

# Gross signs and histopathology of *Ostrea puelchana* infected by a *Bonamia exitiosa*-like parasite (Haplosporidia)

Marina A. Kroeck\*

Laboratorio de Parasitología e Histopatología de Moluscos Bivalvos LABPAT-IBMP (SENASA LA 0116), Instituto de Biología Marina y Pesquera 'Alte. Storni', (8520), San Antonio Oeste, Rio Negro, Argentina

**ABSTRACT:** Haplosporidian microcells belonging to the genus *Bonamia* parasitise various species of oysters around the world. In Argentina, *Bonamia* sp. was the causative agent of mass mortality among *Ostrea puelchana* cultured in San Antonio Bay (San Matías Gulf), and it was detected in natural beds inside San Matías Gulf. In order to describe the gross and histopathological signs caused by *Bonamia* sp. in *O. puelchana*, cultured and wild oysters were sampled and analysed by traditional techniques including heart imprints and histology. Cells of *Bonamia* sp. were observed in connective tissue, free or within haemocytes, in gills and around the digestive gland, stomach, intestine and gonad. Gross signs, histopathological alterations in *O. puelchana*, and *Bonamia* sp. cytological morphology resemble those reported for *B. exitiosa*. However, I propose to treat the Argentinean species as *B. exitiosa*-like until more molecular and ultrastructural studies are conducted to determine the correct taxonomy.

**KEY WORDS:** *Bonamia exitiosa*-like · *Ostrea puelchana* · Histopathology · San Matías Gulf · Patagonia · Argentina

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## INTRODUCTION

Haplosporidian microcells belonging to the genus *Bonamia* parasitise different species of oysters globally. There are 4 species currently acknowledged that have caused epizootic diseases and mortalities around the world: *B. ostreae*, which infects *Ostrea edulis* in Europe, Morocco, USA and Canada (Bower 2007); *B. exitiosa*, which parasitises *O. chilensis* in New Zealand (Hine et al. 2001) and probably *O. angasi* in Australia (Corbeil et al. 2006); *B. roughleyi*, which parasitises *Saccostrea glomerata* in south-east Australia (Cochennec-Laureau et al. 2003); and *B. perspora*, which infects *Ostrea stentina* (= *Ostreola equestris*) in North Carolina (USA; Bishop et al. 2006, Carnegie et al. 2006). Other *Bonamia*-like microcells have also been described parasitising *Crassostrea ariakensis* in North Carolina (Burrenson et al. 2004) and *O. chilensis* in

Chile (Kern 1993, Campalans et al. 2000, Lohrmann et al. 2009). In 1996, mass mortality of cultured *O. puelchana* was reported in San Antonio Bay, Argentina, and *Bonamia* sp. was identified as a possible aetiological agent (Kroeck & Montes 2005). This was the first record of a haplosporidian microcell belonging to the genus *Bonamia* for *O. puelchana* along its geographical distribution (Fig. 1a). An oyster culture area was identified as the focus of bonamiosis, from which the pathogen spread through the natural oyster beds (Fig. 1c), located at the SW and NE of the San Matías Gulf (Kroeck et al. 2008).

To describe the gross and histopathological signs caused by *Bonamia* sp. in *Ostrea puelchana*, cultured and wild oysters were sampled and analysed by traditional techniques including heart imprints and histology. Moreover, to compare to other *Bonamia* spp., a cytological description of *Bonamia* sp. was made.

\*Email: mkroeck@gmail.com

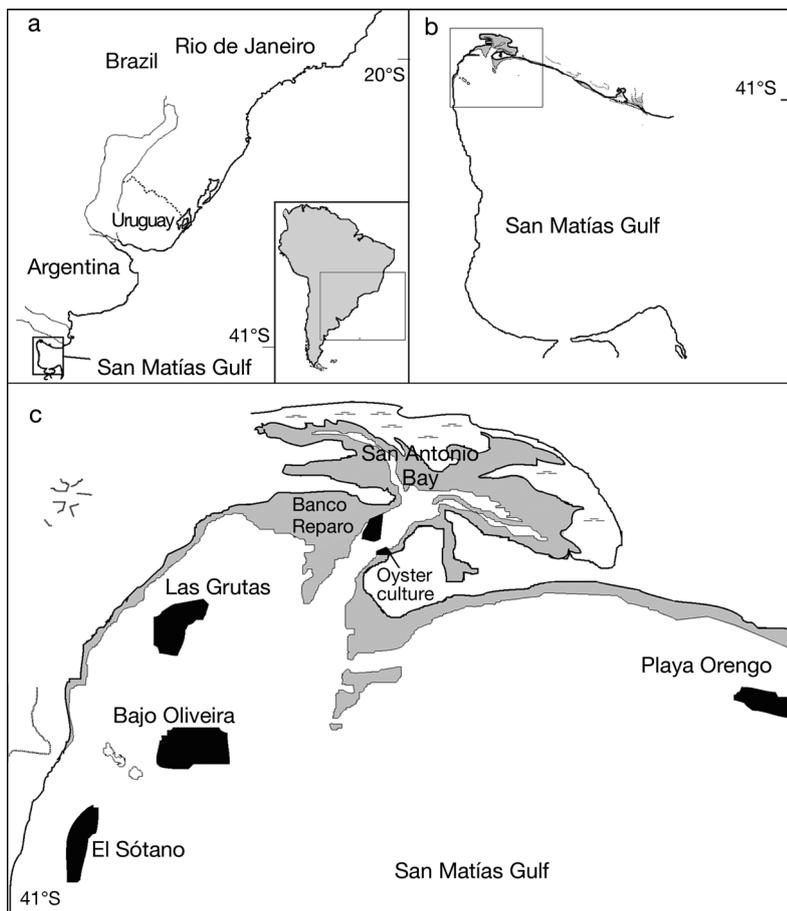


Fig. 1. Study area, oyster culture and sampling sites. (a) Geographical distribution of *Ostrea puelchana*. (b) Location of San Antonio Bay. (c) Natural beds of *O. puelchana* and the oyster culture site

## MATERIALS AND METHODS

Between September 1996 and October 2001, samples of 30 oysters were collected semi-annually, in spring and in late summer to early autumn, on each of the 5 natural beds: Banco Reparó (BR), Las Grutas (LG), Bajo Oliveira (BO), El Sótano (ES) and Playa Orengo (PO). Furthermore, samples of 30 oysters (September 1996) and 60 oysters (October 1997) were collected from oyster culture within San Antonio Bay (Fig. 1c).

Oysters were measured, weighed and opened at the hinge. They were examined for digestive gland colour, apparent condition (fat, medium or watery) and gross abnormalities such as watery cysts, abscesses, tumours and gill lesions. Shell and flesh were separated and weighed, the heart was removed and imprints were made and stained with a 'Tinción 15' Kit (Biopur®LB). Soft tissues were fixed in Davidson's solution, embedded in paraffin, cut in 6 µm thick sections and stained with Harris' haematoxylin–eosin. Heart imprints and histological sections were examined under light microscopy for *Bonamia* sp. cell morphology and histological lesions and/or reaction of the host.

To determine the relationship between histology and gross signs, 300 oysters were collected from BR between May 1997 and November 2001. A chi-squared test ( $\chi^2$ ; Sokal & Rolf 1981) was used to determine the relationship between histological and gross signs in the presence of *Bonamia* sp. The measurements of cell size were calculated as the means ( $\pm$ SD) of the longest dimension and the dimension at right angles to it, divided by 2.

## RESULTS

In total, 1288 *Ostrea puelchana* were collected and analysed; of these, 1198 came from 5 natural beds, and the 90 remaining were collected from the commercial cultivation. Parasite prevalence of wild and cultured oysters was 21% and 63.3%, respectively.

### Gross signs associated with *Bonamia* sp. infection

Gross abnormalities such as watery cysts, abscesses and watery cysts and tumours were not observed.

Table 1. *Ostrea puelchana*. Prevalence (%) of *Bonamia* sp. in relation to gross signs and haemocytic infiltration. (Total no. oysters = 300; total no. infected = 93; thus, total prevalence = 31%)

	Gross signs				Gill lesions	Haemocytic infiltrations
	Digestive gland colour Normal	Digestive gland colour Pale	Apparent condition Fat/medium	Apparent condition Watery		
No. oysters (%)	262 (87.3)	38 (12.7)	242 (80.7)	58 (19.3)	70 (23.3)	230 (76.7)
Prevalence	26.3	4.7	24.3	6.7	9.7	21.3

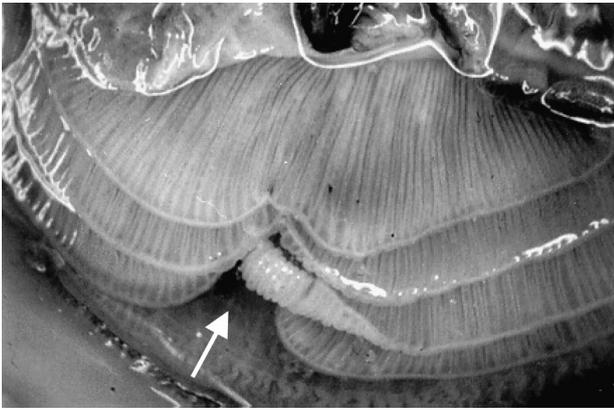


Fig. 2. *Ostrea puelchana*. Gill lesions (arrow)

From the sub-sample of 300 oysters collected at BR, 93 (31.0%) were infected by *Bonamia* sp. Abnormal clear or pale colouration of the digestive gland was observed in 38 (12.7%) oysters, and 4.7% of them were heavily parasitised (Table 1). This pale colouration was independent ( $0.69$ ;  $p > 0.05$ ) of *Bonamia* sp. presence and in moderate/low infections was not observed. Oysters with watery condition (19.3%) were spawning or spent, and a low percentage of them (6.7%) were parasitised (Table 1). The presence of infection was independent ( $0.41$ ;  $p > 0.05$ ) of the condition (fat, medium or watery) of *Ostrea puelchana*. Some oysters (70/300) presented gill lesions (Fig. 2), and 9.7% of them were parasitised (Table 1). The presence of gill lesions was not independent of the presence of the parasite ( $5.7$ ;  $p < 0.05$ ); however, the degree of association was low ( $C = 0.138$ ).

#### Histopathological alterations associated with *Bonamia* sp. infection

Histological examination revealed the presence of marked haemocyte infiltration in connective tissue of the mantle, gills and digestive gland, with parasitised and non-parasitised haemocytes (Figs. 3 & 4). From the sub-sample of 300 oysters collected at BR, 156 showed haemocyte infiltration, of which 89 (29.7%) were parasitised (Table 1). The presence of haemocyte infiltration was not independent of the presence of the parasite ( $103.12$ ;  $p < 0.05$ ), with a relatively high degree of association ( $C = 0.548$ ).

During spawning periods (November to December and February to March), haemocytes accumulated around and inside the gonads (Fig. 3a). Entry of *Bonamia* in haemocytes into the gonad occurs particularly during reabsorption of ovocytes in April to May. Infected haemocytes and free *Bonamia* between ovocytes could be observed (Fig. 4d).

Haemocyte infiltration was most common in the vesicular connective tissue (VCT) around the digestive gland, particularly around stomach, intestine and digestive diverticulae (Fig. 3b,d). Infiltration was accompanied by disassociation of VCT containing cell debris, broken haemocytes with picnotic nuclei and parasitised granular haemocytes (Fig. 4a–c).

Gills infected with *Bonamia* sp. showed haemocyte infiltration of the connective tissue and, less frequently, hypertrophy of the filaments (Fig. 3c). In heavily infected oysters, haemocytes spread from the suprabranchial area into the gills, sometimes causing rupture of the epithelium and release of parasitised and non-parasitised haemocytes (Fig. 3e).

#### Cytological description of *Bonamia* sp.

Parasitised haemocytes had an eccentric nucleus, and usually 1 or 3, but up to 7 or 8, parasites were located inside a parasitophorous vacuole (PV) in the cytoplasm (Figs. 4a,b & 5).

*Bonamia* sp. cells are spherical or ovoid and range in size from  $3.7$  to  $4.2$   $\mu\text{m}$ . They have a basophilic, and sometimes eosinophilic cytoplasm and a central circular nucleus.

Four stages of *Bonamia* sp. were observed:

(1) Uninucleate form. This cell form was observed both in light infections and in highly parasitised oysters. It was distinguished by its small size ( $2.7 \pm 0.3$  to  $3.1 \pm 0.3$   $\mu\text{m}$ , mean  $\pm$  SD), irregular and spherical shape, with a central nucleus (Fig. 4c,d). Basophilic uninucleate cells occurred from September to November and were rarely observed in late summer to early fall samples. Otherwise, eosinophilic uninucleate cells were observed in March and April, mainly during reabsorption of ovocytes of wild oysters. However, they were also observed during the months in which high mortality of farmed oysters occurred in September 1996 and October 1997. In semi-thin sections, they were seen as dense and clear forms (Fig. 5a).

(2) Bi-nucleate form. This form was distinguished by its 2 nuclei, slightly larger size ( $3.3 \pm 0.2$  to  $4.0 \pm 0.3$   $\mu\text{m}$ , mean  $\pm$  SD), ovoid but sometimes spherical shape and slightly more eosinophilic cytoplasm (Fig. 4b). Occurrence of this stage was only observed in April and August.

(3) Plasmodial-like form. This stage was observed only in April and August samples. It was distinguished by its irregular shape, eosinophilic cytoplasm with 3, rarely 4, nuclei and was slightly larger ( $4$  to  $5$   $\mu\text{m}$ ) than bi-nucleate form (Figs. 4c & 5).

(4) Vacuolate-like form. This form was distinguished by a poorly delineated nucleus, with cytoplasm around a central vacuole. The nucleus was eccentric, giving

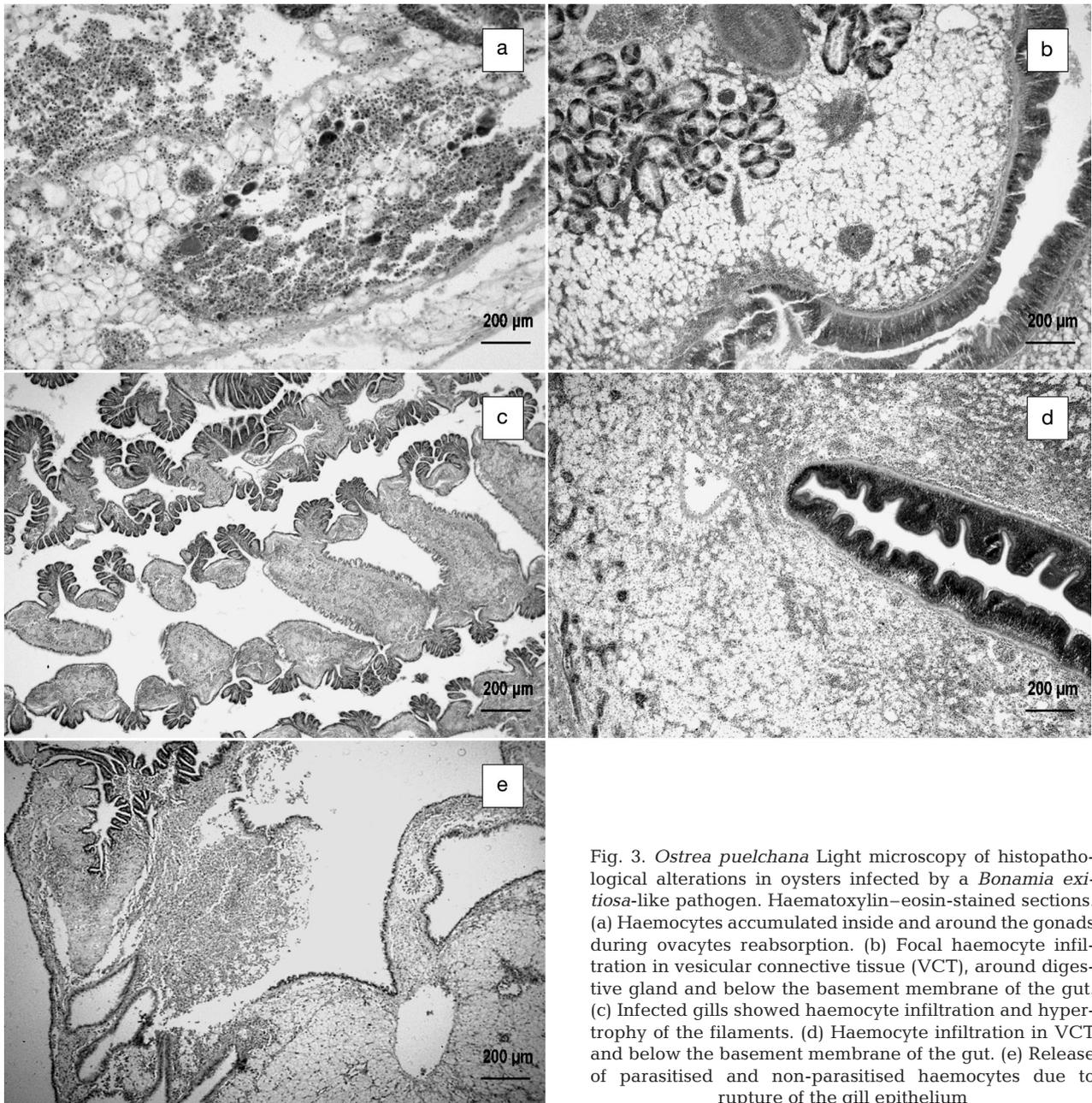


Fig. 3. *Ostrea puelchana* Light microscopy of histopathological alterations in oysters infected by a *Bonamia exitiosa*-like pathogen. Haematoxylin–eosin-stained sections. (a) Haemocytes accumulated inside and around the gonads during ovocytes reabsorption. (b) Focal haemocyte infiltration in vesicular connective tissue (VCT), around digestive gland and below the basement membrane of the gut. (c) Infected gills showed haemocyte infiltration and hypertrophy of the filaments. (d) Haemocyte infiltration in VCT and below the basement membrane of the gut. (e) Release of parasitised and non-parasitised haemocytes due to rupture of the gill epithelium

the cell section the appearance of a signet ring (Fig. 5a,d). This cell form was only observed in April, in the semi-thin sections.

## DISCUSSION

### Gross signs and histopathological alterations associated with *Bonamia* sp. infection

In all *Ostrea puelchana*, gross signs such as watery cysts, abscesses or tumours were not observed. Al-

though some parasitised oysters had pale colouration of the digestive gland and a watery condition were observed, these external signs were not associated with *Bonamia* sp. presence. This has also been reported for *O. chilensis* in New Zealand parasitised by *Bonamia exitiosa* (Dinamani et al. 1987, Hine & Jones 1994) and for *O. edulis* in Galicia (Spain) infected by *B. ostreae* (Montes 1992).

Gill lesions were observed in 23.3% of the analysed oysters, but only 9.7% of them were parasitised. Dinamani et al. (1987) found a similar percentage (10%) for infected oysters with frayed and badly eroded gills. A

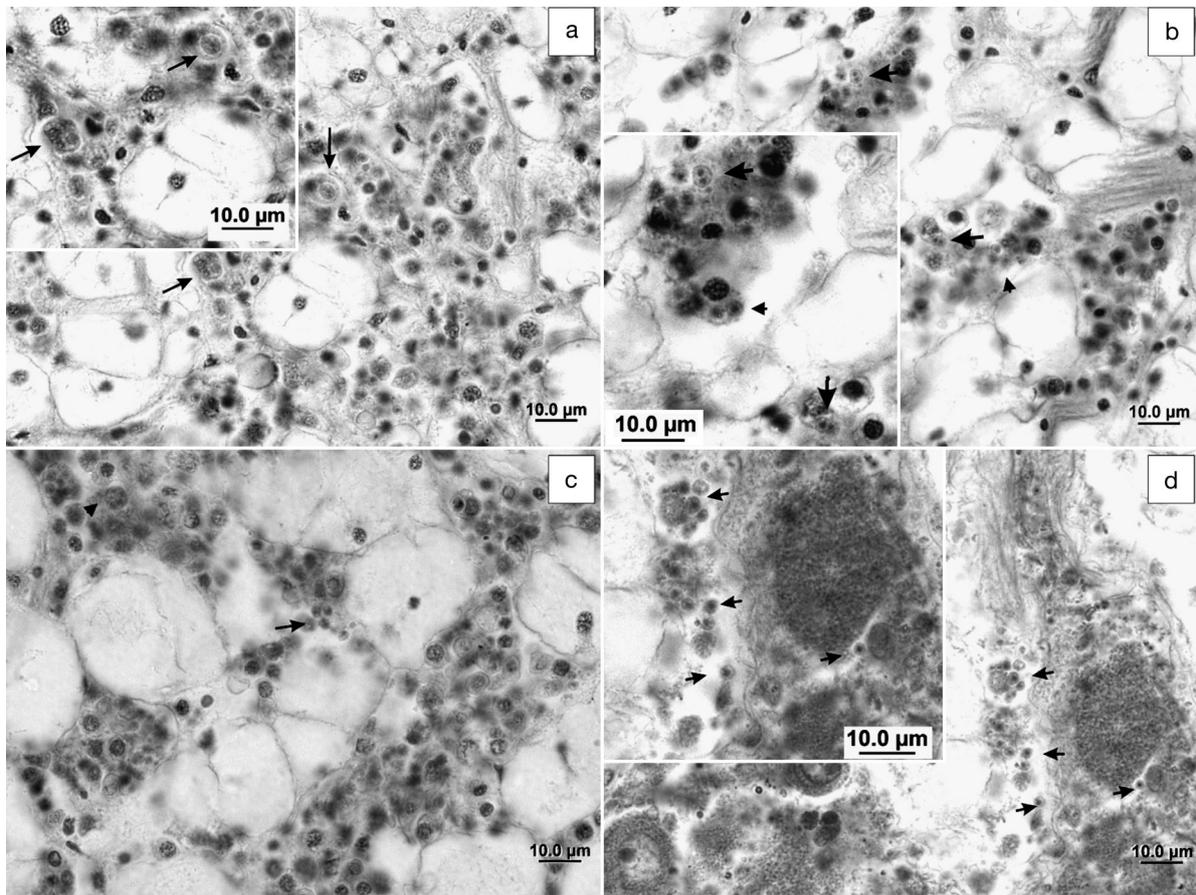


Fig. 4. *Ostrea puelchana*. Light microscopy of the *Bonamia exitiosa*-like pathogen in haematoxylin–eosin-stained sections. (a) Haemocytes with eccentric nucleus infected by 1 *B. exitiosa*-like cell inside a parasitophorous vacuole (PV; arrows). Insert: Uniuucleate cell with an indented or concave region of the parasite surface at higher magnification. (b) Uniuucleate (small arrow) and free binucleate (large arrow) cell forms. Insert: Two uniuucleate (small arrow) cell forms inside the same PV and free binucleate (large arrow) cell forms at higher magnification. (c) Free uniuucleate (arrow) and plasmodial-like (arrowhead) forms. (d) Free uniuucleate cell forms (arrows) between ovocytes. Insert: The same cell forms at higher magnification

yellowish band at the ending of gill lesions was described for *Ostrea edulis* (Tigé et al. 1980); however, this band was not observed in *O. puelchana*. Moreover, several authors (Tigé et al. 1980, Grizel et al. 1988, Mc Ardle et al. 1991, Montes 1992) reported gill lesions as characteristic of bonamiosis caused by *Bonamia ostreae* in *O. edulis*. However, although in *O. puelchana* gill lesions were the only external signs significantly associated with the presence of the parasite, the degree of association was low. Therefore, frayed or eroded gills cannot be considered as pathognomonic for infection by *Bonamia* sp. in *O. puelchana*. These symptoms may also have been due to other causes, such as congenital, environmental or mechanical factors.

Prevalence of *Bonamia* sp. and the percentages of haemocytic infiltrations are dependent events with a relatively high degree of association. The differences

between parasite prevalence and percentages of infiltration values are probably due to some stress problems that cause these tissue reactions (Hine et al. 2002), or due to a 'latent period' as in *Ostrea edulis* parasitised by *B. ostreae* (Culloty et al. 2003). These tissue reactions could also be due to other infections such as viral infections (Da Silva et al. 2005).

The most important microscopic signs of *Bonamia* sp. infection in *Ostrea puelchana* are (1) dense cellular accumulations due to haemocytic infiltration in all connective tissue (systemic infiltration), (2) dissociated appearance of connective tissue containing lysed haemocytes with picnotic nuclei and cells debris (Kroeck & Montes 2005; Figs. 2 to 4), and (3) intracellular localisation of *Bonamia* sp. (1–7 parasites per haemocyte) or parasites lying free between cells in connective tissue (Figs. 4 & 5).

The same histopathological alterations have been reported for *Ostrea edulis*, *O. angasi* and *O. chilensis*

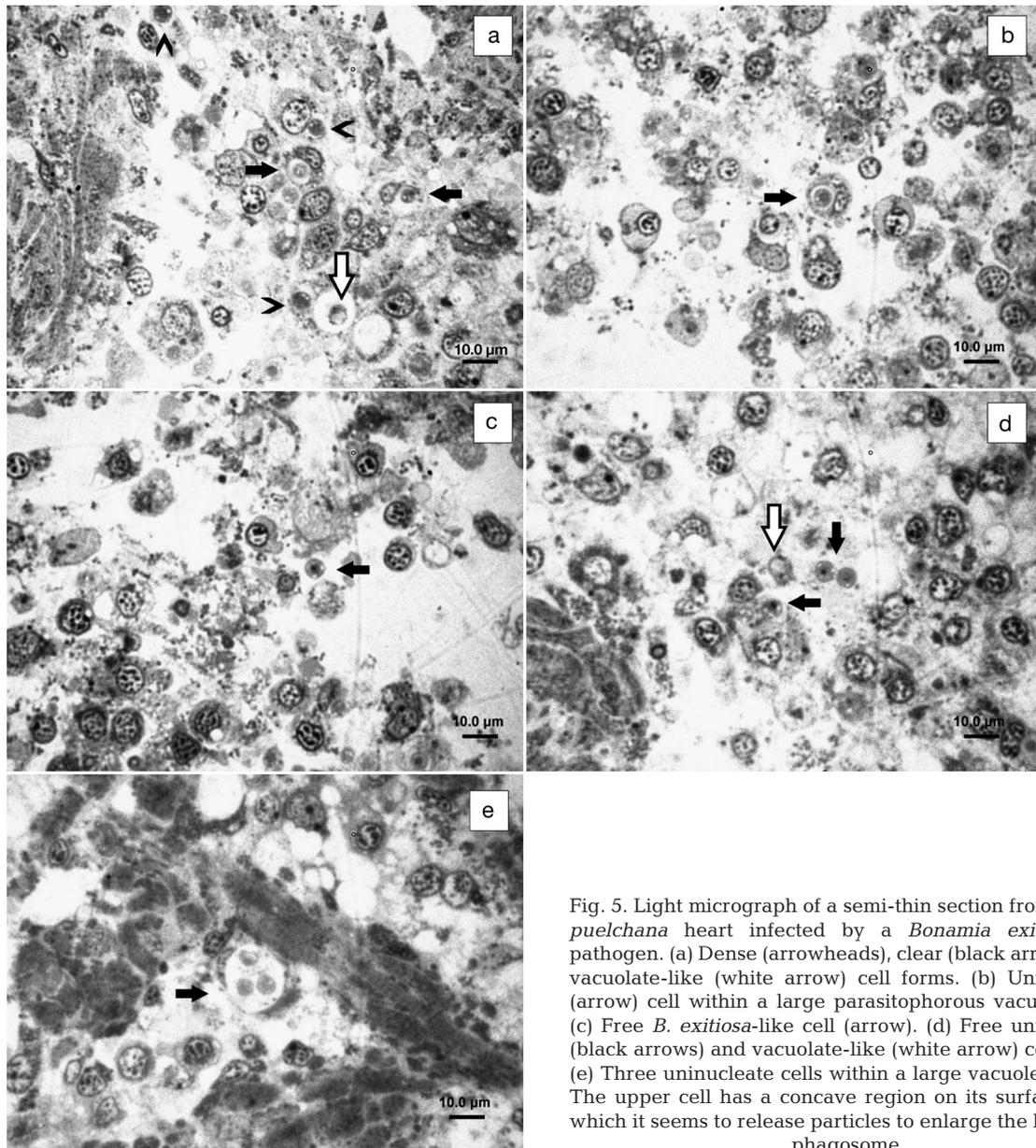


Fig. 5. Light micrograph of a semi-thin section from *Ostrea puelchana* heart infected by a *Bonamia exitiosa*-like pathogen. (a) Dense (arrowheads), clear (black arrows) and vacuolate-like (white arrow) cell forms. (b) Uninucleate (arrow) cell within a large parasitophorous vacuole (PV). (c) Free *B. exitiosa*-like cell (arrow). (d) Free uninucleate (black arrows) and vacuolate-like (white arrow) cell forms. (e) Three uninucleate cells within a large vacuole (arrow). The upper cell has a concave region on its surface, from which it seems to release particles to enlarge the host phagosome

parasitised by *Bonamia ostreae* and *B. exitiosa* (Dinamani et al. 1987, Grizel et al. 1988, Mc Ardle et al. 1991, Montes 1992, Hine et al. 2001). Similar conditions have been observed in tissue and cells of other *Ostrea* spp. parasitised by *Bonamia* spp. (Poder et al. 1982, Balouet et al. 1983, Bucke & Feist 1985, Elston et al. 1986, Farley et al. 1988, Friedman et al. 1989, Hine 1991a,b, Chagot et al. 1992, Friedman & Perkins 1994, Hine & Wesney 1994, Montes et al. 1994).

In previous studies, conducted on samples of farmed oysters collected in September 1996 and October 1997, the occurrence of *Bonamia* sp. in the gonad was never observed (Kroeck & Montes 2005). However, further

studies (Kroeck et al. 2008), carried out between September 1996 and October 2001 on wild oysters, revealed the presence of this pathogen during spawning peaks, particularly inside gonads during ovocyte reabsorption. Like *Ostrea chilensis* (Hine, 1991a), *O. puelchana* spawn in early summer to autumn (November to December and February to March; Morriconi & Calvo 1979), and in April most of the oysters are spawned with accumulation of parasitised and non-parasitised haemocytes around and inside the gonads. Hine (1991a,b) reported that *B. exitiosa* infections display a seasonality closely following *O. chilensis* gametogenesis and reproductive cycle. Parasites proliferate

when haemocytes migrate into the gonad to resorb unspawned gametes (Dinamani et al. 1987, Hine 1991a,b, Carnegie & Cochenec-Laureau 2004). The similarity of the gonadal development and gonadal infection patterns in both ostreids could explain the fact that *Bonamia* cells were not observed within the farmed *O. puelchana* gonads in September 1996 and October 1997.

### Comparison of cytological and developmental stages of Argentinean *Bonamia* sp. to other known *Bonamia* spp.

Different developmental stages have been reported for haplosporidian microcells belonging to the genus *Bonamia* (Pichot et al. 1980, Dinamani et al. 1987, Hine 1991b, Hine et al. 2001, Cochenec-Laureau et al. 2003, Carnegie et al. 2006, Abollo et al. 2008, Lohrmann et al. 2009). Uninucleate small (<5 µm) cells are characteristic of microcell haplosporidians. These cells may be electron dense or electron clear (Pichot et al. 1980, Dinamani et al. 1987, Hine 1991a,b) with eccentric nuclei in *B. ostreae* (Friedman et al. 1989) or central nuclei, as is characteristic of *B. exitiosa* (Hine et al. 2001).

Argentinean *Bonamia* sp. uninucleate cells resemble *B. exitiosa* in the central circular nucleus and size ( $3.1 \pm 0.3$  µm and  $3.1 \pm 0.4$  µm, respectively; Hine 1991a,b, Hine et al. 2001, Abollo et al. 2008). Also, they resemble each other in an indented or concave region of the parasite surface (Fig. 4a; see also Hine 1991b: Figs. 1 & 6; Hine & Wesley 1994: Figs. 14 & 15; Hine et al. 2001: Fig. 13), and particle release appeared to coincide with host phagosome membrane enlargement, forming a PV around the parasite (Figs. 4a & 5e; see also Hine 1991b: Fig. 8; Hine & Wesley 1994: Fig. 16). Moreover, Argentinean *Bonamia* sp. and New Zealand *B. exitiosa* resemble each other in the occurrence and seasonality of basophilic and eosinophilic uninucleate forms, as well as their seasonal prevalence pattern (Hine 1991a, Kroeck et al. 2008).

The occurrence of binucleated cells was reported in *Bonamia ostreae* *in vitro* culture assays (Comps 1983). Hine et al. (2001), Abollo et al. (2008), Lohrmann et al. (2009) and Carnegie et al. (2006) also reported the occurrence of binucleated cells for *B. exitiosa*, Chilean *Bonamia* sp. and *B. perspora*, respectively. Argentinean *Bonamia* sp. binucleated cells resemble those of *B. perspora* in size ( $4.0 \pm 0.3$  µm and  $4.0 \pm 0.9$  µm, respectively) and in their spherical but more often ovoid shape (Fig. 4b). Moreover, *Bonamia* sp. binucleated cells co-occurred with uninucleated cell forms (Fig. 4b), as in *B. perspora* (Carnegie et al. 2006).

The occurrence of plasmodial cells has been observed regularly in *Bonamia exitiosa* (Hine 1991b, Hine et al. 2001). *B. ostreae* plasmodia occur rarely only in moribund or post-mortem oysters (Brehélin et al. 1982), similar to *B. roughleyi* plasmodia (Cochenec-Laureau et al. 2003). The *Bonamia* sp. plasmodial-like form has been observed only in April and August samples. It resembles *B. exitiosa* in *Ostrea edulis* (see Abollo et al. 2008: Fig. 1) and *B. perspora* small plasmodia (see Carnegie et al. 2006: Fig. 1). It was extracellular, and distributed throughout oyster connective tissue as in *B. perspora* (Carnegie et al. 2006).

The *Bonamia* sp. vacuolated-like form was only observed in April in the semi-thin sections (Fig. 5a,d) and resembled *B. exitiosa* in having a poorly delineated nucleus with cytoplasm around a large vacuole (perhaps a vacuolous mitochondrion; see Hine et al. 2001: Figs. 13 & 14).

Gross signs and histopathology in *Ostrea puelchana*, as well as cytology and developmental stages of *Bonamia* sp. resemble those reported for *B. exitiosa*. The Argentinean species is considered to be *B. exitiosa*-like, until more molecular and ultrastructural studies determine the correct taxonomy.

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