the organism was an Ascetosporan very similar to *Bonamia ostreae*, described by Pichot et al. (1980) in the European flat oyster *Ostrea edulis*. Material subsequently obtained from 2 surveys of the oyster beds carried out in September 1986 and January 1987, provided further information on the morphology and pathology of the parasite.

This paper reports the first results of our observations on the parasite, its fine structure and variation of form, together with the histopathology.

MATERIAL AND METHODS

The first sample (ca 20 specimens) came from the western beds of Foveaux Strait (Fig. 1) in June 1986, and was used for preliminary diagnosis. Later, a detailed survey of the beds was carried out in September 1986 to assess the extent and effects of the disease, and more than 1200 oysters were collected from 24 stations covering a wide area. The survey was repeated in January 1987 when a similar coverage and sampling strategy was adopted, but some 'hot spots' were covered by duplicate tows. For light microscopy, an oblique section, about 3 mm thick, was cut from below the labial palps and across the body of the oyster, and fixed in Davidson's fluid and routinely processed for histology. Sections were cut at about 5 µm and stained with eosin and haematoxylin; for confirmatory diagnosis, Lendrum's phloxine-tartrazine stain (Lendrum 1947) was found to be extremely useful. For TEM, thin slices of tissue were fixed routinely in 2.5 % glutaraldehyde buffered in 0.1 M cacodylate (pH 7.5) (though some of the early samples were fixed in 2 % glutaraldehyde buffered with filtered seawater) post-fixed in Os₂O₄ and stained with uranyl acetate/lead citrate and examined in a Philips 201 EM.

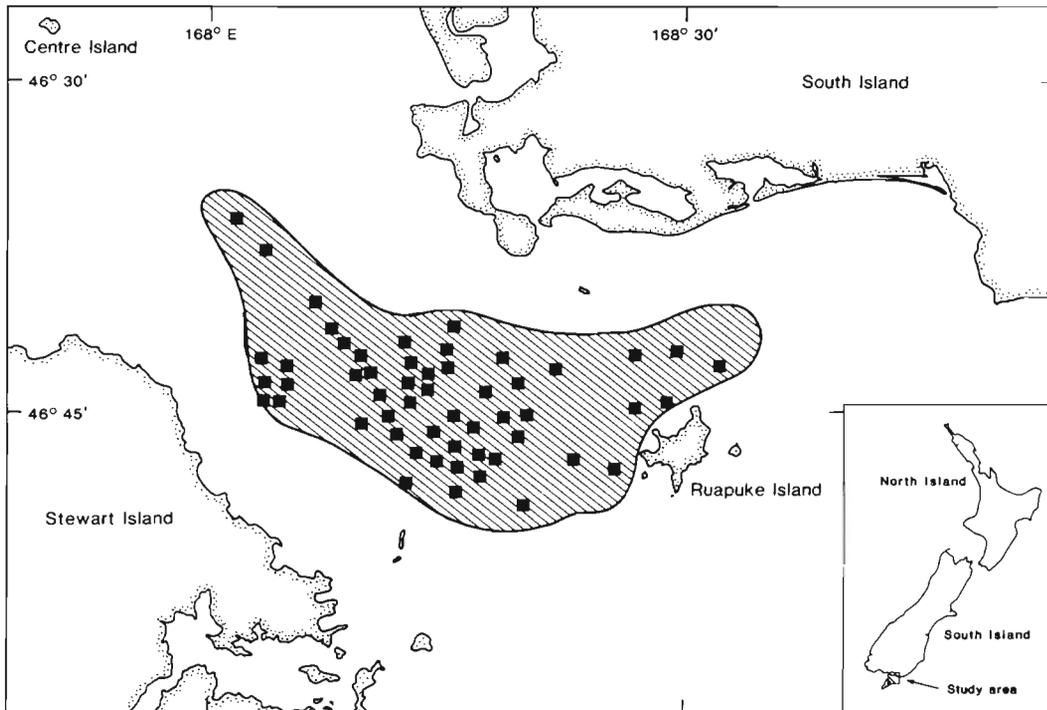


Fig. 1. Foveaux Strait, New Zealand, showing the location of major commercial oyster beds (shaded area) and sampling stations (black squares)

RESULTS

A characteristic histological feature of *Tiostrea lutaria* infected with the parasite was the dissociated appearance of the sub-epithelial layer of mantle connective tissue (Fig. 2) which was infiltrated by groups of parasitized granular haemocytes. Haemocytes had an eccentric nucleus and usually 1 or 2, but up to 10, parasites occurred in the cytoplasm. The parasite was basophilic, spherical or ovoid, 3 to 5 μm in diameter, with a rounded nucleus. In medium to heavy parasitic infections, several ruptured granulocytes containing remains of pycnotic nuclei and cell debris, with the parasites lying free in connective tissue, were observed. In the June and September 1986 samples, parasitized haemocytes were found only occasionally in sub-surface blood sinuses and in the Leydig tissue around the viscera, or invading gonadal follicles.

In the January samples, however, the oysters had heavy infections in connective tissue, around the gonad, the follicular lumen, the digestive diverticula and intra-branchial spaces. A large number of parasites were extracellular in sub-epithelial blood sinuses and in the Leydig tissue. Parasites around basal lamina of digestive tubule epithelia (Fig. 3) were only occasionally found to penetrate the lamina and to lie within or between epithelial cells. Hyalinocytes were not infected, though in most parasitized oysters there was a marked reaction, characterized by dense proliferation

and infiltration of haemocytes in the connective tissue around the viscera (Fig. 4).

The oyster showed no external symptoms of parasitosis though, occasionally, emargination and scalloping of the gill lamellae were observed in a few oysters. In 2 oysters taken in January, the gills were frayed and badly eroded and histology revealed infiltration of gill tissue by *Bonamia ostreae*.

Fine structure

'Lighter forms'

In the June and September samples, the most frequently observed form of the parasite was an electron-lucent rounded or ovoid body, 3 to 5 μm in diameter, referred to as the 'lighter' form. It was bound by a unit membrane as well as a plasmalemma of similar thickness of the host cell, to give it the appearance of a double-membrane bound body (Fig. 5). A large nucleus (1.5 to 2.8 μm), coarsely granular, with a distinct peripherally located nucleolus (Fig. 6a) was present. The nuclear outline was occasionally irregular and was often punctuated by dense granular material close to or outside the nuclear membrane. Occasionally a section of the endoplasmia reticula (ER) cisterna was juxtaposed to the outer nuclear membrane and a large dense body in the cytoplasm. A bundle of microtubules was frequently found within the nucleus.

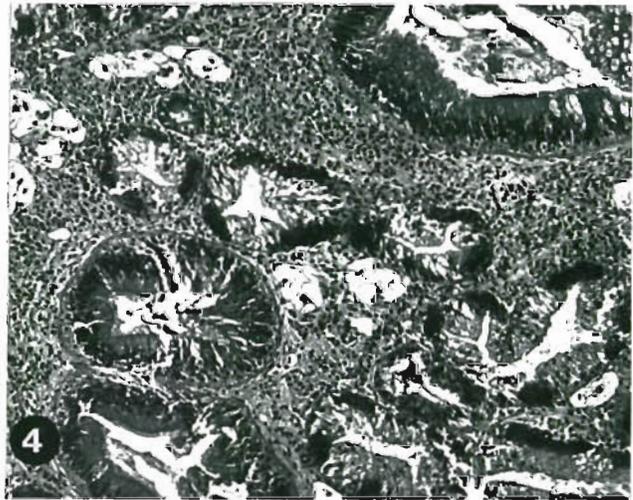
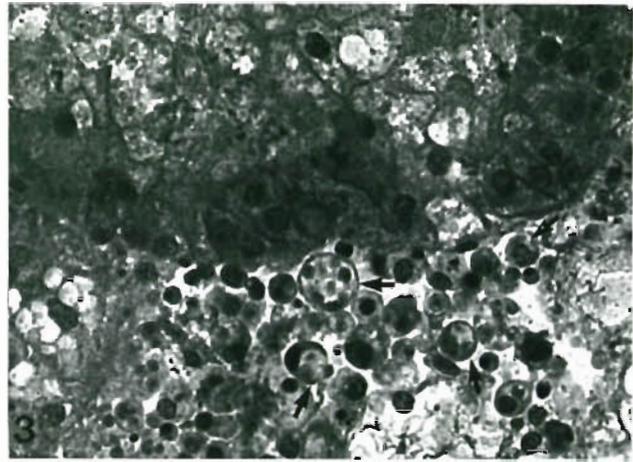
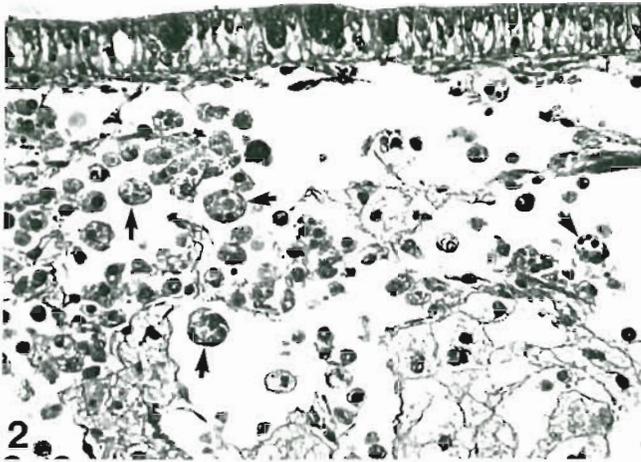


Fig. 2 to 4. *Tiostrea lutaria*. Micrographs. Fig. 2. Sub-epithelial layer of mantle showing *Bonamia ostreae*-infected haemocytes (arrows); $\times 750$. Fig. 3. *B. ostreae*-infected granulocytes (arrows) around digestive tubule; $\times 750$. Fig. 4. Hyalinocyte infiltration of Leydig tissue around digestive diverticula; $\times 750$

Usually, a single large vesicular mitochondrion, up to $1.8 \mu\text{m}$ across, was present near the nucleus; it was typically saucer-shaped and partly curled around the nucleus, with a few tubular or shelf-like cristae. In the majority of the lighter forms, a few ER cisternae were present close to the periphery, and occasionally arrays of flattened cisternae occurred close to dense bodies and vesicles and may have constituted Golgi bodies.

Haplosporosomes were characteristically present, scattered throughout the cytoplasm, and appeared to be of 2 types: the most common was an electron-dense spherical body, 80 to 100 nm, with an electron-lucent ring around it; the other type was rounded, 100 to 130 nm in diameter, had a less dense core and a lighter cortical zone, with a distinct electron-dense ring separating the 2 layers. The latter appeared to be associated with Golgi or ER cisternae. A single large globule, granular body or inclusion in the cytoplasm, 400 to 700 nm in diameter, was found in most lighter forms. It was not membrane-bound, but occurred close to Golgi lamellae and the mitochondrion.

Several intermediate forms, varying between uni-

nucleate bodies with intranuclear microtubules to diplokaryote stages (Fig. 6) and bipartite bodies were frequently observed. The diplokaryotic stage appeared to have heterochromatin in the daughter nuclei, with a layer of electron-dense material between the 2 outer nuclear membranes separating the daughter nuclei and a section of ER membrane (Fig. 6), and with more than one mitochondrion. Daughter nuclei also had prominent nucleoli.

'Lighter' forms were not detected in any of the oysters examined in January.

'Dense' forms

These were often spheroid or irregularly ovoid cells, 3 to $>5 \mu\text{m}$ in size, with a more granular and electron-dense cytoplasm, which were found only rarely in June and September samples, but constituted the dominant and common forms in the January sample.

Two broad types of the dense form have been identified: (1) the 'classic dense' type, that occurred in the

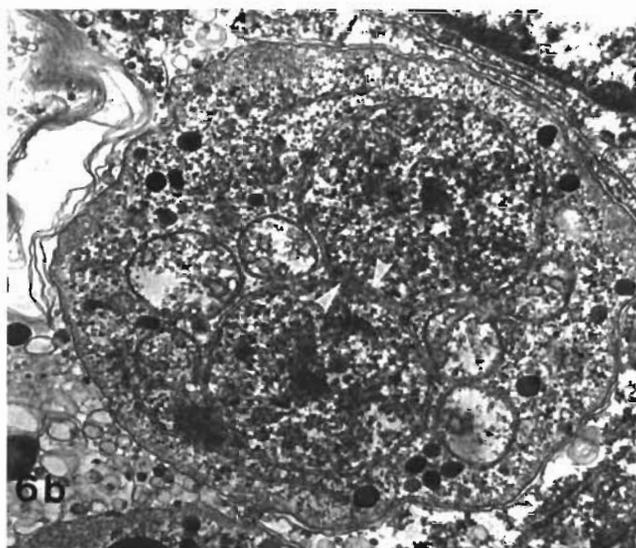
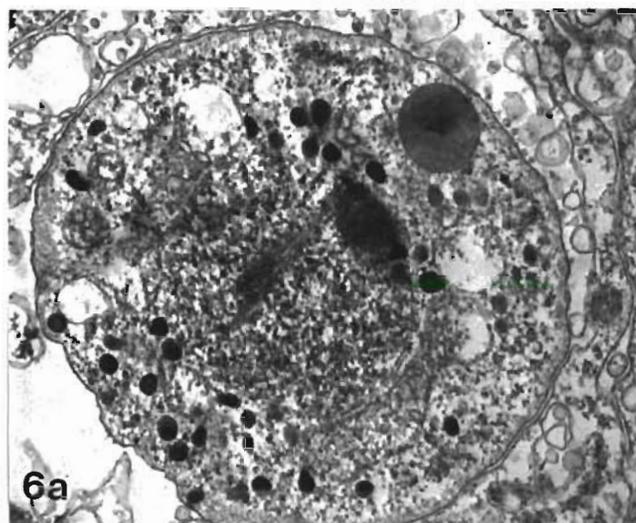
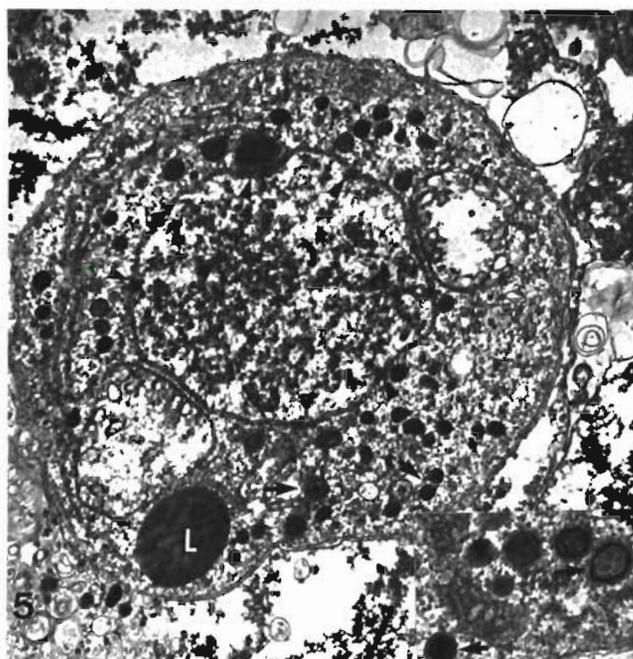


Fig. 5 & 6. *Bonamia ostreae*. Electron-micrographs of 'lighter' form. Fig. 5. Note haplosporosomes (arrows), large globule (L), and sites in nuclear membrane (arrowheads) where dense granular material passes into cytoplasm; $\times 19000$; inset: 2 types of haplosporosomes (arrows); $\times 21000$. Fig. 6. (a) Intranuclear microtubules; (b) divided nucleus with electron-dense material (arrowheads) between daughter nuclei; $\times 26000$

January sample, and (2) a second type ('Type 2'), which only superficially resembled the 'classic' form but showed peculiar features, that occurred mostly in the September sample.

'Classic' dense form. The parasite, 2 to 3 μm in diameter, had a clearly defined outer cell membrane, an eccentric nucleus, with a peripheral nucleolus, and large vesicular mitochondria with annular cristae (Fig. 7). In addition to a small number (10 to 13) of haplosporosomes, 1, or occasionally 2, large dark globular bodies (500 to 600 nm) were usually present. The cytoplasm was richly granular, except for a lucent fringe around the perimeter, and a few ER cisternae were detected.

Less common 'dense' form (Type 2: Fig. 8). This was observed occasionally in the June and September samples; generally it had a somewhat fuzzy outer cell membrane, which appeared uneven, and the

peripheral cytoplasm was demarcated either as a narrow band of dense granular material or as a lighter 'ectoplasm' zone of fine granular structure. The nucleus was large and had aggregates of chromatin material and occasionally contained several nucleoli. Closely surrounding the nucleus were long, attenuated mitochondria, usually dumb-bell shaped with club-like ends and string-like middle sections, and with numerous annular cristae arranged concentrically. In sections, the rounded cristae appeared more lucent against a dense matrix. An array of flattened ER cisternae, several layers thick, was often found around one pole of the nucleus in many of the dense forms. The general cytoplasm was coarsely granular, ribosome-rich, and appeared to have a greater number of haplosporosomes than the other forms, mainly of the dense spherical type (80 to 100 nm). A smaller array of flattened cisternae, with electron-dense material

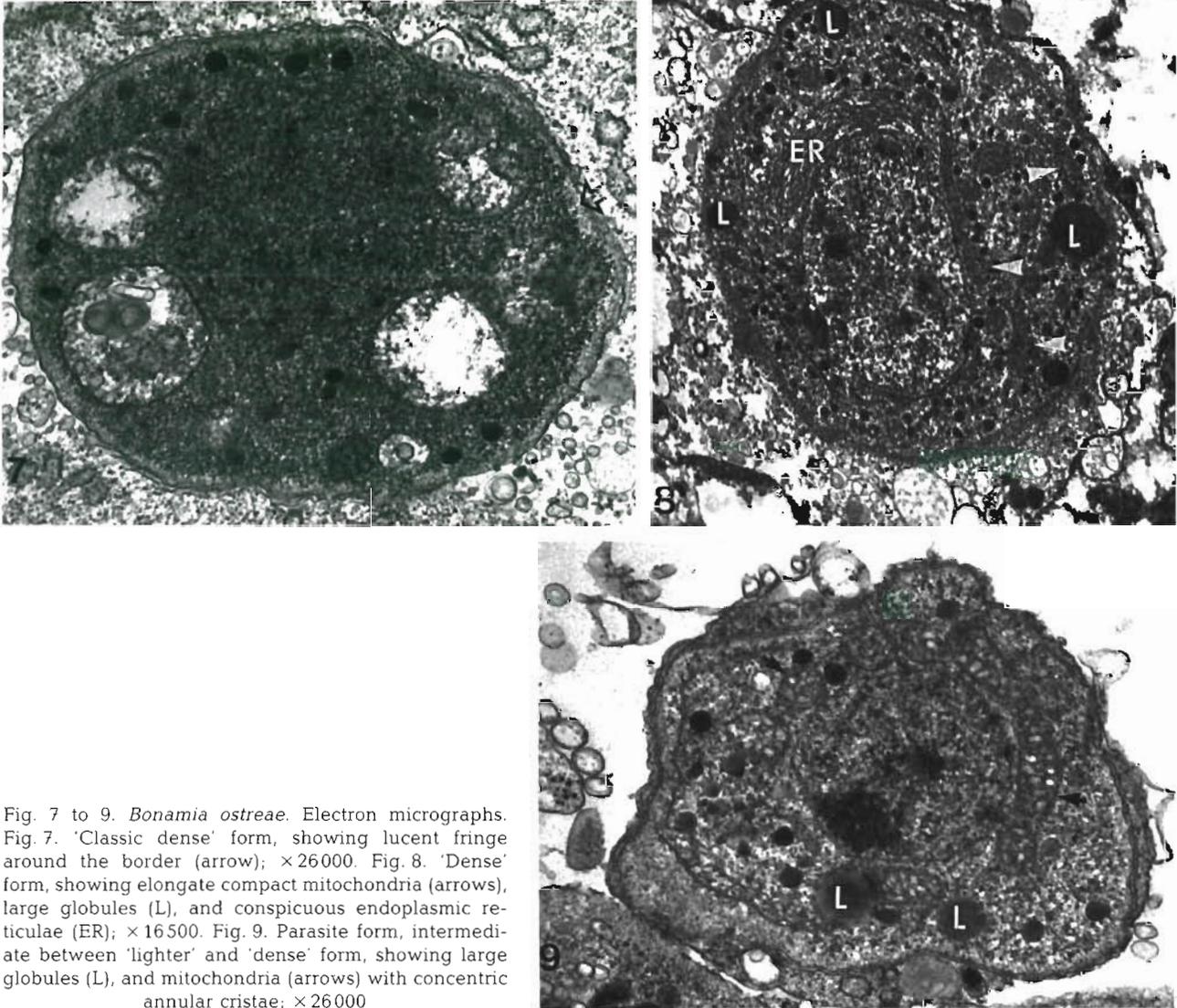


Fig. 7 to 9. *Bonamia ostreae*. Electron micrographs. Fig. 7. 'Classic dense' form, showing lucent fringe around the border (arrow); $\times 26000$. Fig. 8. 'Dense' form, showing elongate compact mitochondria (arrows), large globules (L), and conspicuous endoplasmic reticulae (ER); $\times 16500$. Fig. 9. Parasite form, intermediate between 'lighter' and 'dense' form, showing large globules (L), and mitochondria (arrows) with concentric annular cristae; $\times 26000$

between the lamellae, suggested the site of a Golgi body. Another characteristic of these dense forms was the presence of more than one large globule 300 to 700 nm in diameter, and of varying electron density, scattered in the cytoplasm.

Variation

Variation in the morphology of the dense form was common and appeared to be related to changes within the cytoplasm. When the cell outline was irregular, the cortical layer of the cytoplasm appeared as a lucent fringe and there was an increase in the number of large globules; whether these represented structural characteristics or anomalies is not certain. In some of the dense forms observed in the January sample, the mitochondria

were less compact (Fig. 9) and had well-defined annular cristae surrounded by a denser matrix. Some of these forms appeared to be intermediate in character between the lighter form and the classic dense form.

Host cell

Non-parasitized blood granulocytes had a large ovoid nucleus, numerous rounded mitochondria placed in a group close to the nucleus, with tubular cristae set in a dense matrix, and a large area of lucent cytoplasm occupied by vesicular structures, smooth membranes and irregular granules (Fig. 10). Granular haemocytes harbouring the parasite were enlarged, with the nucleus shifted peripherally, and often irregular in outline. An endocytotic membrane enveloped the parasite and there

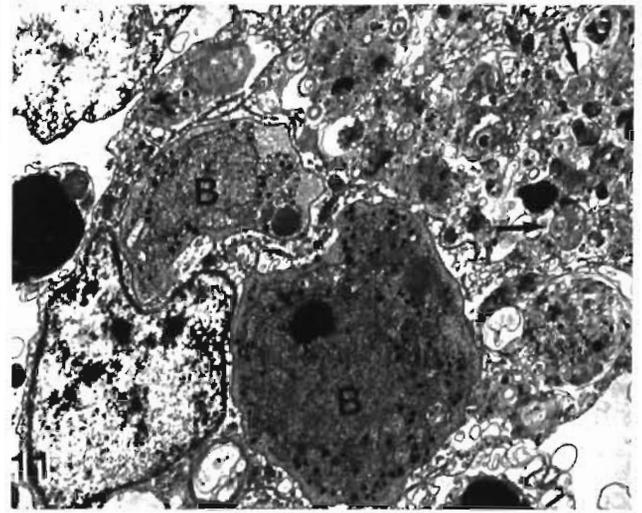
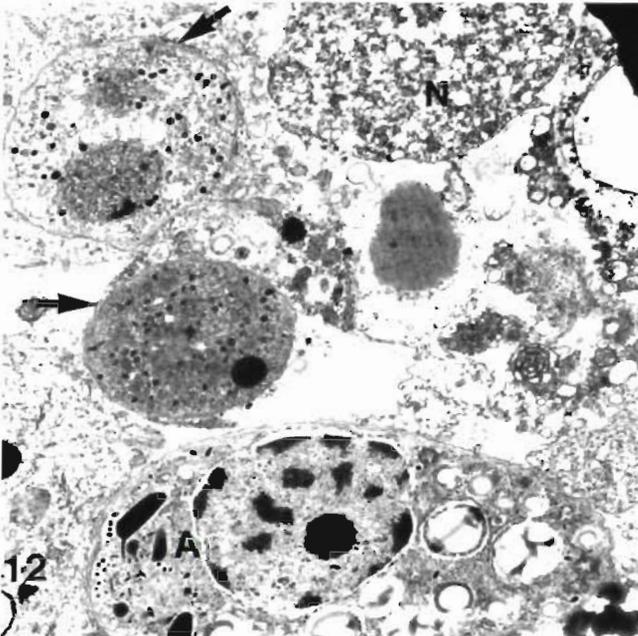
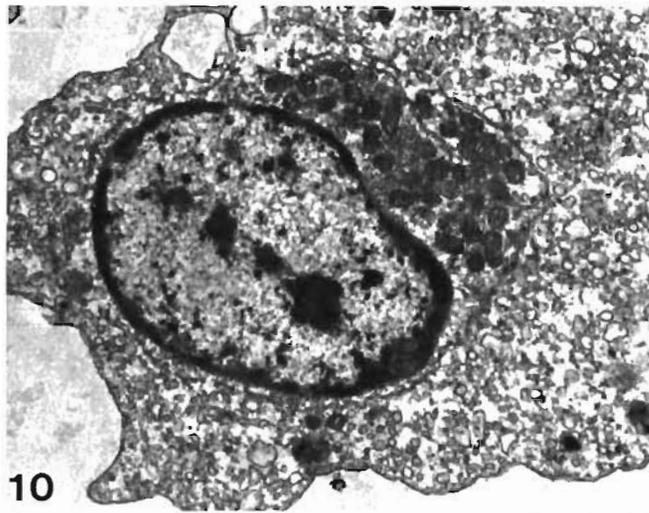


Fig. 10 to 12 *Tiostrea lutaria*. Electron micrographs. Fig. 10. Normal granular haemocyte, showing rich array of mitochondria close to the nucleus; $\times 15000$. Fig. 11. Partially degenerating granulocyte, showing 2 parasites (B), and debris of cell organelles and myelinated structures (arrows); $\times 15000$. Fig. 12. Infected granulocytes, showing host cell nucleus (N), and 2 *Bonamia ostreae* (arrows) and a merozoite of an Apicomplexan (A); $\times 15000$.

was a decrease in the number of mitochondria and smaller vesicles. In heavily infected granulocytes, the cytoplasm appeared coarse and contained a large number of irregular vesicles, globules and myelinic figures (Fig. 11). Several granulocytes contained apparent debris of phagocytosed parasites and others appeared to be undergoing degenerative changes. In the January samples, several *Bonamia*-infected haemocytes were also invaded by merozoite stages of an apicomplexan (Fig. 12), which will be described elsewhere.

DISCUSSION

The material examined in this study included morphologically distinct forms and probably developmen-

tal stages of the parasite as well. During the September (early spring) survey, the dominant type of the parasite was a 'lighter' form, nearly identical to the type recorded for *Bonamia ostreae* (Pichot et al. 1980, Balouet et al. 1983), which we regard as a vegetative intracellular phase that undergoes multiplication by binary fission within the granulocyte. This phase of the parasite was largely restricted to sub-epithelial spaces and superficial blood sinuses. The frequent occurrence of intranuclear microtubules, dumb-bell shaped nuclei and mitochondria, together with diplokaryote stages and recently divided cells, points to a rapidly dividing vegetative phase consistent with *B. ostreae* (Balouet et al. 1983). In contrast, the January (mid-summer) survey revealed a denser form, very similar to the common and frequently occurring type described for *B. ostreae*

(Comps et al. 1980, Pichot et al. 1980, Balouet et al. 1983), and which was the most dominant and often the only form of the parasite present in the dredge oyster.

Bonamia ostreae at this stage had penetrated deeper into connective tissue surrounding the gonad follicles and the digestive diverticula, as well as the gut and the gills. In light microscope preparations, larger numbers of the parasite were found 'free' in the Leydig tissue, in blood sinuses and follicular lumen. Thus, between one season and the next, there was not only a change in the form of the parasite (from 'lighter' forms to 'dense' forms) but a shift from superficial locations to deeper sites of underlying tissue. Whether this represented a response to changes in host physiology or is a developmental phase of the parasite itself remains unclear. In the host, however, following spawning in spring, there is a resorbing phase in mid-summer (December/January) when large numbers of phagocytes invade gonadal follicles and clear away unshed gametes. The mobilization of large numbers of granulocytes in this process in the oyster could account for the presence of parasitized haemocytes also within the follicles and its transport to other tissue through blood vessels and sinuses. The 'dense' forms of the parasite may represent an infective stage when food reserves are being built up. Though a spore stage has not been identified, the presence of free (exocytotic) stages of the parasite, in association with identifiable excretory sites of the host, may suggest a pre-sporulation or an infective phase. Stages of the parasite, for example, have been detected occasionally in the digestive tubule lumen and in outer gill epithelium, whence the parasite may be voided to the outside.

The occurrence of a 'dense' form and a few other forms that appear to be intermediate in character between the classic 'dense' form and the 'lighter' form awaits further study in order to understand their significance to the parasite's life cycle. Some 'dense' forms appear to have accumulated reserve material and active organelles: the mitochondria are elongated, compact, often dumb-bell shaped, with well formed cristae set in a dense matrix; they are placed close to the nucleus and the ER cisternae, to suggest a high level of metabolic activity; the ER is often several lamellae thick and there are 3 or more large dense bodies/inclusions in the cytoplasm which appear to have different types of reserve material.

Conversely, it remains to be verified whether changes seen in a few 'dense' forms (Type 2) are indicative of degenerative effects, probably as a result of host-cell reaction. A similar dense form in a closely related haplosporidan parasite in *Ostrea edulis*, described by Bonami et al. (1985, 'F4 stage'), has been considered by the authors as probably a degenerative stage. Bonami et al. (1985) have suggested that the

'dense' form could probably represent a spore stage, very similar to the dense plasmodial stage ('P4') of the parasite, found in the gill epithelia. They put forward the view that degenerative changes taking place in the 'dense' forms ('F4' and 'P4') are probably a result of viral infection and hyperparasitism, and point to this as an example of fragility in the parasite life cycle. We have not detected any sign of viral activity in our material, and it is suggested that host resistance and reaction may also account for some degenerative changes.

The absence of a spore stage in *Bonamia ostreae* and presumed closely aligned haplosporidan parasites of oysters has posed problems in the classification of these organisms within the Ascetospora. Bonami et al. (1985) suggest that it is possible that developmental cycle does not include a spore stage in these species, though as Elston et al. (1986) suggest, cryptic stages may be present, especially in cases where there are inflammatory lesions and haemocyte proliferation in the oyster, but no sign of the parasite.

In general, the possession of haplosporosomes, the division by endomitosis and the presence of diplokaryotic cells are characteristics which the New Zealand species shares with other ascetosporans and *Bonamia ostreae*. Also, similarities in form, size, morphology and fine organelle details exist between the parasite found in the New Zealand oyster and *B. ostreae* described in the flat oyster *Ostrea edulis* in Europe and in America. However, differences exist between the New Zealand form and *B. ostreae*, in certain key organelles: number of haplosporosomes and large globules, morphology of mitochondria, smaller nucleus:cytoplasm ratio. There are also changes of form from one season to the next, with the domination of the 'lighter' form in the spring in the New Zealand species, which is in contrast with the European species (Pichot et al. 1980, Balouet et al. 1983).

For the present it is proposed to treat the New Zealand species distinct from *Bonamia ostreae*. Recent evidence (Elston et al. 1986) suggests that the disease originated from *Ostrea edulis* stock in the USA and spread to Europe; how and when it came to affect natural populations of New Zealand oysters is not yet clear.

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