Histological survey of four species of cultivated molluscs in Chile susceptible to OIE notifiable diseases

Catastro histológico de cuatro especies de moluscos cultivados en Chile susceptibles a enfermedades de declaración obligatoria a la OIE

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Resumen.- Se examinaron moluscos cultivados en Chile para detectar la presencia de enfermedades de declaración obligada a la OIE (Office International des Epizooties: Organización Mundial de Sanidad Animal). Se tomaron muestras de tejidos, en invierno y en verano, de las especies susceptibles que se cultivan en el país, los abalones *Haliotis discus hannai* y *H. rufescens*, y las ostras *Crassostrea gigas* y *Ostrea chilensis*. Se realizaron cortes histológicos de todos los moluscos obtenidos y para el caso de ostra chilena, se procesó además tejido para observación en microscopio electrónico de transmisión (MET) para describir al protozoo tipo *Bonamia* presente en los hemocitos de algunas ostras. El único patógeno de declaración obligada a la OIE que se encontró fue *Xenohaliotis californiensis* en *H. rufescens*, bacteria que se encontró formando inclusiones intracelulares en epitelios digestivos, principalmente post-esófago y glándula digestiva. La descripción ultraestructural del protozoo tipo *Bonamia* sp. indica que se parece en algunos aspectos y difiere en otros, de las especies *B. ostreae* y *B. exitiosa*, pertenecientes al listado de enfermedades de moluscos de declaración obligada de la OIE.

Palabras clave: Enfermedades de moluscos, *Bonamia* sp., OTR en abalones, *Xenohaliotis californiensis*

Abstract.- Shellfish cultured in Chile were analyzed to detect the presence of diseases notifiable to OIE (Office International des Epizooties: the World Organization for Animal Health). Tissue samples of the susceptible species, the abalones *Haliotis discus hannai*, *H. rufescens*, and oysters *Crassostrea gigas*, and *Ostrea chilensis* cultured in Chile were collected in winter and summer. Histological sections were performed of all species; furthermore, tissue from the Chilean oyster (*O. chilensis*) was processed for transmission electron microscopy (TEM) to describe the *Bonamia*-like protozoan detected in the haemocytes of some oysters. The only pathogen from the list of notifiable diseases of the OIE was *Xenohaliotis californiensis* in *H. rufescens*. This bacterium formed intracellular inclusions in digestive epithelia, mainly in the post-esophagus and digestive gland. The ultrastructural description of the *Bonamia* -like protozoan shows it is similar in some features and different in others from the species *B. ostreae*. and *B. exitiosa* included in the list of notifiable diseases of the OIE.

Key words: Mollusc diseases, *Bonamia sp*., RLOs in abalones, *Xenohaliotis californiensis*

Introduction

Mollusc culture is an important economic and social activity in Chile, being practised both on native and exotic species. The main native cultured species are scallops (*Argopecten purpuratus* Lamarck, 1819*)*, mussels (*Mytilus chilensis* (Hupé, 1854)*, Choromytilus chorus* (Molina, 1782), *Aulacomya atra* (Molina, 1782)) and the flat oyster (*Ostrea chilensis* Philippi, 1845*)*. Farmed exotic species are red abalone (*Haliotis rufescens* Swainson, 1822), Pacific abalone (*Haliotis discus hannai* Ino, 1953), and the Pacific oyster (*Crassostrea gigas* Thunberg, 1795*)*.

The OIE (Office International des Epizooties: the World Organization for Animal Health) listed diseases of molluscs are: Infection with *Bonamia ostreae, B. exitiosa*, *Marteilia refringens*, *Perkinsus marinus*, *P. olseni*, *Xenohaliotis californiensis* and abalone viral mortality (OIE 2008[1]). The cultivated molluscs in Chile susceptible to these pathogens are the oysters *O. chilensis* and *C. gigas*, as well as the abalones *H. discus hannai* and *H. rufescens*. Chile is an OIE member and as such, is obliged to notify the occurrence of any of the listed diseases.

Considering the increasing trade of molluscs, and the international necessity for surveillance and zoning, the fisheries governmental agency (Subsecretaría de Pesca) developed two lists of high risk diseases for molluscs in

[1] OIE. 2008. Aquatic Animal Health Code. Chapter 1.2.3. Diseases listed by the OIE. [on-line] <http://www.oie.int/eng/normes/fcode/en_chapitre_1.2.3.htm>

Chile, which were released in January 2002. These lists are assessed each year by a group of national experts, and modified, if necessary. List 1 includes all OIE listed diseases, excluding those known to be present in Chile. On the other hand, list 2 includes the OIE listed diseases that are present in Chile, and/or other infections considered of high risk in the country.

Information on protistan or prokaryotic parasites is scarce, and only some of it has been published. Haemic neoplasia is a pathological condition of still unknown origin, described from *Ostrea chilensis* (Mix & Breese 1980, Rojas *et al.* 1999) and *Mytilus chilensis* (Campalans *et al.* 1998); a *Bonamia*–like parasite was described from *O. chilensis* (Kern 1993[2], Campalans *et al.* 2000). Rickettsiales-like organisms (RLOs) have been reported from scallops (*Argopecten purpuratus)*, with no evidence of damage to the host (Lohrmann *et al.* 2002).

The two areas concentrating 98% of the mollusc production were studied, in an attempt to get a better understanding of the current health status of Chilean cultivated molluscs.

Material and methods

Four mollusc species were studied: *Haliotis rufescens* (Swainson, 1822), *H. discus hannai* (Ino, 1953), *Crassostrea gigas* (Thunberg, 1795) and *Ostrea chilensis* (Philippi, 1845). Mollusc cultivation in Chile is carried out in two zones, one in the northern part of the country, between latitudes 27º and 32ºS, and the other one in the south, between latitudes 41º and 42ºS (Fig. 1). The number of individuals of each species sampled from these sites for each season is shown in Table 1.

Two samplings were performed for all species: spring-summer 2003 (December-January) and fall-winter (April-August) 2004. The intended sample size was 60 specimens by location; however, this was not always possible. Therefore in each case a minimum of 20 individuals from each farm in the same location was sampled. An additional sampling of *O. chilensis* was performed in August 2004 (winter).

Table 1

Sampling locations and number of specimens collected from each species in summer, autumn and winter

Localidades de muestreo y número de especímenes recolectados de cada especie en verano, otoño e invierno

Location	Species	Summer Nº sampled	Autumn Nº sampled	Winter Nº sampled	Total sampled
Northern zone					
Caldera	*H. rufescens*	62		59	121
	H. discus hannai	60		60	120
Guanaqueros	*H. discus hannai*	60		60	120
Tongoy	*C. gigas*	57		63	120
Los Molles	*H. rufescens*	49		60	109
Southern zone					
Calbuco	*O. chilensis*	60	60	60	180
	C. gigas	86		34	120
Ancud	*O. chilensis*	60	60	70	190
Dalcahue	*C. gigas*	87		50	137
	H. rufescens	38		50	88
Chonchi	*H. rufescens*	112		70	182

[2]Kern FG. 1993. Shellfish health inspections of Chilean and Australian oysters. Journal of Shellfish Research 12: 366 [Abstract]

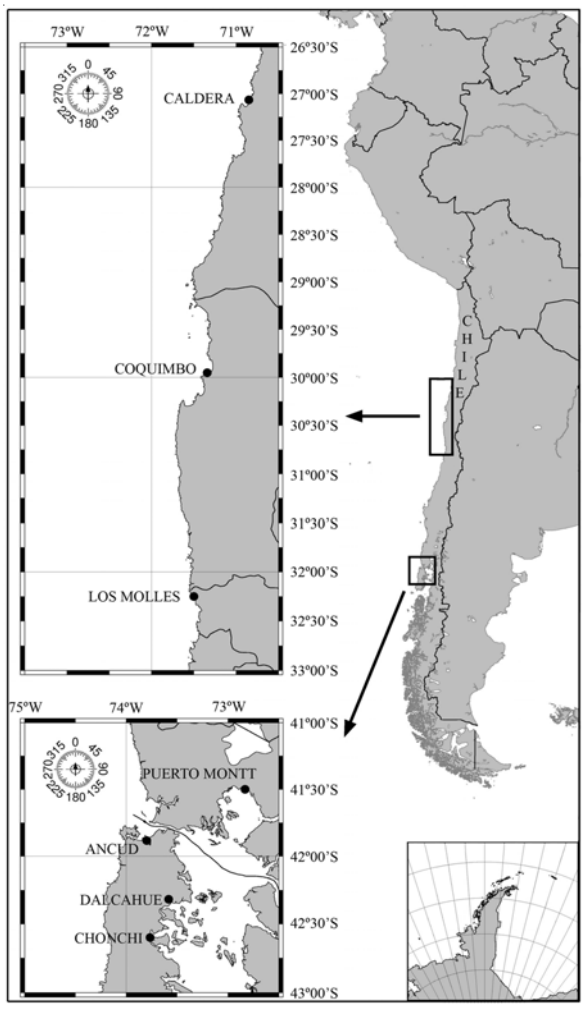

Figure 1

Location of the sampling zones

Ubicación de las zonas de muestreo

Histology

Soft tissues were removed from the shell and sectioned, including digestive gland, gills, mantle and kidney, placed in tissue embedding cassettes, and fixed in Davidson´s fluid for 24 hours (Shaw & Battle 1957). They were then transferred to 70% ethanol, and further processed for histology. Five µm thick sections were stained with Harris haematoxylin and alcoholic eosin, and carefully analyzed for any putative pathogens. Photographs were taken with an Eclipse E600 microscope.

Transmission Electron Microscopy (TEM)

Small, 1 mm^3 pieces of digestive gland and gills were fixed for one hour in 3% glutaraldehyde in 0.2 M cacodylate buffer pH 7.4 with 1.75% NaCl at room temperature. Tissues were washed three times in cacodylate buffer and post-fixed for two hours in 1% reduced OsO_4 (1:1 mixture of 3% aqueous potassium ferrocyanide and 2% OsO_4). After washing three times with the same buffer, they were rinsed in distilled water, stained for 1 hour with 2% aqueous uranyl acetate, dehydrated in ethanol, washed in acetone, and embedded in Medcast (Pelco). Semi-thin sections, 1 µm thick, were cut on a Reichert Ultracut S microtome, and stained with toluidine blue. Ninety nm thin sections were cut with a diamond knife, collected on copper grids, and stained with 2% aqueous uranyl acetate and lead citrate (Reynolds preparation: mix 1.33 g lead nitrate, 1.76 g sodium citrate and 30 mL distilled water for 30 min, add 8 mL of 1N NaOH, and distilled water to make 50 mL, pH 12). The sections were observed with a Zeiss EM 900 electron microscope at 50 kV, and photographs were taken.

Measurements of ten *Bonamia* sp. cells were taken from TEM photographs.

Sex categories

The sex categories of Chilean oysters were determined following Siddiqui & Ahmed (2002): a) Males: follicles containing only male gonadal tissue, b) Female: follicles containing only female gonadal tissue, c) Ambisexual: follicles containing 50% male and 50% female gonadal tissue, d) Predominantly male: follicles containing more than 50% male gonadal tissue, e) Predominantly female: follicles containing more than 50% female gonadal tissue, f) Indifferent: no gonadal tissue to be distinguished.

Statistical analysis

A pathogen prevalence comparison (*X. californiensis* in red abalone, and *Bonamia* sp. in the Chilean oyster) was carried out among the different seasons and locations where infected abalones or oysters were detected, applying the Mann Whitney rank sum test (Canavos 1988). For analyzing if infection with *Bonamia* sp. was sex-related in oysters a Chi-square test for independence was applied (Canavos 1988).

Results

Two pathogens similar to OIE listed ones were detected: Rickettsiales-like organisms (RLOs) in red abalone (*H. rufescens)*, and a haemocytic parasite in Chilean oyster (*O. chilensis*). *C. gigas* and *H. discus hannai* were free from the pathogens included in the list of notifiable diseases of the OIE.

The findings in red abalone (*H. rufescens*) corresponded to basophilic inclusions with rickettsiales-like organisms (RLOs) observed in the post-esophagus

(Fig. 2a,b) and digestive gland of red abalones. In severe infection cases, different degrees of metaplasia of the digestive gland epithelia were also observed (Fig. 2c).

Table 2 shows the prevalence of RLOs infection in red abalones, and the mean measurements of the 500 red abalones sampled. Eighty four abalones, with a mean size ranging from 44.0 mm (± 11.87) to 62.0 mm (± 14.24), harboured RLOs. Seventy four infected abalones had undetermined sex (immature), six were males, and four were females.

The prevalence of RLOs in summer and winter was significantly different in Dalcahue (P <0.05), however, not so in Chonchi (P = 0.076). There were no significant differences in annual prevalence of RLOs between Dalcahue and Chonchi (P = 0.009).

The parasite *Bonamia* sp. was detected as intracellular inclusions in haemocytes, always associated to haemocytic infiltrations, especially in gills (Fig. 3a,b), and also in connective tissue around gonads, digestive gland, and mantle. Table 3 shows the prevalence for the

Table 2

Prevalence of *X. californiensis* infection and size of red abalones (N° infected/N° examined) for each zone in summer and winter

Prevalencia de infección con *X. californiensis* y talla de abalones rojos (Nº infectados/Nº examinados) para cada zona en verano e invierno

Location	Summer		Winter		Total
	N°+/N° examined	Average size (mm) (± SD)	N°+/N° examined	Average size (mm) (± SD)	
Caldera	0/62	---- / 27 (± 4.14)	0/59	---- / 13 (± 1.81)	0/121
Los Molles	0/49	---- / 25 (± 6.65)	0/60	---- / 35 (± 3.46)	0/109
Dalcahue	0/38 (0%)	---- / 54 (± 21.04)	18/50 (36%)	62 (± 14.24) / 49 (± 19.68)	18/88 (20%)
Chonchi	35/112 (31%)	44 (±11.87)/ 47 (±16.19)	31/70 (44%)	47 (± 7.40) / 42 (± 8.95)	66/182 (36%)
Total	35/261 (13%)		49/239 (21%)		84/500 (17%)

Table 3

Prevalence of *Bonamia* sp. and size of *Ostrea chilensis* (N° infected/N° examined) for each zone in summer, autumn and winter

Prevalencia de *Bonamia* sp. y tamaño de *Ostrea chilensis* (N° infectadas/ N° examinadas) para cada zona en verano, otoño e invierno

Location	Summer		Autumn		Winter		Total
	N°+/N° examined	Mean size (mm) (± SD)	N°+/N° examined	Mean size (mm) (±SD)	N°+/N° examined	Mean size (mm) (±SD)	
Calbuco	5/60 (8%)	59 (± 4.83)/ 62 (± 6.78)	2/60 (3%)	75 (± 4.95)/ 73 (± 5.74)	6/60 (10%)	69 (± 3.76)/ 69 (± 3.46)	13/180 (7%)
Ancud	2/60 (3%)	58 (± 5.66)/ 64 (± 5.98)	0/60 (0%)	-----/ 60 (± 11.41)	0/70 (0%)	----/ 57 (± 15.47)	2/190 (1%)
Total	7/120 (6%)		2/120 (2%)		6/130 (5%)		15/370 (4%)

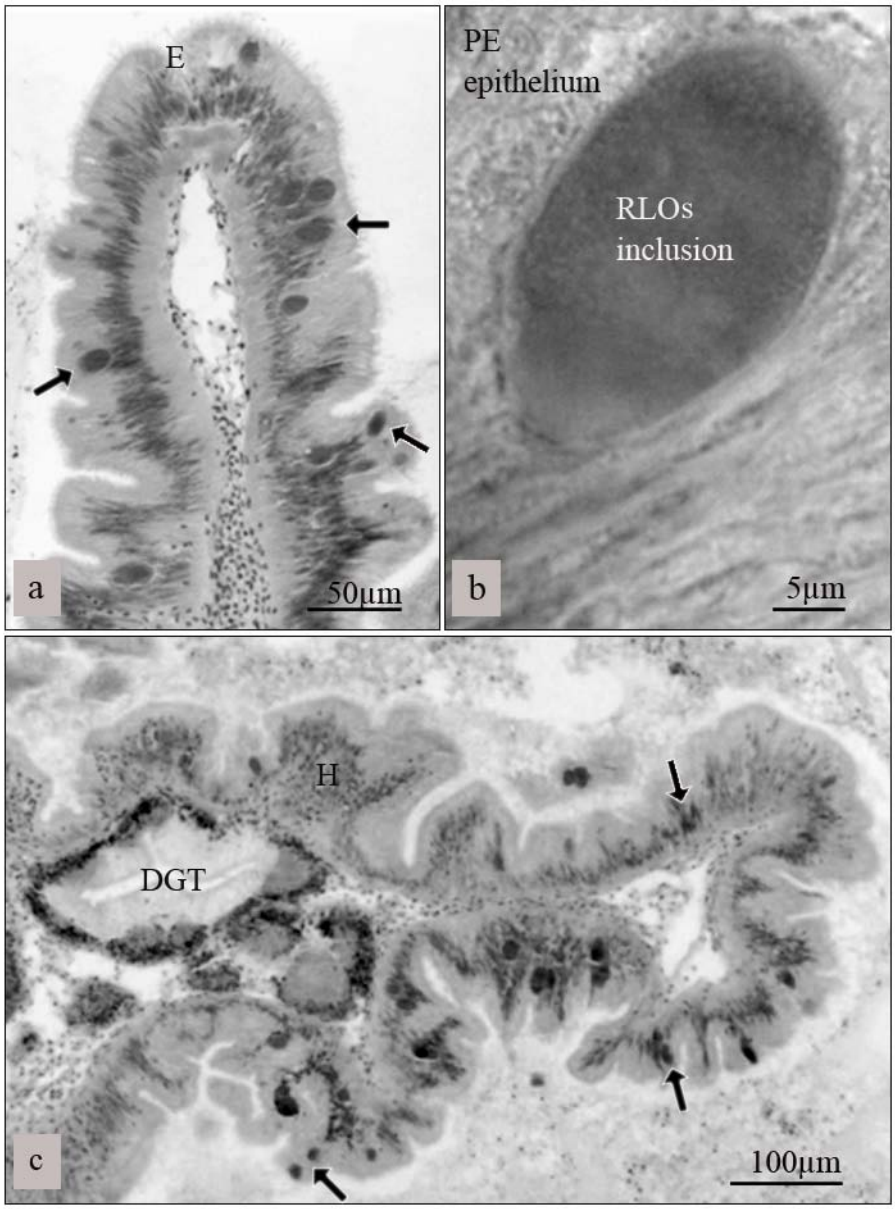

Figure 2

a) Inclusions with rickettsiales-like organisms (RLOs) (arrows) in post-esophagus (PE) epithelium (E) of red abalone. b) High power image of one inclusion with *X. californiensis* RLOs. Note the very finely granular homogenous texture of this RLOs inclusion. c) Metaplasia of digestive gland in red abalone. One digestive tubule can still be recognized (DGT). Arrows point to inclusions with RLOs (only few are labelled). H: haemocytes. Stain: H & E

a) Inclusiones con organismos tipo rickettsiales (OTR) (flechas) en epitelio (E) de post-esófago (PE) en abalón rojo. b) Imagen de una inclusión con organismos tipo rickettsiales (OTR) de *X. californiensis*. Nótese la textura finamente granular de esta inclusión con OTRs. c) Metaplasia de glándula digestiva en abalón rojo. Hay un túbulo digestivo que aún puede ser reconocido (DGT). Las flechas apuntan a inclusiones con OTR (sólo unas pocas están marcadas). H: hemocitos. Tinción: H & E

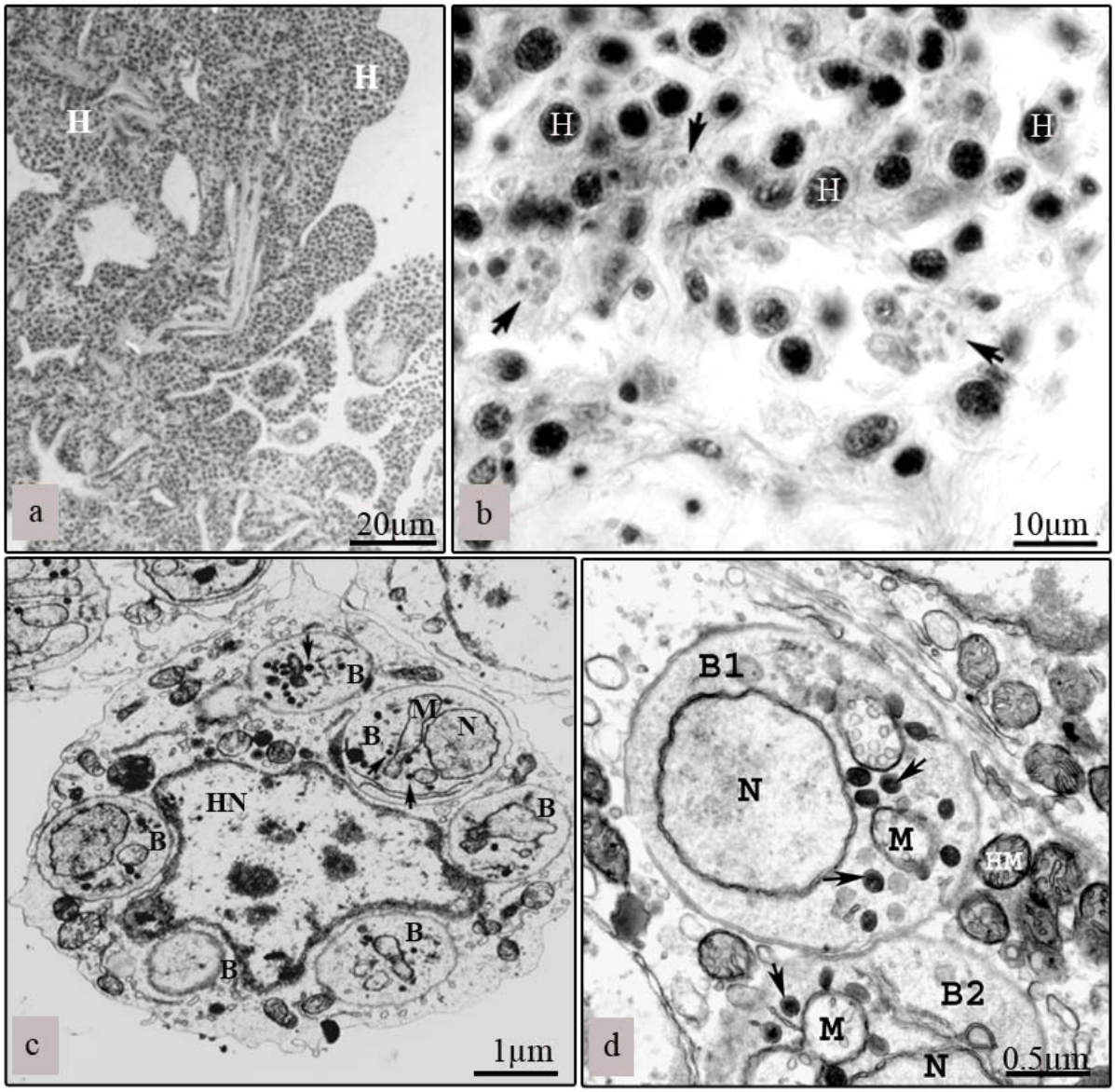

Figure 3

a) Heavy gill haemocytic infiltration in Chilean oyster infected with *Bonamia* sp. H: haemocytes. b) Higher magnification of Fig. 3a showing haemocytes (H) with *Bonamia* sp. cells in their cytoplasm (arrows). Stain: H & E. c) Transmission electron microscopy (TEM) image of one whole haemocyte with six *Bonamia* sp. (B) cells. HN: haemocyte nucleus; N: nucleus; M: mitochondrion. d) TEM image of *Bonamia* sp. cell (B1) and part of another one (B2) inside a haemocyte citoplasm. N: nucleus, M: mitochondrion, HM: haemocyte mitochondrion; Arrows: haplosporosomes

a) Masiva infiltración con hemocitos en branquia de ostra chilena infectada con *Bonamia* sp. H: hemocitos. b) Ampliación de la Fig. 3a mostrando hemocitos (H) con células de *Bonamia* sp. en su citoplasma (flechas). Tinción: H & E. c) Microscopía electrónica de transmisión (MET) de un hemocito completo con seis células de *Bonamia* sp. (B). HN: núcleo de hemocito; N: núcleo; M: mitocondria. d) MET de una célula de *Bonamia* sp. (B1) y parte de otra (B2) ubicadas en el citoplasma de un hemocito. N: núcleo; M: mitocondria; HM: mitocondria de hemocito; Flechas: haplosporosomas

Table 4

Sex categories of all Chilean oysters sampled, and of those infected with *Bonamia* sp.

Categorías sexuales del total de ostras chilenas muestreadas, y de aquellas infectadas con *Bonamia* sp.

Sex category	*Bonamia* presence		
	Negative	Positive	Total
Ambisexual	16	0	16
Female	112	6	118
Indifferent	11	0	11
Male	27	2	29
Predominantly Female	102	2	104
Predominantly Male	87	5	92
Total	355	15	370

three sampling periods and the two culture sites. A low power TEM image of one haemocyte with six *Bonamia* cells is shown in Fig. 3c. In Fig. 3d one *Bonamia* cell and a small section of another one can be observed. The *Bonamia* cells were slightly ovoid, with mean measurements of 176-147 nm (range 141-269 and 115 to 202 nm). The nucleus was electron lucent, with a spherical/ovoid shape, sometimes slightly irregular (Fig. 3c,d). The haplosporosomes were spherical/ ovoid, in variable number, and often very close to the mitochondria, which had dilated cristae (Fig. 3d).

Fifteen out of 370 oysters showed infection with *Bonamia* sp.: 13 from Calbuco (Puerto Montt), and 2 from Ancud (Chiloé). The total of sampled oysters had an average shell length of 64 mm (± 10.71), and the infected oysters a size of 65 (± 7.50) mm (Table 3). The sex categories of all sampled oysters as well as the sex categories of the infected oysters are shown in Table 4.

There were no significant differences in prevalence of *Bonamia* sp. infections between the seasons of the year ($P = 0.243$), however, there were differences between localities, being significantly higher in Calbuco ($P = 0.003$). The infection of *Ostrea chilensis* with *Bonamia* sp. was independent of host sex ($P = 0.589$).

Discussion

The results of this study show that molluscs cultivated in Chile are mostly free from OIE listed pathogens. The only notifiable OIE listed pathogen found was the rickettsial-like organism (RLO) *Xenohaliotis californiensis* that causes the foot withering syndrome in abalones. A suspected *Bonamia* microcell was found to be a true *Bonamia*, however, the correspondence to any of the two OIE listed species (*B. ostreae* and *B. exitiosa*) or if it is a new species is yet to be determined.

The rickettsial *X. californiensis* is an intracellular bacterium that lives in gastrointestinal epithelia of abalones, causing degeneration or metaplasia of the digestive gland. The abalones catabolize their own foot muscle proteins, which leads to atrophy of the foot, and often also to death (Gardner *et al.* 1995, Friedman *et al.* 2000). In this study the same type of basophilic intracellular inclusions described for black or for red abalone were observed, as well as metaplasia, which is characteristic of *X. californiensis* infection in red abalone (Moore & Robbins 2000).

This pathogen was present in both sexes, and in a broad size range. It was found only in abalones from the southern zone in both summer and winter samplings. However, subsequent analysis of red abalones (2005-2008), showed that this pathogen is also present in the northern zone (unpublished observations). As this pathogen is disseminated in the country, it has been included in List 2 of high risk diseases in Chile, and the farmers are obliged to histologically monitor the presence of this pathogen twice a year.

The prevalence of the *Bonamia*-like protozoan from Chilean oysters was relatively low (up to 6%) compared to prevalences of *Bonamia exitiosa* reported from the same oyster species in New Zealand (up to 51%). The lowest prevalence was observed in April (autumn), being highest in summer and winter. Meanwhile, the infection caused by *B. exitiosa* in Chilean oysters from New Zealand shows peak prevalence in April (autumn) during the post spawning and egg resorption period, and a second, lower peak in August (winter). The latter is characterized by low numbers of infected haemocytes in the oyster (Hine 1991) which differs from the results of this study, where high numbers of infected haemocytes were observed.

The infection of *O. chilensis* with *Bonamia* sp. was not related to seasonality. December, January and February are peak months when larval release coincides with a period of weight loss of the oysters (Solís 1967). This situation of diminished physiological condition of the host may favor the presence of the parasite in summer. According to the same study, a second period of low oyster weight is observed in winter, which may be associated to the increased presence of the parasite in individuals analyzed in August. It may be suggested the presence of this protozoan is more related to the depressed physiological condition of the host than to reproductive or environmental factors. Similarly, the prevalence of *B. ostrea* in *Ostrea edulis* is not influenced by the seasonal

temperature changes or the host reproductive cycle (Grizel *et al.* 1988, Montes 1991, Cáceres-Martínez *et al.* 1995).

The infection of *O. chilensis* with *Bonamia* sp. was independent of host sex as also occurs in *O. edulis* infected by *B. ostreae* (Culloty & Mulcahy 1996), and in *O. puelchana* infected by *Bonamia* sp. (Kroeck *et al.* 2008).

This study showed the presence of the protozoa always involved a severe haemocytic infiltration in the connective tissue of the mantle, gills and digestive gland. The same inflammatory response has been described for *O. edulis* infected with *B. ostreae* and *B. exitiosa* (Balouet *et al.* 1983, Abollo *et al.* 2008), and *Ostrea puelchana* infected with *Bonamia* sp. (Kroeck & Montes 2005).

The differences of prevalence of *Bonamia* sp. in both culture areas may be explained by the differences in size of the oyster farms. Calbuco in Puerto Montt (annual prevalence 13/180) is a large-scale farm and Ancud, in Chiloé (annual prevalence 2/190) is a small-scale farm. Since smaller stocks have reduced release of infective stages of *Bonamia*, the transmission of the parasite is more difficult (Hine 1996).

The taxonomic affiliation of *Bonamia* sp. in the Chilean oyster (*Ostrea chilensis*) is not clear. The molecular studies confirmed that it is indeed a *Bonamia* species (Campalans & Campalans[3]); however, it is still not clear if it corresponds to any of the known *Bonamia* species or if it is a new *Bonamia* species (Arzul *et al.* 2005[4]).

The ultrastructural description of *Bonamia* sp. in *O. chilensis* shows that the studied parasite belongs to the *Bonamia* genus. It is smaller than *B. exitiosa*, the parasite of *O. chilensis* from New Zealand (NZ), being more similar to *B. ostreae* in size. Lohrmann *et al.* (2009) provided a detailed ultrastructural description of this parasite in *O. chilensis* from Chile, and compared it with other known *Bonamia* species. Some features, such as number of mitochondria and of lipid droplets along with the small size, are similar to *B. ostreae*. Features such as the presence of circles of smooth endoplasmic reticulum, confronting cisternae, are similar to *B. exitiosa*, and are only found in NZ, Chilean and Australian *Bonamia*. Another common characteristic of Chilean and NZ *Bonamia* is an elevated number of haplosporosomes, and a well developed, nucleus apposed, Golgi body, which indicates active haplosporogenesis. Further studies, both ultrastructural and molecular, are needed to clarify if the Chilean *Bonamia* corresponds to any of the known *Bonamia* species, or is a new species.

Even though all samples from the four studied species were very carefully analyzed for the presence of any of the known OIE listed pathogens, the only true OIE listed pathogen detected in Chile was *Xenohaliotis californiensis*, infecting red abalone.

Acknowledgments

This research was financed by FIP grant Nº 2003-27. We thank Fidel Vargas from the TEM facilities of Universidad de Valparaíso for his skillful technical assistance. We are very grateful to three anonymous reviewers who improved this manuscript with their highly valuable comments.

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Recibido el 17 de abril de 2009 y aceptado el 4 de agosto de 2009