Vol. 51: 49–60, 2002 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published August 15

Severe apicomplexan infection in the oyster *Ostrea chilensis*: a possible predisposing factor in bonamiosis

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ABSTRACT: Histological examination of 6455 oysters *Ostrea chilensis* from Foveaux Strait south of New Zealand over a 5 yr period showed >85% contained apicomplexan zoites, irrespective of season. Zoites occurred around the haemolymph sinuses and the digestive diverticulae at all intensities of infection; occurrence in the sub-epithelium, Leydig tissue and gills/mantle increased with increasing intensity of infection. Many (>35%) oysters were heavily infected, and most of them had severely damaged tissues. Heavy infections affected gametogenesis; 1% of lightly infected oysters had empty gonad follicles lacking germinal epithelium compared with 2% of moderately infected oysters and 9% of heavily infected oysters. Of oysters with empty gonad follicles, 75% were heavily infected with zoites. The parasite spread from the haemolymph sinuses and moved between Leydig cells, causing their dissociation and lysis. Some zoites were intracellular in Leydig cells. Lesions contained many haemocytes phagocytosing zoites, leading to haemocyte lysis and causing a haemocytosis. Fibrosis occurred to repair lesions in a few oysters. The zoites had a typical apical complex with 2 polar rings and 84 sub-pellicular microtubules. Prevalence and intensity of concurrent *Bonamia exitiosus* infection was related to the intensity of zoite infection, with only 3.8% of *B. exitiosus* infections occurring in the absence of zoites, 20.0% occurring in light zoite infections, 30.9% in moderate zoite infections, and 45.4% when oysters were heavily infected with zoites. The converse was not the case, as 75.3% of zoite infections occurred in the absence of *B. exitiosus* infection, including 51.1% of moderate to heavy zoite infections. There was a statistically significant association between intensities of *B. exitiosus* and of zoites ($p < 0.0001$). Zoites may increase the susceptibility of oysters to *B. exitiosus* by occupying and destroying haemocytes, and by destroying connective tissue cells and utilising host glycogen reserves. The parasite may be heteroxenous, with other stages in the terebellid polychaete *Pseudopista rostrata.*

KEY WORDS: Apicomplexan · Zoites · Oysters · *Ostrea chilensis* · *Bonamia exitiosus* · Susceptibility · Gametogenesis

INTRODUCTION

There have been few descriptions or reports of apicomplexans in bivalves, although the infrequent pathogenicity of this group may have resulted in underreporting of these organisms. *Pseudoklossia* spp. infects the digestive glands of polyplacophorans (Debaisieux 1919) and the kidneys (Léger & Duboscq 1915, 1917, Debaisieux 1919, Tigé et al. 1977, Leibovitz et al. 1984, Bower 1992, Rekkarinen 1993, Desser & Bower 1997a,b, Desser et al. 1998) and ovagonia (Buchanan 1979) of various bivalves. Apicomplexan-like organisms have also been reported from the intestinal epithelium of clams *Tapes philip-*

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pinarum by Bower et al. (1992), scallops *Argopecten irradians* by Whyte et al. (1994), and oysters *Crassostrea virginica* by Meyers (1981). Azevedo (1989) described merozoites of *Cryptosporidium* sp. from the gills of clams *Ruditapes decussatus*. Morado et al. (1984) reported an unidentified coccidian from the kidneys of clams *Protothaca staminea*, and subsequently Desser & Bower (1997a,b) erected a new genus, *Margolisiella*, to accommodate the monoxenous parasite in *P. staminea*. They moved 4 monoxenous *Pseudoklossia* spp. into the new genus, thus separating them from heteroxenous *Pseudoklossia* spp. (Desser & Bower 1997b). *Margolisiella tellinovum* (Buchanan 1979, Desser & Bower 1997b), *M. haliotis* (Friedman et al. 1995, Desser & Bower 1997b), *Pseudoklossia glomerata* (Léger & Duboscq 1915), *Cryptosporidium* sp. (Azevedo 1989) and *M. kabatai* (Morado et al. 1984, Desser & Bower 1997a,b), may cause serious damage to their hosts.

Mass mortalities occurred among oysters *Ostrea chilensis* in Foveaux Strait on the south coast of New Zealand from late 1985 to 1993. The epizootics reduced the population of commercial-sized oysters (>58 mm diameter) by 91% of the stocks present in 1975 (Doonan et al. 1994). Additional to *Bonamia exitiosus*, the suspected primary pathogen, oysters were parasitised by an apicomplexan (Hine 1991a). The present study was conducted because subsequent examination showed that oysters lightly infected with, or free of *B. exitiosus*, but heavily parasitised by the apicomplexan, suffered severe tissue damage. The tabulated data presented here were taken from the same oysters as those examined for *B. exitiosus* (Hine et al. 2001) by Hine (1991a), with further general observations on the apicomplexan in 4710 oysters.

MATERIALS AND METHODS

Samples of dredged oysters *Ostrea chilensis* were collected for detailed examination at irregular intervals between September 1986 and May 1989 (see Table 1), and samples of a further 4710 oysters taken between September 1986 and June 1991 were examined for the presence or absence of coccidians. Oysters were opened, and a 3 mm section across the body was fixed in Davidson's fixative, processed for light microscopy and stained with haematoxylin and eosin (H & E), periodic acid-Schiff (PAS), or Lendrum's phloxin-tartrazine (Lendrum 1947). Slides for detailed examination were scanned at ×390 and the presence or absence of the apicomplexan was recorded for each tissue. Each infected oyster was classified into one of 5 grades of intensity of infection: Grade 1—light; a few (<5) parasites present, only observed after extensive searching of the section. Grade 2—light to moderate; a few parasites observed in most tissues. Grade 3—moderate; parasites readily observed, especially around the haemolymph sinuses. Grade 4—moderate to heavy; parasites abundant in all tissues, and many lesions evident. Grade 5—heavy; parasites abundant and tissues congested, connective tissue (Leydig) cells (CTC) destroyed.

To study the effects of the parasite on reproduction and gametogenesis, gonads were classified into 5 groups: Group A—male gonads ranging from those containing epithelial spermatogonia, free spermatogonia, spermatocytes and spermatozoa and a few epithelial ovagonia to gonads with no female elements but packed with spermatozoa. Group B—spawned male gonads, empty except for a few spermatozoa and/or phagocytes, sometimes with a few epithelial ovagonia; no free ovacytes. Group C—female gonads with free ovacytes and ova, with a few residual spermatocytes often present between ova. Group D—spawned female gonads, empty except for fragments of ova or residual eggs, and phagocytosing haemocytes; epithelial spermatogonia often present. Group E—follicles empty, neither spermatogonia nor ovagonia present, and germinal epithelium sometimes not apparent.

Data on the intensity of concurrent *Bonamia exitiosus* infection in relation to reproductive stages was scored as detailed in Hine (1991a).

In most months, a few (<8%) oysters contained abnormally proliferative spermatogonia, and they were excluded here. These were diagnosed by the Registry of Tumors in Lower Animals (RTLA), Smithsonian Institution, Washington, DC, as seminomas/dysgerminomas (RTLA Accession Nos. 5161 to 5164). Sterile oysters containing sporocysts of *Bucephalus* sp. (<2% mo^{-1}) were also excluded.

Only 1 developmental stage of the apicomplexan was observed in oysters, and therefore possible intermediate hosts were considered. The only invertebrate living abundantly and in proximity to infected oysters was the polychaete *Pseudopista rostrata* Hutchings & Smith, 1997 (Terebellidae), which lives in mud tubes on the oyster valves and surrounding structures. Therefore, 50 *P. rostrata* were collected from an infected farm site and processed for routine H&E histology.

For transmission electron microscopy, small (1 mm^3) samples of oyster digestive gland were fixed for electron microscopy in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 to 2 h, washed twice in buffer, post-fixed for 1 h in 1% OsO_4, sectioned, stained for 10 min in 5% uranyl acetate and for 5 to 6 min in 5% lead citrate, and examined on a Philips 201 or Philips 420ST electron microscope.

Table 1. *Ostrea chilensis.* Prevalence, intensity and tissue distribution (% occurrence) of zoites in monthly samples

	Month/year									
	9/86	1/87	4/87	5/87	6/87	8/87	11/87	4/88	12/88	5/89
No. of oysters examined	198	196	100	105	180	197	198	194	182	195
Prevalence (%)	94	96	97	98	96	97	98	97	99	98
Mean intensity	2.9	2.8	3.2	3.0	3.1	3.5	2.5	3.1	3.2	2.6
Mantle/gills	27	29	62	41	39	39	16	44	69	50
Suprabranchial and around kidneys	89	80	92	83	87	93	81	90	97	90
Sub-epithelial	58	56	80	72	58	79	63	78	83	70
Gonad	0	0	2	3	0	0	0	0	0	0
Kidney wall or lumen	4	0	12	20	0	0	0	2	0	2
Haemolymph sinus and diverticulae	97	96	100	95	97	99	99	98	98	93
Around lower gut	29	28	42	29	28	48	37	53	57	34

Table 2. *Ostrea chilensis.* Distribution (% occurrence) of zoites in different areas of the oyster at different levels of intensity (Grades 1 to 5) of infection

Intensity grade	Mantle/gills	Suprabranchial kidneys	Sub-epithelia	Haemolymph sinus diverticulae	Around lower gut
1	3	44	15	87	8
2	18	79	36	100	14
3	31	91	74	99	38
4	66	97	89	100	58
5	81	100	99	100	84

RESULTS

Occurrence and tissue distribution

The only apicomplexan developmental stage observed in the oysters consisted of elongated bodies (4.3–5.3 × 6.2–9.8 µm, n = 50) resembling merozoites and sporozoites. As their genesis by merogony or sporogony was not observed, they are herein called zoites (Lee et al.1985). Prevalence ranged between 94% in September 1986 and 99% in December 1988 (Table 1), and was never <85% in 4710 other oysters examined from field sites between 1986 and 1990. There was no apparent trend in the mean intensity of infection (Grades 1 to 5; Table 1). Tissue distribution showed similar prevalence in connective tissue cells (CTC) around the suprabranchial sinuses and kidneys, and the haemolymph sinuses and digestive diverticulae in all months (Table 1). Excluding gonad and kidney, occurrence in other tissues was related to intensity of infection (Tables 1 & 2); for example, the relatively low prevalence in gills in November when intensity was lowest. Kidney epithelium and gonad infections were only observed in April, May and September (Table 1).

Effect on reproduction and gametogenesis

The reproductive pattern of *Ostrea chilensis* observed over the sampling period is shown in Table 3. Oysters were developing as females in January (1987), but had spawned or ovacytes had been absorbed by April (1987 and 1988) and May (1987 and 1989), when ovaries contained haemocytes phagocytosing residual ova. Male

Table 3. *Ostrea chilensis.* Occurrence (%) of each reproductive group (A to E) in each monthly sample. n: number of oysters sampled

Month/year	n	Groups				
		A	B	C	D	E
9/86	192	70	4	10	11	5
1/87	192	3	55	23	11	7
4/87	92	4	18	9	67	1
5/87	101	16	10	15	54	5
6/87	178	42	3	13	36	6
8/87	195	67	5	12	15	2
11/87	196	35	53	11	0	1
4/88	190	18	6	16	53	7
12/88	178	12	56	20	7	5
5/89	190	46	2	13	38	1

Table 4. *Ostrea chilensis*. Occurrence (%) of each reproductive group (A to E) in relation to degree (Grades 0 to 5) of zoite infection in each monthly sample

Month/year	Absent or light (Grades 0–2)					Moderate (Grade 3)					Heavy (Grades 4–5)				
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
9/86	79	3	11	6	1	82	3	8	3	3	50	5	10	26	10
1/87	1	61	24	9	4	4	49	23	18	6	4	56	22	6	13
4/87	0	5	18	77	0	15	15	7	63	0	0	28	5	65	2
5/87	22	5	16	51	5	11	14	21	54	0	14	11	8	58	8
6/87	48	0	26	26	0	46	2	11	40	2	34	6	6	39	15
8/87	64	9	9	18	0	65	5	15	16	0	69	3	12	13	3
11/87	31	55	14	0	0	36	54	10	0	0	44	47	3	0	6
4/88	21	6	10	60	4	8	7	22	59	3	23	5	15	44	13
12/88	17	51	22	10	0	10	61	19	10	0	12	54	21	3	12
5/89	55	3	10	31	0	40	2	14	45	0	37	0	19	42	2

spawning occurred between September (1986) and November (1987), probably nearer the latter, as in November 1987, 35% of oysters were unspawned males. Occurrence of reproductive stages with absent or light (Grades 0 to 2; 32% of total), moderate (Grade 3; 33% of total) and heavy (Grades 4 and 5; 35% of total) zoite infections is shown in Table 4. Although there were differences between lightly and heavily infected groups in some months (less males among heavily infected oysters in September 1986; more post-male spawning oysters among heavily infected oysters in April 1987), the only uniform trend was the greater proportion of Group E oysters among heavily infected oysters. Only 1% of lightly infected oysters had empty (Group E) follicles, and 2% of moderately infected oysters were Group E, but 9% of heavily infected oysters had empty follicles. Overall, 4% of oysters were in Group E and 75% of these were heavily infected with zoites.

Zoite infection in relation to *Bonamia exitiosus* infection

Intensity of concurrent *Bonamia exitiosus* infection was related to the intensity of zoite infection, with only 3.8% of *B. exitiosus* infections occurring in the absence of zoites, 20.0% of *B. exitiosus* infections occurring in light zoite (Grades 1 and 2) infections, 30.9% in moderate zoite (Grade 3) infections , and 45.4% when oysters were heavily infected (Grades 4 and 5) with zoites (Table 5). The converse was not the case, as 75.3% of zoite infections occurred in the absence of *B. exitiosus* infection, including 51.1% of moderate to heavy zoite infections (calculated from Table 5). The mean intensity of *B. exitiosus* was related to the intensity of zoite infection, but the mean intensity of infection with zoites showed less of a correlation with the intensity of *B. exitiosus* (Table 6). However, there was a statistically significant association between intensities of *B. exitiosus* and of zoites (chi-squared test, $p < 0.0001$). Zoites and *B. exitiosus* were never observed together in the same haemocyte.

Table 5. *Ostrea chilensis*. Intensity of *Bonamia exitiosus* (n = 443) infection in relation to intensity of zoite (n = 1722) infection

Zoite intensity	*B. exitiosus* intensity						
	0	1	2	3	4	5	Total
0	117	8	2	2	3	2	134
1	179	27	6	3	6	1	222
2	236	20	8	6	9	2	281
3	441	59	23	23	23	9	578
4	305	45	16	28	23	17	434
5	135	23	8	15	11	15	207
Total	1413	182	63	77	75	46	1856

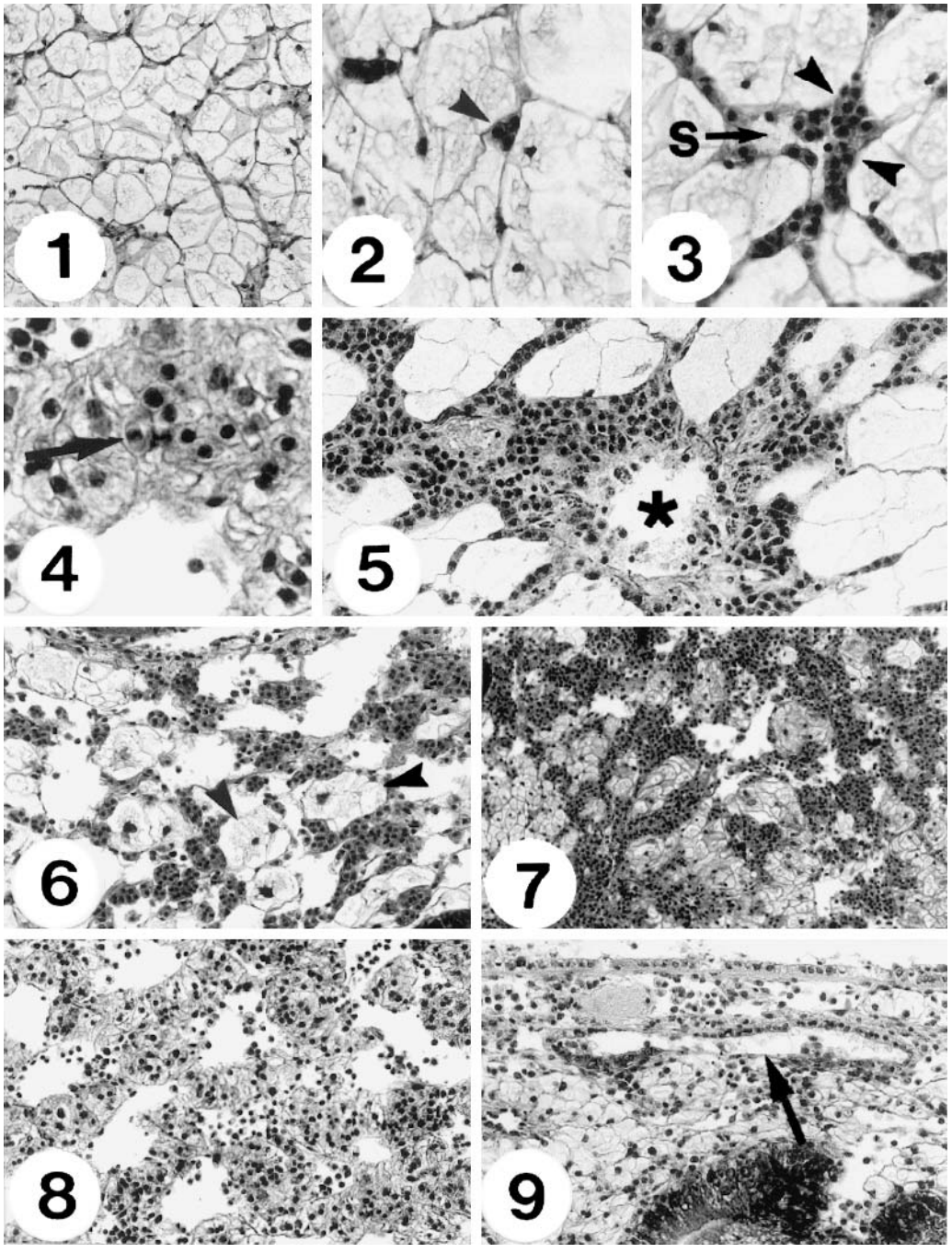
Histopathology

Histological observations were based on zoite-infected, *Bonamia exitiosus*-free oysters. The CTC of healthy oysters are shown in Fig. 1. They are voluminous cells with a small indistinct nucleus and scattered glycogen granules, surrounded by a few haemocytes.

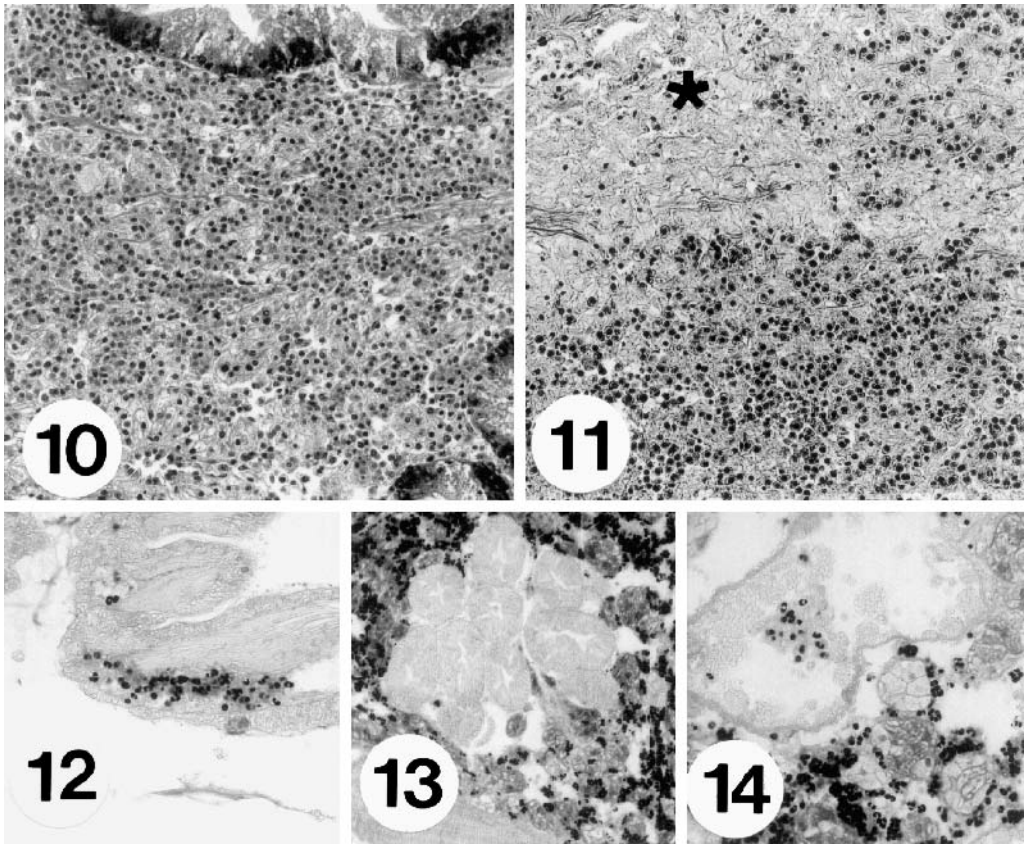
Table 6. *Ostrea chilensis*. Mean intensity (Grades 1 to 5) of each parasite in relation to the intensity of the other

Zoite intensity	Mean *Bonamia exitiosus* intensity	*Bonamia exitiosus* intensity	Mean zoite intensity
0	0.30	0	2.74
1	0.35	1	2.96
2	0.36	2	3.10
3	0.54	3	3.52
4	0.78	4	3.20
5	0.98	5	3.80

At all intensities of infection, zoites were either intercellular between CTC or in small groups within haemocytes. In a few Grade 3 infections and most Grade 4 and 5 infections, zoites were occasionally intracellular in CTC. Schizogony was not observed. In Grade 1 (Fig. 2) and Grade 2 (Fig. 3) infections, zoites were intercellular between CTC, near the haemolymph sinuses only. While most zoites were intercellu-



Figs. 1 to 9. Histology of the development of the apicomplexan infection. Fig. 1. Normal connective tissue cells (CTC) showing their voluminous content and small nuclei (×220); Fig. 2. Grade 1 infection showing small groups of zoites (arrowhead) between the CTC (×375); Fig. 3. Grade 2 infection showing zoites (arrowheads) around a space (S) between CTC (×395); Fig. 4. Grade 3 infection in which zoites are intracellular within CTC (arrow), and some CTC have dissociated, leaving a space (×475); Fig. 5. Grade 3 infection showing zoites spreading from a haemolymph sinus (*) (×285); Fig. 6. Grade 3 infection isolating CTC islets (arrowheads; ×195); Fig. 7. Grade 4 infection showing reduction in islet size (×125); Fig. 8. Empty spaces between CTC in a Grade 4 infection; note the large number of zoites intracellular in CTC (×165); Fig. 9. Empty gonad follicle lacking a germinal epithelium in a Grade 4 infection (×220)



Figs. 10 to 14. *Figs. 10 & 11:* Grade 5 zooite infections; Fig. 10. Almost total replacement of CTC in a Grade 4 infection (×190); Fig. 11. Extensive fibrosis (*) replacing CTC in a Grade 5 infection (×190); *Figs. 12 to 14:* PAS-stained sections; Fig. 12. Gill section showing zoites in gill CTC, but not in epithelium (×170); Fig. 13. Zoites in the digestive gland CTC, but not in digestive epithelia (×80); Fig. 14. Zoites in CTC, and a few in the gonad (×125)

lar, some were intracellular within CTC (Fig. 4). In Grade 3 infections, zoites were numerous around blood sinuses, particularly the suprabranchial sinuses, in a radiating or stellate pattern (Fig. 5), and among the CTC of the sub-epithelium and in the gills or mantle. Haemocytosis occurred in some Grade 3, and all Grade 4 and 5 infections, causing congestion of CTC and haemolymph sinuses by infiltrating haemocytes. In 14% of Grade 3 infections, zoite infiltration around the CTC tissue resulted in their separation into islets (Fig. 6). Infection of the renal epithelium was often associated with epithelial hyperplasia.

In Grade 4 infections (24% of oysters examined) zoites were abundant in and among CTC, and in 83% of sections zoites were associated with CTC damage. In the sub-epithelium and around the gonads, gut and digestive diverticulae, the islets of CTC became progressively smaller as zoite numbers increased (Fig. 7). CTC also detached from each other, leaving spaces that were empty or contained zoites in and around large numbers of haemocytes (Fig. 8). The detachment of CTC appeared to be related to the presence of haemocytes, rather than zoites. Haemocytes containing several zoites had pycnotic nuclei. In Grade 4 and 5 infections, gonad follicles were often empty (Fig. 9), but a few ovagonia or spermatogonia were usually present on the germinal epithelium. The oysters were small and thin compared with uninfected oysters from shells of the same size.

Two patterns were apparent in Grade 5 infections. In ~90% of Grade 5 oysters the CTC had been destroyed, leaving a stroma of necrotic debris, parasites and haemocytes surrounding isolated gonad, gut and digestive diverticulae (Fig. 10). Replete haemocytes with pycnotic nuclei were common. The digestive diverticulae either appeared spongy, with spaces between epithelial cells, or the epithelial cells were flattened. Gonad follicles were often empty and lacked a germinal epithelium. The gills, while congested with parasites, lacked lesions or other damage. Oysters were small, thin and watery.

In ~10% of Grade 4 and 5 infections, damage to the CTC had been replaced by eosinophilic fibrous tissue, binding the gut, digestive diverticulae, empty gonad

follicles and surface epithelium closely together (Fig. 11). There was no indication of subsequent regeneration of CTC. These oysters were small with hardened tissues. Oysters intermediate between the 2 patterns, with fibrosis in some areas, were also common.

The distribution of zoites was most easily determined by PAS staining (Figs. 12 to 14). They occurred in the CTC of the gills (Fig. 12), not in the gill or digestive epithelia (Fig. 13), and seldom in the gonad (Fig. 14).

Ultrastructure

Ultrastructurally, zoites contained all the structures previously reported from apicomplexan zoites. They were elongated and elliptical in outline (Figs. 15 & 16), 7.9 µm (range 6.2 to 9.8 µm) long and 4.9 µm (4.0 to 5.3 µm) wide (n = 50), with a round nucleus occupying almost the whole width of the cell, halfway down its length. The pellicle surface had irregular angular ridges, with 84 sub-pellicular microtubules (Fig. 17) running from the level of the nucleus anteriorly to attach to 2 polar rings. The conoid was not clearly seen (Fig. 18), and pre-conoidal rings were not observed. The rhoptries were angular or squarish at their posterior ends (Figs. 15, 16, & 18). Rhoptries were not serially sectioned and counted, but up to 6 rhoptry ducts passed through the conoid. Micronemes passed from the apical complex (Fig. 18) to near the anterior surface of the nucleus (Fig. 19), but were occasionally posterior to the nucleus (Fig. 16). Flattened Golgi cisternae arose from the anterior surface of the nucleus (Fig. 19), or Golgi-like cisternae with osmiophilic content occurred in association with osmiophilic dense vesicles (Figs. 20 & 21). Although not observed, these dense vesicles may coalesce to form the dense granules. An apicoplast or plastid was not observed. Amylopectin granules, while largely posterior, were also observed anterior to the nucleus (Fig. 15). Hollow thick-walled structures (Fig. 20) were frequently seen posterior to (and less frequently anterior to) the nucleus (Fig. 16). They may be sections through 1 or more mitochondria, but they could not be identified. The micropore, situated next to the nucleus, consisted of thickened outer and inner layers of the pellicle (Fig. 22). An osmiophilic mass was common on the surface of intracellular zoites (Fig. 23).

Small areas of heterochromatin, and granular heterochromatin, were scattered throughout the nucleus (Figs. 15 & 16). Intrahaemocytic zoites sometimes contained extranuclear centrocones and microtubules (Fig. 24), suggesting schizogony by semi-open pleuromitosis, but schizogony was not observed. Bundles of intranuclear microtubules (Figs. 15 & 16), 25 nm in diameter, were also frequently observed. Intrahaemocytic zoites lay directly in the cytoplasm, which appeared ragged, and when haemocytes were replete with zoites, the nucleus was compressed and pycnotic (Fig. 25). Several zoites were seen in CTC, but were never tightly packed, as in haemocytes (Fig. 26).

Examination of polychaetes

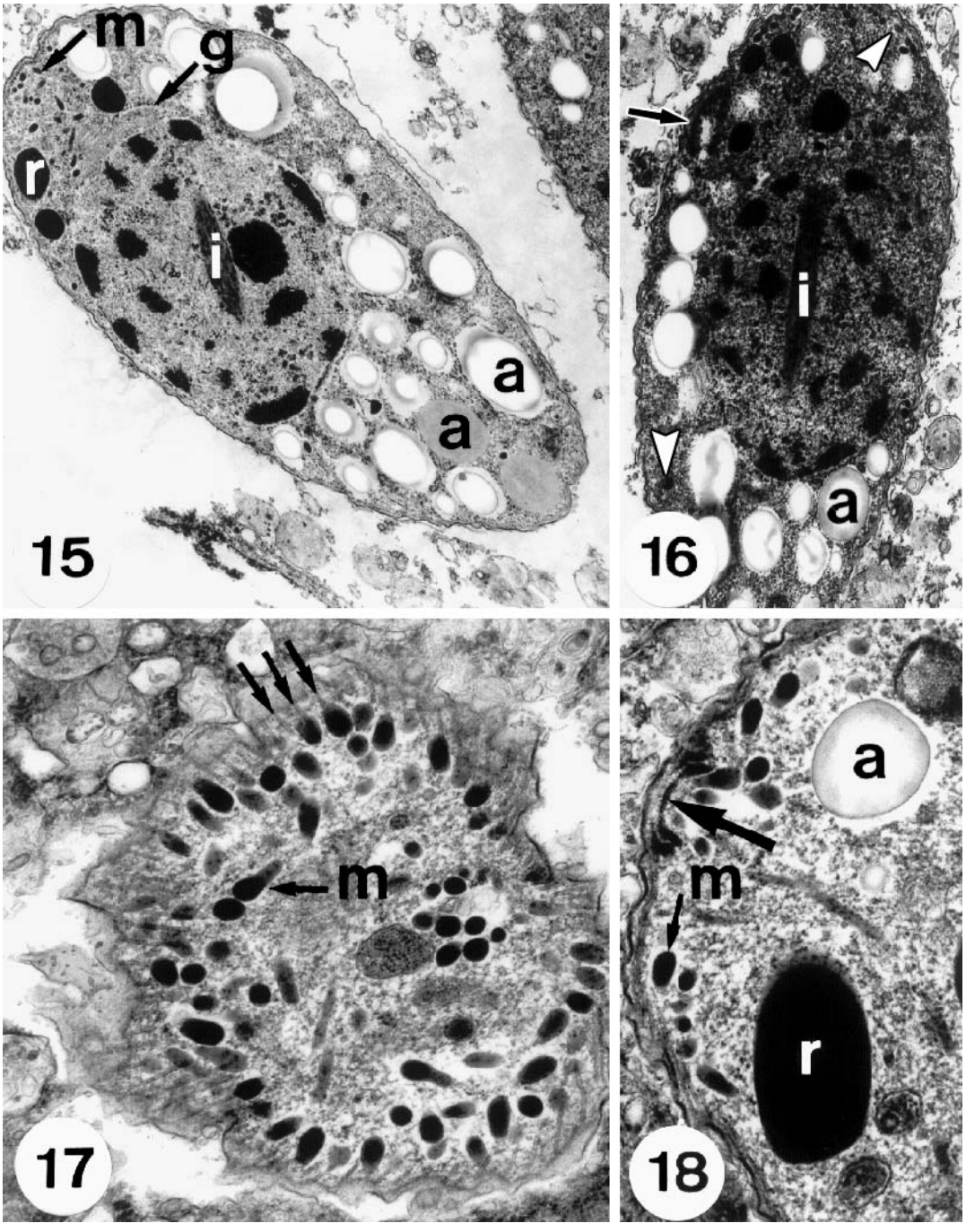
All the *Pseudopista rostrata* examined were heavily infected with coelomic gregarines that ranged from small (15 × 40 µm) to large (55–80 × 90–100 µm) pale eosinophilic trophozoites, larger (100–115 × 270–310 µm) deeply eosinophilic gamonts, and slender sporozoites (9 × 80 µm). The gamonts were observed in syzygy. In <80% of *P. rostrata*, macrogamonts (70–80 × 150–195 µm) and microgametocytes (55–70 µm in diameter), shedding microgametes (3 µm in diameter) infected epithelial cells of the digestive tract.

DISCUSSION

The only apicomplexan stage observed in oysters are termed zoites (Vivier & Desportes 1990) as they have not been observed to arise by sporogony or merogony. Apicomplexan merozoites, tachyzoites, bradyzoites and sporozoites lack any consistent ultrastructural features that distinguish them from each other (Dubey et al. 1998), and their general structure is conserved both ultrastructurally and at the molecular level (Speer et al. 1999, Tomley & Soldati 2001). The zoites in this study lack the oocyst, sporocyst, sporozoite configuration, and are unlike the *Cryptosporidium* merozoites described by Azevedo (1989) and merozoites of *Aggregata eberthi* reported by Heller (1969). The zoites do not resemble the heteroxenous *Pseudoklossia* spp. in development or tissue site, or any other apicomplexans reported from molluscs, and therefore they cannot be classified at present. Zoites are common in *Ostrea chilensis* all around New Zealand, at low intensity (Grades 1 or 2), but high prevalence (Hine unpubl. data). The occurrence of only 1 stage in the oyster suggests that the parasite is heteroxenous, in which case its other host is likely to be an invertebrate in close contact with oysters. It was not possible to determine whether the sexual stages of an apicomplexan in the gut epithelium of *Pseudopista rostrata* were those of the coelomic gregarine or the oyster zoite. This may be resolved by developing a specific probe from oyster zoites, and using it for *in situ* hybridization with the polychaete apicomplexans.

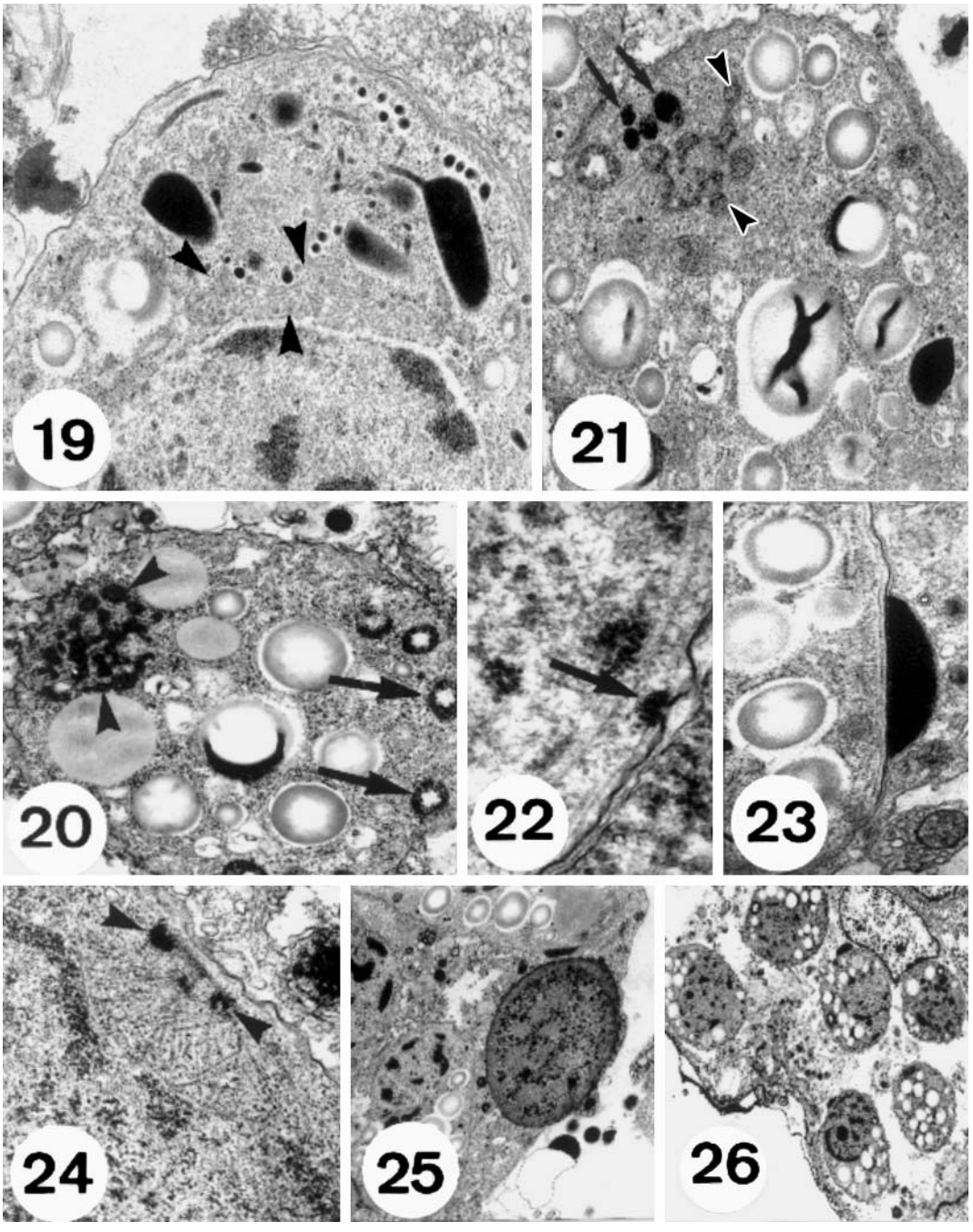
Zoites were initially intercellular between CTC, but in heavier infections were intracellular in CTC, causing separation and lysis or degeneration of these glyco-

gen-containing cells of the digestive gland. This may release glycogen, making it available to be metabolised into amylopectin, the energy reserve of zoites. Their relationship with haemocytes was unclear, but they were less frequently intrahaemocytic than *Bonomia exitiosus*, and zoites never occur in haemocytes in heart smears (unpubl. obs.). Apicomplexan zoites may actively invade host cells by using their conoids, rhop-



Figs. 15 to 18. Zoite ultrastructure. Fig. 15. Zoite with anterior micronemes (m), rhoptries (r), central nucleus with anterior attached Golgi (g), intranuclear microtubules (i), and amylopectin (a) granules (×17 200); Fig. 16. Similar zoite to Fig. 15, showing thick-walled structures anteriorly (arrow), anterior and posterior micronemes (arrowheads), and intranuclear microtubules (i) (×17 600); Fig. 17. Section through the anterior of a zoite at the level of the micronemes (m), showing 84 sub-pellicular microtubules (arrows; ×39 300); Fig. 18. Section through the edge of a conoid (arrow), showing micronemes (m), rhoptry (r) and an amylopectin granule (a; ×66 650)

tries, micronemes and dense granules (Dubremetz et al. 1998), but these organelles appeared intact in intrahaemocytic zoites, suggesting that entry into host cells was passive. However, intrahaemocytic zoites lay in direct contact with host cell cytoplasm, not in phagosomes. The observation of intranuclear tubules and semi-open pleuromitosis in intrahaemocytic zoites suggests that schizogony or merogony may occur in



Figs. 19 to 26. Zoite organelles. Fig. 19. Anterior of a zoite showing micronemes, rhoptries and flattened Golgi cisternae (arrowheads) near the nucleus (×26 875); Fig. 20. Transverse section of a zoite and Golgi cisternae with osmiophilic content (arrowheads), also profiles of thick-walled structures (arrows; ×26 220); Fig. 21. Golgi cisternae with osmiophilic content (arrowheads) associated with dense vesicles (arrows; ×31 200); Fig. 22. The micropore (arrow; ×54 200); Fig. 23. Amorphous mass lying on the parasite surface (×31 460); Fig. 24. Extranuclear centrocones (arrowheads) and spindles (×53 120); Fig. 25. Portion of a haemocyte replete with zoites showing the ragged haemocyte cytoplasm and pycnotic haemocyte nucleus (×4260); Fig. 26. Putative CTC with intracellular zoites (×3110)

haemocytes, but division was never observed. Zoites were not observed in the gut lumen or in epithelia, and therefore their pathway into the oyster is unknown. In very light, and therefore presumably early, infections, zoites occurred in CTC around the haemolymph sinuses. Their subsequent distribution was related to crowding of CTC, forcing zoites into the CTC of more peripheral areas such as the gills, and possibly by seasonal changes in the distribution of haemocytes within the host.

The occurrence of zoites in the kidney in April and May coincides with the peak prevalence of *Bonamia exitiosus* in the kidney in April and May (Hine 1991a), which may reflect normal movement of haemocytes to the kidney in those months. Similarly, *B. exitiosus* enters the gonad in April in haemocytes that invade and absorb residual ova. The parasite is shed through the gonad in April and May (Hine 1991a), the same months that zoites were observed in the gonads. *B. exitiosus* proliferates rapidly and packs the gonad whereas zoites were rarely observed in the gonad and then only at low numbers. Possibly, similar numbers of haemocytes infected with *B. exitiosus* and with zoites enter the gonads, but *B. exitiosus* proliferates rapidly, while zoites do not.

The apicomplexan had a considerable impact on the oyster population. Over a third (35%) of infections were Grades 4 or 5, and some of Grade 4, and all of Grade 5, infections had CTC damage that appeared irreversible. However, the fibrosis in <10% of Grade 4 and 5 oysters indicated that tissue repair was possible, and these oysters may have survived as a stunted part of the population. The occurrence of 75% of Group E oysters among those classified as heavily (Grades 4 and 5) infected, and the lack of germinal epithelium among many Group E oysters, suggests heavy infection by the parasite may make oysters sterile. These follicles differed markedly from those of recently spawned oysters (Groups B and D) in the degeneration of the germinal epithelium, and in a lack of correlation between their occurrence and spawning seasons. Similar inhibition of gametogenesis has been reported from oysters (*Crassostrea virginica*) infected with *Haplosporidium nelsoni*, the plasmodium of which, like the zoites, occurs intercellularly (Barber et al. 1988a, Ford & Figueras 1988, Ford et al. 1990, Barber 1996). Although reduction in fecundity with *H. nelsoni* infections may be due to reduced feeding (Newell 1985), glycogen is also depleted to meet the energetic burden of parasite infection (Barber et al. 1988b).

Heavy infection by zoites caused severe tissue damage, similar to that seen in heavy *Bonomia exitiosus* infections. Although zoites caused severe disease in the absence of *B. exitiosus*, *B. exitiosus* most often caused severe disease when moderate to heavy zoite infections occurred concurrently (Table 5). Concurrent infections are likely to be synergistically detrimental to the host for 2 reasons. Firstly, both pathogens infect haemocytes and therefore in concurrent infections haemocytes would become more rapidly depleted than if the same infection levels occurred separately. Secondly, each parasite would exacerbate the energy reserve problems caused by the other, particularly in summer/autumn (January to April), during the peak spawning period (Jeffs & Hickman 2000), and peak prevalence and intensity of *B. exitiosus* infection (Hine 1991a). Glycogen, the main energy reserve of oysters, is converted to lipid during ovagenesis to provide larvae with an energy reserve. In *Ostrea chilensis*, <25% of female oysters absorb unspent ova (Jeffs & Hickman 2000), so that the energy reserve is recycled. However, in *B. exitiosus*-infected females, the parasite enters the ovary in the haemocytes that phagocytose the ova, and it utilises ovarian lipid for rapid proliferation (Hine 1991a,b), therefore depriving the host of that energy reserve. In the absence of zoite infection, the host may be able to recover from spawning or the loss of its lipid reserves, using its glycogen reserves. If, however, zoites concurrently infect the oyster and utilise the CTC glycogen, the oyster loses both its energy reserves simultaneously, making recovery from spawning unlikely. Concurrent heavy infections with both parasites may kill the host by energy depletion, whether or not spawning occurs at the same time.

Examination of sections of oysters sampled in 1964 showed that *Bonamia exitosus* occurred in Foveaux Strait oysters at that time (Hine 1996). As *B. exitiosus* has a well-defined annual pattern of infection (Hine 1991a,b), suggesting a long-standing host-parasite association, it appears likely that it is enzootic in the region. The event that started the 1985 to 1993 epizootic is unknown. It has been thought to be due to high densities of oysters facilitating easier direct transmission of *B. exitiosus* between oysters, environmental factors acting directly or indirectly on the oysters weakening their resistance to infection, or the emergence of a virulent new strain of *B. exitiosus*.

If it is conservatively assumed from Table 5 that only zoite Grade 5 *Bonamia exitiosus*-free (n = 135) oysters would have died (7.3% of the total oysters), but all Grade 3 to 5 *B. exitiosus*-infected oysters (n = 198) would have died (10.7% of the total oysters), then proportionately 40.5% of mortalities would be due to zoite infections only, and 59.5% of mortalities would be due to *B. exitiosus* and zoite infections. If Grade 3 to 5 zoite infections do act synergistically with *B. exitiosus*, then of the 198 Grade 3 to 5 *Bonamia*-infected oysters, 164 (82.8%) also had moderate to severe (Grades 3 to 5) zoite infections and these oysters would have died of combined zoite-*B. exitiosus* infections. Thus of the

total mortalities, 40.5% would be due to zoites only, 49.2% would be due to combined zoite/*B. exitiosus* infections, and 10.2% to *B. exitiosus* only. This interpretation may be simplistic, as oysters apparently could tolerate much higher intensity levels of zoites, than those of *B. exitiosus.* However, zoites may play an important role in *B. exitiosus* pathogenesis.

Acknowledgements. I would like to thank Bruce Hearn for providing infected oysters and polychaetes, and Dr Geoff Read for identifying the polychaetes. Dr Brian Bull kindly helped with the statistical analysis. I am also grateful to Marie-Loue Vinet and Dr Ben Diggles for helping with completion of the plates, and Karen Reader, formerly of the Electron Microscope Unit, Victoria University of Wellington, for help with electron microscopy.

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Editorial responsibility: Albert Sparks, Seattle, Washington, USA

Submitted: December 8, 2001; Accepted: January 23, 2002
Proofs received from author(s): July 23, 2002