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Histopathological assessment of the health status of *Mytilus chilensis* (Hupé 1854) in southern Chile

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ABSTRACT

Mussel (*Mytilus chilensis*) farming is an important economic and social activity in the south of Chile. With a landing of 302,642 tons in year 2016, Chile is the second mytilid producer after China, and the first exporter worldwide. To be better prepared to protect this industry, the health status of a total number of 478 mussels *(M. chilensis)* from the Los Lagos Region, in the south of Chile, was assessed. Baseline information on symbionts, parasites and pathological conditions of both cultivated and natural mussels was collected using histology. Organisms detected included: intracellular microcolonies of bacteria (IMC), located in the epithelium of the gill and of digestive gland tubules; a microsporidian resembling *Steinhausia mytilovum* hosted by oocytes; two gill ciliates; a copepod; and a digenean trematode. Pathological conditions included neoplasia, hemocyte aggregates within digestive epithelia, lipofuscin-like pigments in various tissues, and gill epithelium desquamation. The prevalence of each finding was assessed and compared statistically between sites and between cultured and natural populations. The infection intensity of them was low, and no OIE listed parasite was detected. Of all the findings of this study, those that could be of concern are the IMC, neoplasia, and the *Steinhausia mytilovum*-like microsporidian. This study provided baseline data necessary for the design of a larger, seasonal sampling, which will allow to assess the feasibility of a permanent monitoring program to protect the huge mussel cultivating industry of the Los Lagos Region in Chile.

1. Introduction

Mussel (*Mytilus chilensis*) farming is an important economic and social activity in the south of Chile. With a landing of 302,642 tons in year 2016, this industry currently represents the second most important activity of the national aquaculture, only surpassed by the farmed salmon industry (Sernapesca, 2016). Growth rates of the industry have led to an increasing number of cultivation centers, with 99.98% of this activity concentrated in the Region of Los Lagos, covering 1120 centers registered to date. The mussel culture industry includes a fully integrated supply chain, with activities ranging from seed collection, growth (fattening) harvesting and processing of the product, generating over 17,000 jobs. At a global scale, Chile is the second mytilid producer (after China), and the main exporter of frozen (mainly) and canned product (Programa Estratégico Regional Industria de la Mitilicultura, 2015).

The mussels are cultured on ropes suspended from long-lines, and the spat is provided by the existent natural beds.

A concern in any livestock production is disease; the stress of being at high population density in aquaculture may decrease immune functions, and therefore also the resistance to disease (Bower and Figueras, 1989; Morley, 2010; Rybakov and Kholodkovskaya, 1987; Sindermann and Rosenfield, 1967; Sweet and Bateman, 2015). On the other hand, the closeness of the hosts favors the transmission of parasites, mainly those transmitted horizontally and with direct life cycles (Murray and Peeler, 2005; Nowak, 2007).

No abnormal mortalities of mussels have been reported in Chile to date, however, there have been mortality events reported elsewhere in the world (Bower and Figueras, 1989; Li and Clyburne, 1979; Munford et al., 1981; Polsenaere et al., 2017). It is vital to know what the "normal" parasites are of any species that is cultured to have a point of comparison if a disease outbreak occurs. In this sense, numerous

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surveys have been undertaken for assessing the prevalence of parasites and other pathological conditions in mussels (primarily *Mytilus edulis* and *M. galloprovincialis*) from various regions around the world. Surveys of *M. galloprovincialis* have been accomplished in Spain (Figueras et al., 1991b; Villalba et al., 1997), Greece (Karagiannis et al., 2013; Karagiannis and Angelidis, 2007), Italy (Carella et al., 2018; Matozzo et al., 2018) and in The Slovenian Adriatic Sea (Gombac et al., 2014; Kovačić and Pustijanac, 2017). In the UK, *M. edulis* and *M. galloprovincialis* have been examined from a well-documented hybrid zone (Bignell et al., 2011; Bignell et al., 2008; Lynch et al., 2014), *M. edulis* in Finland (Sunila, 1987) and in Canada (Belvin et al., 2007).

Very little is known about the health state of *M. chilensis* in Chile; disseminated neoplasia was reported in this species from Chiloé by Campalans et al. (1998). Cremonte et al. (2015) examined 175 individuals of *M. chilensis* from the Region of Los Lagos in Chile, describing the presence of intracellular bacteria-like colonies in the digestive gland and gills, *Paravortex*-like turbellaria in the intestine, and both ciliates and copepods attached to the gills. Disseminated neoplasia was also present at low prevalence.

A histological survey was undertaken on healthy *M. chilensis* from culture centers and natural beds to describe which parasites and pathological conditions are present, their prevalence and distribution in the three broad areas of cultivation of Los Lagos Region. These initial findings will serve as future reference should a mortality event occur, and will feed the design of a larger-scale, seasonal sampling. The final aim is to assess the feasibility of a permanent monitoring program, to protect the massive mussel aquaculture industry of southern Chile.

2. Materials and methods

2.1. Samples

2.1.1. Sample size

The sample size in the study area was obtained assuming maximum variability for the prevalence (P = Q = 0.5) and the finite population correction factor was ignored. The sample size formula, is given by

$$n = Z^2_{1-\alpha/2} PQ/d^2$$

where PQ = 0.25 is the variance value for the prevalence parameter; d = 0.045 is the magnitude of error and Z = 1.96 the 0.975 point of the standard Normal distribution. A total sample size of 478 was obtained; a number of 437 was allocated proportionally to the number of culture centers in these areas, Calbuco with 21%, Castro with 54% and Quellón with 24% of them. The samples were taken from one culture center in each area during July and August (Austral winter) 2016: Calbuco (41°50′S; 73°.3′W), Castro (42°21′S; 73°32′W) and Quellón (43°8′S; 73°45′W). A smaller sample size of 41 was allocated to two natural intertidal beds, El Manzano (42°1′S; 72°39′W) and La Mora (43°11′S; 73°44′) (Fig. 1).

Adult mussels (mean shell length ranging from 5,4 to 6,4 cm) were sampled for histology by severance of the posterior adductor muscle prior to placing the whole mussel into Davidson's seawater fixative (Shaw and Battle, 1957) for 24 h. Mussels were transferred into 70% ethanol prior to further processing. Following the measurement of shell length, a transverse-oblique section (≈4–5 mm) was obtained from each mussel, to include all tissues of interest (gills, digestive gland, nephridia, foot, gonad and mantle) and placed into a histocassette. Samples were processed for histology using standard protocols. A 5 µm section was cut using a rotary microtome, stained with Harris hematoxylin and eosin (H & E), and Gram stain for bacteria (Howard et al., 2004).

2.2. SEM of histological sections

For scanning electron microscopy (SEM), selected wax blocks were sectioned at 5 µm, mounted on coverslips, and prepared for scanning electron microscopy (SEM). They were dewaxed in three changes of xylene, passed through three changes of 100% ethanol, critical point dried using CO_2, and ion sputtered with gold (Lohrmann et al., 2002). They were viewed in a Hitachi SU 3500 scanning electron microscope, and images were saved. Measurements of intracellular bacteria were recorded directly with the SEM.

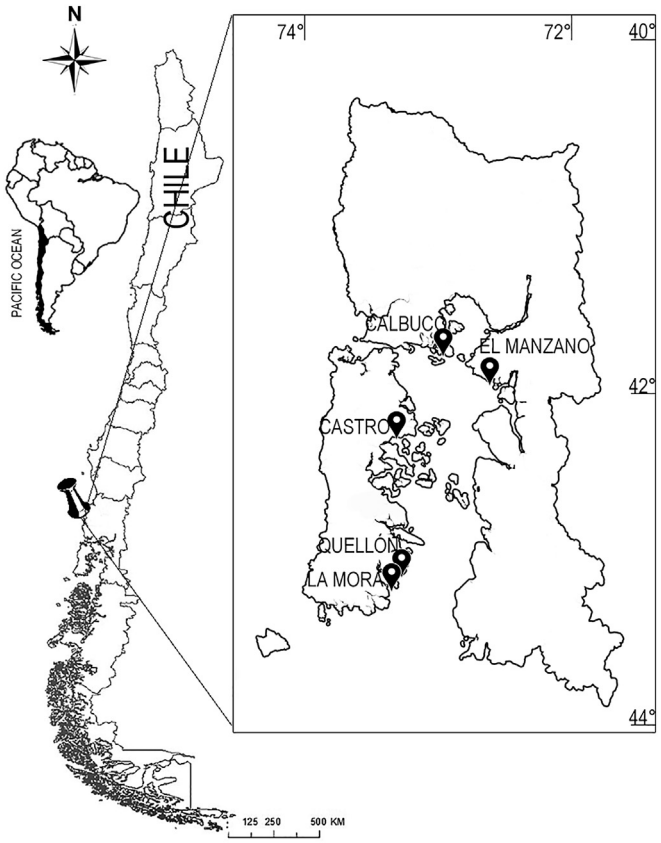

Fig. 1. Map of the Region of Los Lagos in southern Chile, Pacific coast of Southamerica. *Mytilus chilensis* were sampled from three culture areas, Calbuco, Castro and Quellón, and from two natural beds, El Manzano and La Mora. The total mytilid cultivation region corresponds to approximately 25.347 km^2.

2.3. Histological evaluation

Histological slides were observed using a Zeiss Axiostar microscope with all images being captured using an attached Canon Powershot 620 or an EOS Rebel T6 camera. Data corresponding to individual mussels were recorded using Microsoft Excel, to include: shell length, symbionts (prokaryotes, protistan or metazoan), defensive reactions and other conditions. The prevalence (presence of a given symbiont or pathological condition/number of examined specimens) was assessed. The mean intensity of infection (number of a particular symbiont or microcolony of parasites present in the whole tissue section/number of infected specimens) was assessed for the most abundant findings (Bush et al., 1997). The intensity of neoplasia was graded based on the number of neoplastic cells present and the area occupied by them: Grade I, incipient, a small number of cells present in branchial hemolymph vessel or sinus; grade II, neoplastic cells invading connective tissue and hemolymph sinuses around digestive system, nephridia and mantle; grade III, connective tissue and hemolymph sinuses completely invaded by neoplastic cells, visible on the whole organism.

2.4. Statistical analysis

2.4.1. Prevalence

Comparison of the prevalences between culture sites, between natural beds, and between cultured and natural mytilids was assessed. Two methods, the classical Wald-type asymptotic Gaussian (Vollset, 1993) and the Wilson score (Wilson, 1927), were used to calculate the confidence intervals for the prevalence of symbionts and other conditions in cultivated and natural *Mytilus chilensis*. For low prevalences the classical method failed, because the calculated lower limit can be below zero. Conversely, for prevalences approaching 100%, the upper limit may exceed 100%. To avoid this type of aberrancy the alternative Wilson Score method was used.

A chi-squared test of homogeneity was used to determine if two different culture areas are similar in prevalence and a $100(1-\alpha)$ per cent confidence limits for difference between two prevalences were calculated. Confidence limits with Wald-type asymptotic method for the difference between two prevalences, and the lower and upper confidence limits with Newcombe-Wilson (Newcombe, 1998) hybrid score method were used.

2.4.2. Intensity of infection

Mean intensity and the standard error were obtained to analyze the infection with bacterial inclusions in gills. A one-way Anova model was used to test equal mean intensity among culture areas and natural intertidal beds. A logarithm transformation was necessary for prove of the homogeneity assumption.

3. Results

The results of the histopathological analysis of 478 adult *Mytilus chilensis* from 3 culture areas and two intertidal natural beds corresponded to different types of prokaryotes, protozoa, metazoan and pathological conditions; the prevalences are summarized in Table 1, and illustrated in Figs. 2–5.

3.1. Prokaryotes

Two types of bacterial microcolonies (IMC) were detected, consisting in basophilic intracellular inclusions. The most abundant IMC type was located in the epithelium of the intermediate zone of the branchial filament (Fig. 2A). The bacteria were Gram negative as shown for the branchial IMC (inset in Fig. 2A). The multiplication of the bacteria caused morphological changes to the gill epithelium characterized by a dramatic increase in volume of the affected cells. Host cells seemed to change from being flattened in appearance, to an almost spherical dome (Fig. 2A). Fig. 2B shows an IMC inclusion viewed with scanning electron microscopy (SEM), where individual bacteria could be discerned and measured. Additionally, some fibrillar material, not visible with light microscopy, could be observed between them. The IMC inclusions measured 24.8 ± 7.98 μm in length, and 20 ± 6.95 in width (n = 50), and the rod shaped bacteria measured 936 ± 154.7 nm in length, and 432.9 ± 52.8 nm in width (n = 53).

The prevalence of these IMC was lowest in the natural bed La Mora (14.3%) and highest in the other natural bed, El Manzano (60%) (Table 1), with a significant difference ($p < .007$). In the culture areas it ranged between 32.5 and 41.7%, with no significant differences between them ($p > .181$). The difference between cultivated and natural mussels was not significant ($p > .993$). The mean intensity of infection, was lowest (3.3) in the natural bed La Mora, and highest (25.3) in the other natural bed, El Manzano. The difference in mean intensity of infection between culture areas was not significant (Anova, $p > .16$). However, comparing mussels from natural beds with cultivated ones, a significant difference was detected (Anova, $p < .015$), produced by the natural bed, El Manzano.

The other type of IMC inclusion was located in the digestive tubule epithelium or in the non-ciliated epithelial cells of secondary tubules (Fig. 2C). It was almost spherical, strongly basophilic and denser than the branchial IMCs. The inclusions measured 25.6 ± 5.5 μm × 21.1 ± 4.2 μm (n = 30). SEM showed that the intracellular bacteria were in close proximity to each other (Fig. 2D). Measurements for individual bacteria that were judged to be longitudinal (length) and transverse planes (diameter) were as follows 455.43 ± 40.5 nm in length, and 362.14 ± 32.8 nm in width (n = 14).

The digestive gland IMC were present in two of the culture sites only, with a low prevalence of 1.7% and 1.5% in Calbuco and Castro respectively. The intensity of infection was too low to be assessed, and they were absent in natural beds.

3.2. Protists

A microsporidian, similar to *Steinhausia mytilovum* was found infecting a single oocyte of an individual mussel from Castro. The spores or sporonts were located in a cytoplasmic vacuole, slightly compressing the oocyte nucleus (Fig. 3A & B). Hemocytic infiltration of the mantle and gonad was associated with the presence of this microsporidian.

An *Ancistrum* –like ciliate and an unidentified ciliate were observed on the gills. The *Ancistrum*–like ciliate had an elongated pear shape, with a very basophilic macronucleus and a small, less basophilic micronucleus, located posteriorly (Fig. 3C). It measured 45.0 ± 8.35 μm in length, and 16.4 ± 3.3 μm in width. It was attached to the gills, but did not appear to cause any defensive reaction. The highest prevalence corresponded to the natural bed El Manzano with 15%, in La Mora it

Table 1
Prevalence (%) of symbionts and other conditions in cultivated and natural *Mytilus chilensis* from the Los Lagos Region in southern Chile.

Symbiont or condition	Calbuco (n = 120)	Castro (n = 197)	Quellón (n = 120)	El Manzano (n = 20)	La Mora (n = 21)
Branchial IMC[a]	32.5	39.1	41.7	60.0	14.3
Digestive gland IMC[a]	1.7	1.5	0.0	0.0	0.0
Microsporidian	0.0	0.5	0.0	0.0	0.0
Ancistrum like ciliates	7.5	12.2	7.5	15.0	0.0
Unidentified gill ciliates	7.5	68.0	9.2	10.0	4.8
Gill copepods	8.0	2.5	2.5	10.0	0.0
Digenean metacercaria	0.0	1.0	0.0	45.0	47.6
Hemocytic infiltration	57.5	60.9	37.5	95.0	100.0
Gonad. hemocytic infiltr	22.5	6.6	15.8	75.0	57.1
Dig. epithelia HAs[b]	95.0	52.3	37.5	95.0	95.2
Lipofuscin like pigments	93.3	42.1	50.0	100.0	95.2
Gill epithel. detachment	67.5	68.5	61.7	45.0	28.6
Neoplasia	10.8	12.2	0.0	0.0	0.0

[a] Intracellular microcolonies of bacteria.
[b] Hemocyte aggregates.

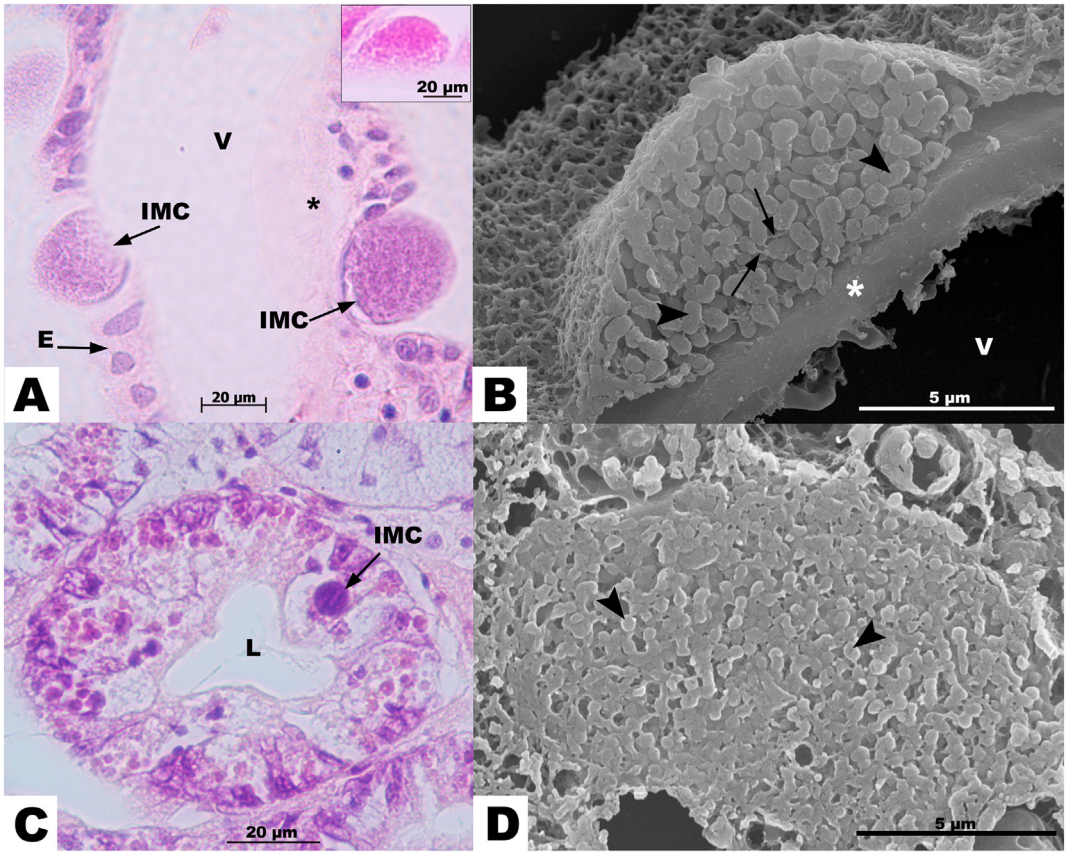

Fig. 2. Intracellular micro colonies of bacteria (IMC) in *Mytilus chilensis*. (A) Two IMC inside epithelial cells (E) of gills, hemolymphatic vessel (V) basal lamina (*) H&E. Inset: Detail of an IMC after Gram staining, showing its Gram-negative character. (B) Scanning electron microscopy (SEM) picturing one IMC inclusion. Individual bacteria can be distinguished (arrowheads), note fibrillar material between them (arrows), hemolymphatic vessel (V), basal lamina (*). (C) Intracellular micro colonies of bacteria (IMC) in a digestive tubule cell, lumen (L) of digestive tubule H&E. (D) SEM of one IMC in digestive tubule epithelium. The inclusion was very compact, with abundant extracellular matrix, only few bacteria could be discerned (arrowheads).

was 0%. The difference in prevalence between cultivated areas and natural beds was not significant ($p > .84$).

The unidentified ciliate measured 68.9 ± 9.15 µm in length, and 32.9 ± 5.98 µm in width, and was not attached to the epithelium. The prevalence was high in Castro (68%), and the lowest in La Mora (4.8%). The difference in prevalence between the cultivated areas was significant ($p = .000$), while it was not between natural beds ($p > .964$).

3.3. Metazoa

Few copepods with very low prevalence (Table 1) were detected associated to gills, however, no defensive reaction was observed (Fig. 3D). The prevalence was highest in El Manzano (10%) and 0 in La Mora. The difference in prevalence between cultivated areas and natural beds was not significant ($p > .544$).

Digenean metacercariae were only found in the intertidal mussels from the natural beds (La Mora and El Manzano). They were encapsulated by a host derived epithelium, immersed in the vesicular connective tissue of the mantle. In some cases a hemocytic infiltration was observed, as well as different degrees of degradation of the metacercariae. Fig. 3E shows one metacercaria exhibiting both suckers, oral and ventral. It was almost completely surrounded by an acellular, hyaline envelope, with the exception of the region in front of the oral sucker. The prevalence was 45.0% in El Manzano and 47.5% in La Mora, the difference was not significant ($p > .964$).

One *Paravortex* like metazoan was detected in the intestine of one mussel from Calbuco (Fig. 3F).

3.4. Other conditions

Inflammatory reactions consisted of hemocytic infiltration and small inflammatory foci. Collectively, these reactions were recorded as hemocytic infiltration and were primarily observed in the digestive gland, gills and plicate membrane. The lowest prevalence was registered in cultured mussels from Quellón (37.5%), and the highest one in the natural bed La Mora (100%). The difference in prevalence between cultivated areas and natural beds was significant ($p = .000$). In gonad, hemocytic infiltration was associated to the degradation of female or male gametes, and was registered as gonadal hemocytic infiltration (Fig. 4A & B). The highest prevalences were recorded in the natural beds, El Manzano with 75%, and La Mora with 57.1%. The difference in prevalence between cultivated areas and natural beds was significant ($p = .00$).

Two types of degenerative changes were reported, lipofuscin like pigments (LLP), and detachment or desquamation of gill epithelium. LLP was present in different organs: plicate membrane, digestive gland, gills and nephridium (Fig. 4C). The lowest prevalence was observed in Castro (42.1%), reaching up to 100% in El Manzano. The difference in prevalence between natural beds was not significant ($p > .980$), it was between natural beds and cultivated areas ($p = .000$).

Fig. 4D shows a transverse section of a branchial filament, where the three zones are indicated, frontal, intermediate, and abfrontal. The detachment of gill epithelium occurred mainly in the abfrontal region of the branchial filament, but sometimes reaching up to the frontal region, the epithelium was separated from the basal lamina (the proteinaceous skeleton) of the branchial filament (Fig. 4D). The lowest

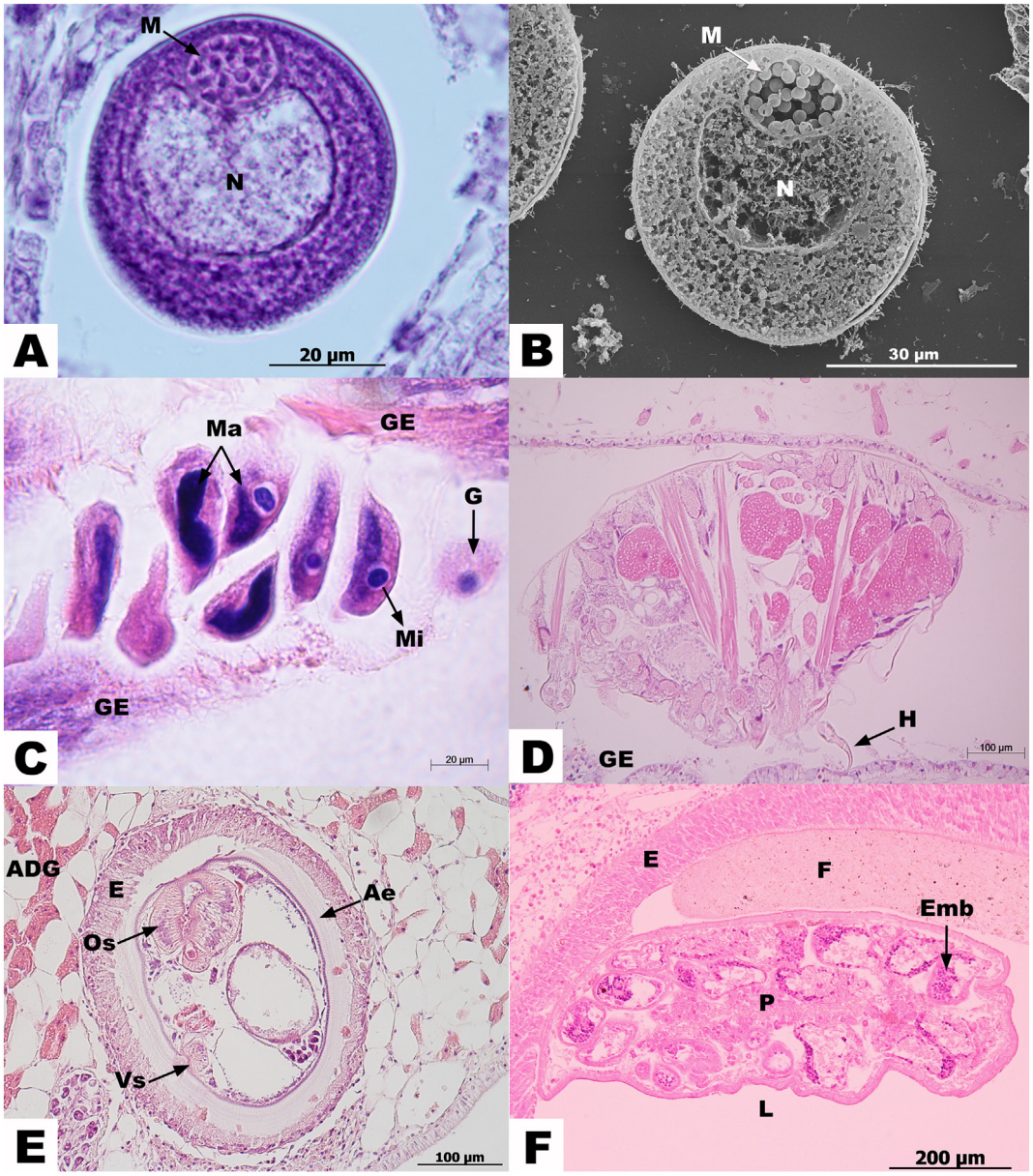

Fig. 3. Protozoa and metazoa in *Mytilus chilensis*. (A) Vacuole with developing spores of a *Steinhausia mytilovum*-like microsporidian (M) in cytoplasm of oocyte, oocyte nucleus (N). H&E. (B) The same oocyte viewed with scanning electron microscopy, vacuole with developing microsporidian spores (M) oocyte nucleus (N). (C) Several *Ancistrum* –like ciliates located on the gill filaments, gill epithelium (GE) macronucleus (Ma) micronucleus (Mi) granulocyte (G). H&E. (D) Copepod attached to the gills, hook (H) gill epithelium (GE). H&E. (E) Encapsulated digenean metacercaria immersed in the mantle connective tissue of *Mytilus chilensis*, adipogranular cell (ADG),mantle derived epithelium (E), acellular envelope (Ae), oral sucker (Os) ventral sucker (Vs) H&E. (F) A *Paravortex*- like turbellarian (P) inside the intestine of *M. chilensis*, intestine epithelium (E) faeces in intestinal lumen (F) intestinal lumen (L) embryos (Emb). H&E.

prevalence was detected in La Mora, with 28.6%, reaching close to 70% in Castro. The difference in prevalence between cultivated areas was not significant ($p > .253$), the same occurred for natural beds ($p > .443$). The difference in prevalence between natural beds and cultivated areas was significant ($p = .000$).

Hemocyte aggregates (HAs), often melanised, were detected in digestive epithelia of intestine, secondary tubules, stomach or style sac, apparently being expelled by the host (Fig. 4E). The prevalences were high, being the lowest one 37.5% in Quellón, and 95% or higher in Calbuco, and the two natural beds. The difference in prevalence between natural beds was not significant ($p > .964$), it was between natural beds and cultivated areas ($p = .000$).

Disseminated neoplasia was detected in the two culture areas of Calbuco and Castro. Variable numbers of anaplastic cells were seen in the hemolymphatic vessel of gills, and in the connective tissue around the digestive gland and intestine, the nephridia, and the mantle (Fig. 4E, F, G & H). The nuclei were bigger and more basophilic than those of normal hemocytes, the cell shape tended to be fusiform, binucleated cells (Fig. 4G) and mitotic figures were observed occasionally (Fig. 4H). The diameter of normal hemocyte nuclei ($n = 50$) was $5.73 \pm 0.56 \times 5.56 \pm 0.51$ µm, neoplastic nuclei were double that size, $11.81 \pm 1.41 \times 11.04 \pm 1.26$ µm ($n = 50$). The difference in the prevalence of disseminated neoplasia was significant between culture areas ($p = .000$) while this pathological condition was not detected in natural beds.

The intensity of neoplasia was rated in three grades, based on the area covered and the relative number of neoplastic cells, accounting for the progression of the disease. Grade I was the most frequent one (76%), and all the grade I cases found in this study (9 for Calbuco and 19 for Castro) consisted in few neoplastic cells located in the

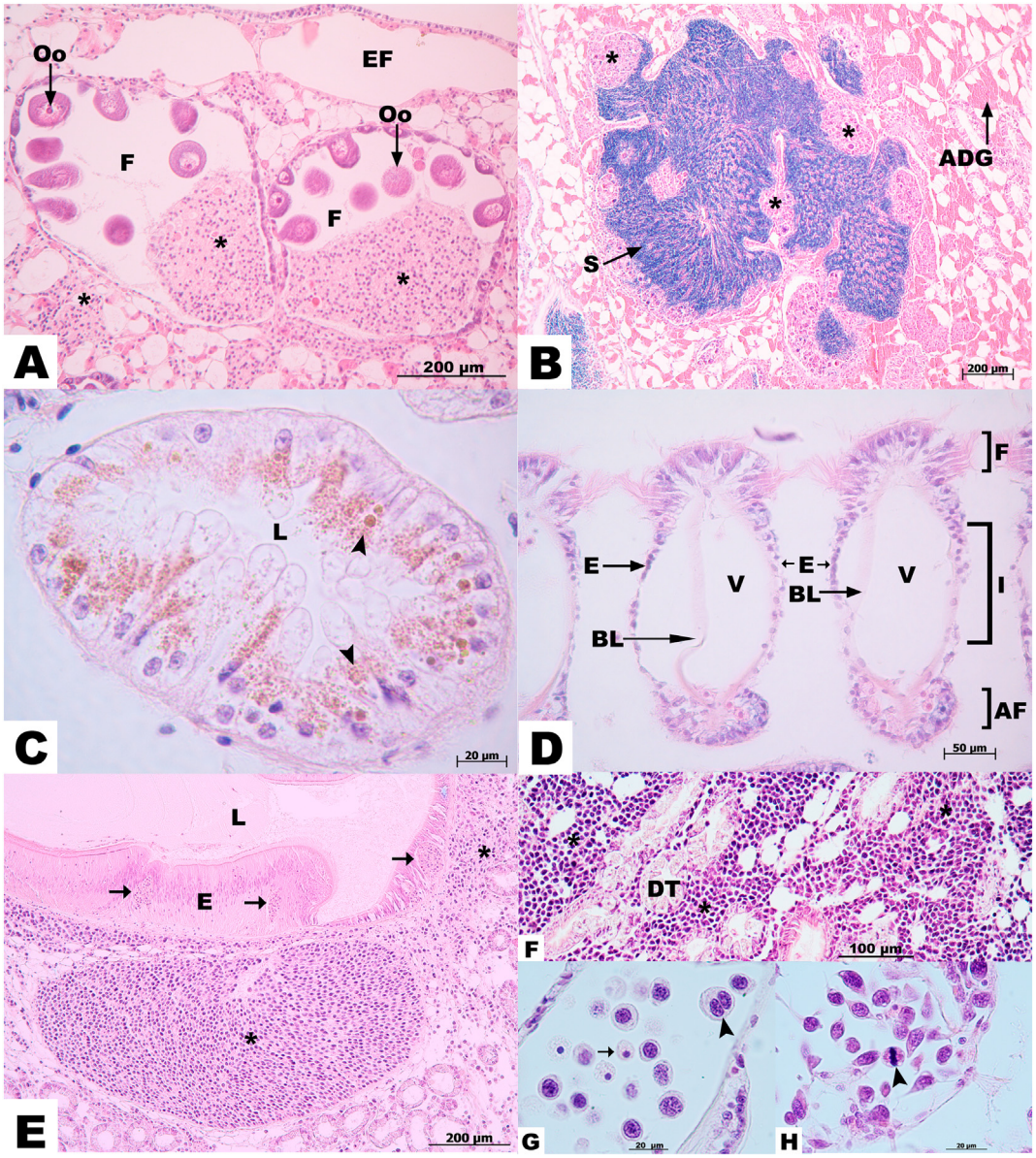

Fig. 4. Other conditions in *Mytilus chilensis.* (A) Hemocytic infiltration (*) in female gonad, follicle (F) oocyte (Oo) empty follicle (EF) note hemocytes are inside and outside the follicle. H&E. (B) Hemocytic infiltration (*) in male gonad, sperm cells (S) adipogranular cell (ADG). H&E. (C) Nephridial (kidney) tubule, lipofuscin pigments in the tubule cells (arrowheads), lumen (L). H&E. (D) Gill epithelium detachment viewed in a transversal section of the branchial filament, frontal zone (F) intermediate zone (I) abfrontal zone (AF) hemolymphatic vessel (V), note that the epithelium (E) is separated from the basal lamina (BL). H&E. (E) Hemocyte aggregates (arrows) in intestinal epithelium (E) intestinal lumen (L), grade III neoplasia, subepithelial group of neoplastic cells (*) in hemolymphatic sinus, and in connective tissue. H&E. (F) Grade III neoplasia, neoplastic cells (*) in the connective tissue of digestive gland, digestive tubule (DT). H&E. (G) Grade I neoplasia in gills, binucleated cell (arrowhead) some normal hemocytes (arrow) (H) Grade II neoplasia, metaphase (arrowhead). H&E.

hemolymph vessel of the gills (Fig. 4G) with no apparent damage to the host tissues. Grade II neoplasia was represented by 2 cases in each locality, in addition to the neoplastic cells in the branchial vessel, some foci of neoplastic cells were seen in a subepithelial location in digestive tissue. In grade III neoplasia (Fig. 4E, F) the vesicular connective tissue around the digestive gland, nephridia, mantle, and in some cases also foot muscle, was invaded by the neoplastic cells, as well as the hemolymph sinuses and vessels. There was no evidence of gametes, while the non-diseased mussels, and those with grade 1 or 2 neoplasia had different stages of gonad maturation. Some normal hemocytes could still be seen, in lower numbers though than in grade II or I neoplasia. Two cases of grade III neoplasia were found in Calbuco, and 3 in Castro.

4. Discussion

The present study established baseline information on the symbionts and other conditions of cultivated *Mytilus chilensis* and mussels from natural beds from the Los Lagos Region in the south of Chile. All the parasites and pathological conditions detected have been described before for other *Mytilus* species, and some of the parasites that could cause disease were not detected, such as the OIE listed protozoan *Marteilia* sp., intestinal copepods, castrating trematodes (such as *Proctoeces* sp.), and Apicomplexan protozoa (coccidian and gregarines). The findings were symbionts, intracellular bacteria in gills and digestive gland, two ciliates on gills, a microsporidian parasite resembling *Steinhausia mytilovum*, and two metazoan parasites. The other conditions were neoplasia, gill epithelium detachment, hemocyte aggregates,

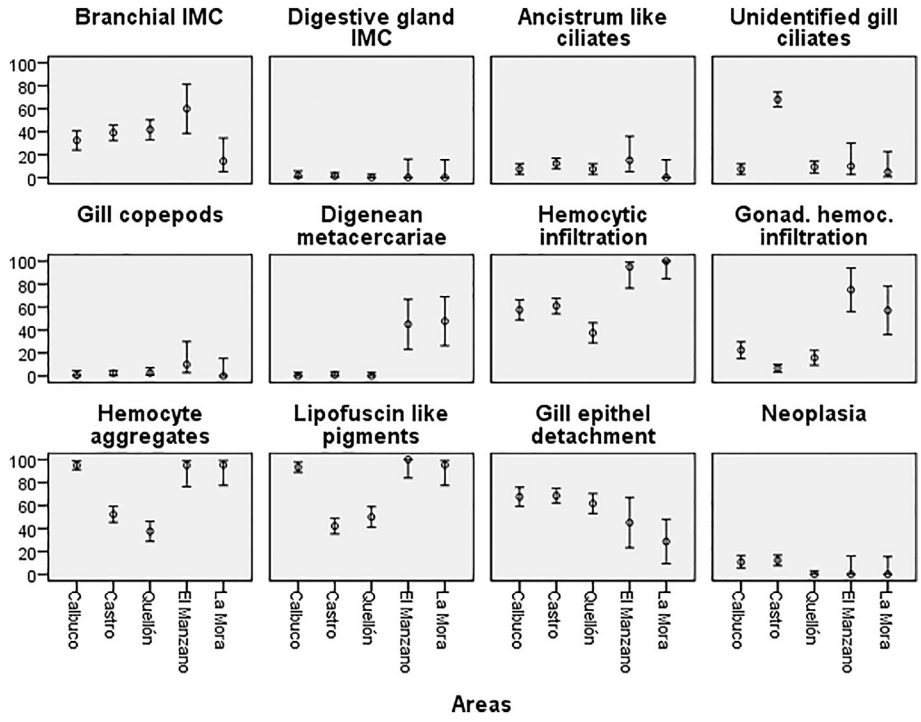

Fig. 5. Prevalences (open circles) and 95% confidence intervals for parasites and other conditions in cultived and intertidal *Mylilus chilensis* from the Los Lagos Region in southern Chile. Calbuco, Castro and Quellón denote three culture areas, El Manzano and La Mora denote two natural intertidal beds.

and lipofuscin pigments.

4.1. Symbionts

4.1.1. Intracellular microcolonies of bacteria (IMC)

Intracellular microcolonies of bacteria are common in molluscs. They have been referred as rickettsiales like organisms (RLOs) or chlamydiales like organisms (CLOs) since the first paper by Harshbarger et al. (1977), but other kind of bacteria may be involved (Cano et al., 2018). In mussels they have been detected in the digestive glands and gills of *Mytilus galloprovincialis* and *M. edulis* (Bower et al., 1994; Comps and Tigé, 1999; Figueras et al., 1991a,b; Sunila et al., 2004; Villalba et al., 1997) in gills of *M. edulis* and *Aulacomya atra* from Argentina (Cremonte et al., 2005), and in gills of *M. chilensis* from the Region of Los Lagos (Chile) with low prevalence and shown to be harmless (Cremonte et al., 2015). In this study also rather low prevalences were found, and although enough IMC inclusions were present for infection intensity to be assessed, it was low as well.

Although most of these intracellular prokaryotic organisms are considered benign, they can in occasions become pathogenic, as it has happened in different bivalves. The histopathological signs associated with mortalities consist in bacterial inclusions of enlarged size, high prevalences (over 70%), and heavy intensity of infection. Mortalities in the sea scallop *Placopecten magellanicus* occurred in Autumn-Winter 1979–80 in Rhode Island, USA, associated to prokaryotic inclusions in gills (Gulka et al., 1983). In N Brittany, France, a mass mortality (ca 40%) of the great scallop, *Pecten maximus* was reported (Le Gall et al., 1988). A more recent mass mortality of *P. maximus* occurred in Lyme Bay, on the SW coast of England, histopathological examination showed a heavy infection of the gills with intracellular microcolonies of bacteria (IMC). The molecular characterization of them using 16S rRNA had a 95% identity with *Endozoicomonas* spp. (Cano et al., 2018). Another intracellular prokaryote, *Candidatus* Xenohaliotis californiensis, infects the digestive system of the abalones, *Haliotis* spp. It is located in the transport tubules of the digestive system, starting with the posterior esophagus, and finally causing metaplasia, transforming the digestive tubules of the digestive gland into transport tubules, where the RLOs can thrive. The abalone is unable to digest its food, and ends up using his foot muscle as fuel, finally dying. The disease it causes is called withering syndrome, an OIE (World Organisation for Animal Health) listed disease, and it is present in the cultivated red abalone *Haliotis rufescens* in Chile (Friedman et al., 2000; Campalans and Lohrmann, 2009; Crosson et al., 2014).

4.1.2. Microsporidian parasite resembling Steinhausia mytilovum

The microsporidian *Steinhausia mytilovum* has been described from *Mytilus galloprovincialis* in Spain (Figueras et al., 1991b; Robledo et al., 1994; Sagristá et al., 1998; Villalba et al., 1997), Greece (Rayyan and Chintiroglou, 2003), Italy (Carella et al., 2018; Ercolini et al., 2008), Morocco (Bhaby, 2015), from *M. edulis* in the East coast of USA (Figueras et al., 1991a; Hillman, 1991; Sunila et al., 2004), in *Mytilus* spp. in France (Comtet et al., 2004) and from the UK (Bignell et al., 2008). The microsporidian parasite detected in this study morphologically resembled *S. mytilovum*, with sporonts in a parasitophore vacuole sitting on the nucleus of the oocyte. To the best of our knowledge, this would be the first record of this parasite for *M. chilensis* in Chile. A strong hemocytic infiltration was associated to the microsporidian, as has been described for *M. galloprovincialis* (Bhaby, 2015; Ercolini et al., 2008; Figueras et al., 1991b; Sagristá et al., 1998; Villalba et al., 1997); hemocytic infiltration was not found by Robledo et al. (1994). Nevertheless, it is not clear yet, if the presence of *S. mytilovum* has an impact in the reproduction of the mytilids. The prevalence of this parasite in females and hermaphrodites of *Mytilus* spp. has occasionally reached up to 35% (Rayyan and Chintiroglou, 2003); 75% (Bhaby, 2015) and 76% (Rybakov and Kholodkovskaya, 1987). However, in most cases prevalences are < 20% (Figueras et al., 1991b; Bower et al., 1994; Comtet et al., 2004), with low intensities of infection. In this study only one mussel was found infected, out of 233 possible hosts (250 females plus 8 hermaphrodites). As this sampling was done in winter, oocytes were not very numerous it has been noted that the prevalence of this parasitic protozoan is higher when abundant mature oocytes are present (Sagristá et al., 1998; Bhaby, 2015).

4.1.3. Copepoda

The parasitic copepod *Mytilicola intestinalis* is only found in *M. edulis* and *M. galloprovincialis* from Europe (Bower, 2009; Bower and Figueras, 1989). It inhabits the intestine, the hooks of their antennae irritate the intestinal epithelium in *M. galloprovincialis* from Portugal (Francisco et al., 2010); and, when in large numbers, it additionally causes intestinal obstruction in Spain (Figueras et al., 1991b; Robledo et al., 1994; Villalba et al., 1997), it has also been found in *Mytilus* spp. from the UK (Bignell et al., 2011; Bignell et al., 2008). Another copepod, *Pseudomycola spinosus*, infects *M. galloprovincialis* and *Mytilus californianus* in Baja California, Mexico, residing in gills, mantle, intestine, stomach and digestive gland (Olivas-Valdez and Cáceres-Martínez, 2002). It causes damage consisting in erosion and rupture of epithelial cells, penetration into the connective tissue of the digestive gland ending in encapsulation, and obstruction of the intestine when in high numbers. However, most copepods inhabit the pallial cavity of their bivalve hosts and are found loosely attached to the gills. Cremonte et al. (2015) described gill copepods, associated to a severe haemocytic infiltration in *Mytilus chilensis* from the Region of Los Lagos. The copepod found in this study, did not seem to elicit a defensive reaction. The histological image also looks different from the copepod found by Cremonte et al. (2015); however it is very similar to the gill copepod *Paranthessius mesodesmatis* found in histopathological slides of the yellow clam *Mesodesma donacium* from the Region of Los Lagos (Lohrmann, unpublished). López et al. (2014) detected *P. mesodesmatis* in this clam in a genetic survey in several localities of the same region, unfortunately no images were provided. The highest prevalence registered in this study was 10% in the intertidal bed El Manzano, with very low intensity, it could be an incidental finding of *P. mesodesmatis,* this is planned to be established in a future molecular study using DNA extracted from the positive tissue blocks.

4.1.4. Trematoda

Different larval trematodes have been reported in *M. edulis* and *M. galloprovincialis* from various locations (Bignell et al., 2008; Bower et al., 1994; Coustau et al., 1993; Figueras et al., 1991a,b; Francisco et al., 2010; Robledo et al., 1994; Sunila et al., 2004; Villalba et al., 1997). The metacercariae detected in this study in the mantle of the intertidal natural mussels strongly resembled the gymnophallids described histopathologically for clam species in Argentina; *Bartolius pierrei* in the clam *Darina solenoides* (Cremonte and Ituarte, 2003), and *Parvatrema* sp. in the clam *Tagelus plebeius* (da Silva et al., 2009; Ituarte et al., 2009). These metacercariae are extrapallial, the mantle epithelium in contact with them suffers a metaplasic change (from cuboidal to cilindrical), surrounds and internalizes them. In this way the metacercariae end up in the mantle tissue, encapsulated by the mantle epithelium, as it was observed in *M. chilensis* from natural beds. The gymnophallid trematode *Parvatrema duboisi* has been reported from *M. galloprovincialis* collected from the Turkish Black Sea coast. It resided in gonad, digestive gland and gills, however, no histology was performed (Ozer and Guneydag, 2014, 2015). Gymnophallid trematodes use molluscs as intermediate hosts, the final hosts are coastal birds (Lauckner, 1983; Ozer and Guneydag, 2015). This would explain why the metacercariae of this trematode were only present in the intertidal mussels, not in the cultivated, subtidal mussels, where birds have no access to them.

4.2. Other conditions

4.2.1. Hemocyte aggregates

Hemocyte aggregates (HAs) correspond to groups of hemocytes with cell debris, crossing the digestive epithelia, apparently on their way out. HAs as those observed in this study have been described as “organised lesions of large aggregations of live and necrotic hemocytes” and called abscesses by De Vico and Carella (2012). Basti et al. (2015) called them thrombi in *M. galloprovincialis* exposed to the toxic dinoflagellate *Heterocapsa circularisquama,* the main organs affected were gills, labial palps, mantle and to a lesser extent digestive gland (Basti et al., 2015). Similar HAs were shown by Sunila (1986) in intestine of *M. edulis* under influence of the outlet of a titanium dioxide plant in the Northern Baltic. She describes it as “a lot of granulocytes and macrophages among the epithelial cells of the intestine wall”, the image corresponds to what we call HAs. In this study HAs were found at high prevalences, up to 95%, and very low intensities in digestive epithelia. As this was only one sampling, and there is no “control”, i. e. *M. chilensis* cultivated in hatchery or from another region, it is difficult to know if this condition is normal for *M. chilensis,* or if it depends on the season, or on other conditions of the culture site (i.e. it could be eliminating microalgal cysts, Apicomplexan oocysts, bacteria).

4.2.2. Lipofuscin-like pigments

Lipofuscin is a brown-yellow material accumulated in lysosomes, it is undegradable by lysosomal hydrolases, and cannot be exocytosed (Brunk and Terman, 2002; Terman and Brunk, 2004). Therefore the lipofuscin content in cells of marine organisms has been attributed to age, (Terman and Brunk, 2004), pollutants (Viarengo et al., 1990) or seasonality (Petrovic et al., 2004; Koukouzika et al., 2009). It has been detected in digestive gland cells (Viarengo et al., 1990; Koukouzika et al., 2009; Raftopoulou and Dimitriadis, 2012), in kidney cells (Bignell et al., 2011), in gill epithelial cells (Gómez-Mendikute et al., 2005). In this study lipofuscin –like pigments were detected in digestive epithelia, in gills, nephridia (kidney) and plicate membrane. The prevalence was highest in one of the culture centres, Calbuco (almost 95%), and in both intertidal natural beds (over 95%). Higher prevalences were to be expected in the natural beds, since the mussels are probably older, and age is one of the causes of lipofuscin accumulation.

4.2.3. Gill epithelium detachment

Gill epithelium detachment is a well documented artifact in fish gills, which happens when a very slight delay occurs between the sacrifice, as it stops breathing, and the fixation of the fish (Speare and Ferguson, 1989; Wolf et al., 2015). Molluscs have an open circulatory system, consistent with their sessile life style, and they stay alive for long periods outside the water, *M. chilensis* lives up to three days. So probably the situation is different for molluscs, as they are still alive when being fixed. To the best of our knowledge there is no published work stating that gill epithelium detachment is an artifact in molluscs. This condition has been described associated to pollution in two mytilids; in *M. galloprovincialis* of the Thermailos Gulf in Greece, it consisted in detachment of frontal or abfrontal cells from the basement membrane. In some cases, total detachment of a wide area of epithelium was observed, this was more intense in the two most polluted stations (Domouhtsidou and Dimitriadis, 2004); and in *M. edulis* exposed to elevated concentrations of copper, detachment of abfrontal and also of endothelial cells was observed (Sunila, 1988).

4.2.4. Disseminated neoplasia

Disseminated neoplasia (DN) has been detected in at least 23 species of marine bivalves worldwide (Carballal et al., 2015). Neoplastic cells are large, with a spherical or ovoid shape, and a high nucleus: cytoplasm ratio, and are found in connective tissue, blood vessels and sinuses of multiple organs (Barber, 2004; Carballal et al., 2015). Although it has not been demonstrated, the most probable origin is hemocytic; the neoplastic cells have similar morphological characteristic to hemocytes, and they are first observed in the circulatory system, where they can be seen along with some normal hemocytes (Elston et al., 1992; Barber, 2004, Campalans et al., 1998; Carballal et al., 2015). Cremonte et al. (2011) described the onset of neoplasia starting from a heavy infiltration of normal hemocytes with a few neoplastic cells, proportion that changed with progression of the disease.

It has been detected in four species of *Mytilus*: *Mytilus trossulus*, *M. edulis*, *M. galloprovincialis* and *M. chilensis*. Significant mortalities have

only been reported in *M. trossulus* from Washington and Oregon, and in British Columbia (Elston et al., 1992). Disseminated neoplasia has been reported at low prevalences from *M. edulis* in Oregon (Farley, 1969; Mix, 1983), British Columbia (Cosson-Mannevy et al., 1984), in Western Long Island Sound (Galimany and Sunila, 2008), the UK (Lowe and Moore, 1978; Green and Alderman, 1983), and Denmark (Rasmussen, 1986). Neoplasia is infrequent in *M. galloprovincialis*, and the prevalences are low when present. In the Slovene Sea it was 1.1% (Gombac et al., 2013), 0.27% in the Rias of Galicia, and 3.4% in Delta de l'Ebre in Spain (Villalba et al., 1997; Carrasco et al., 2008), 0.5% in the Black Sea in Romania (Ciocan and Sunila, 2005); In Italy, Matozzo et al. (2018) found one individual out of a total of 600 in the La Spezia Gulf, and Carella et al. (2013) analysed 10 mussels with DN, retrieved from 3 mussel farms in Naples.

In Chile, neoplasia was first reported by Campalans et al. (1998) in *M. chilensis* from Chiloé Island, with an overall prevalence of 2.4%, ranging from 0 to 17%, no neoplasia was detected in Calbuco. Cremonte et al. (2015) detected 3.3% prevalence of neoplasia in *M. chilensis* from different localities in Chiloé Island. Cultured *M. chilensis* from the Beagle Channel, Argentina, registered a high prevalence of neoplasia, 13.3%. The 4 infected individuals were all female, and they had gonad atrophy (Cremonte et al., 2011). In this study, neoplasia was detected in two of the cultured sites, Calbuco and Castro, with prevalences slightly over 10%. The 5 mussels with grade III neoplasia had no gonads, sex could not be specified, except for one mussel with a few residual sperm cells. Gametic arrest has been associated to DN in *M. chilensis* (Cremonte et al., 2011; Cremonte et al., 2015), *M. edulis* (Farley, 1969; Cosson-Mannevy et al., 1984), and has not been reported for *M. galloprovincialis*.

Hemic neoplasia has been successfully transmitted in *M. edulis* by injecting neoplastic cell homogenate and whole cells, and by cohabitation (Elston et al., 1988). Recently a transmissible type of disseminated neoplasia was reported in the soft shell clam *Mya arenaria* and was also found in other species, including the mytilid *Mytilus trossulus* (Metzger et al., 2015; Metzger et al., 2016). In this study neoplasia was only detected in cultivated mussels, not in natural beds. It is possible to think that the close proximity of cultured mussels on the rope could favor the transmission of the disease. The fact that the initial stage of neoplasia (grade I) corresponding to 70% of the affected mussels from Calbuco and 80% in Castro, was observed in the branchial hemolymph vessel suggests that the gills could be the entry site of the neoplastic cells. Stage III neoplasia was infrequent, this could be due to the age of the cultivated mussels, they might not reach grade III neoplasia before harvest, or they might have died without being noticed.

4.2.5. Are there disease risks for M. chilensis in the Los Lagos Region?

This study is a snapshot of the health status of *Mytilus chilensis*, consisting in one sample taken in winter, covering the three big areas of mussel cultivation and two important natural beds of the Los Lagos Region in southern Chile. None of the parasites was at a high infection intensity, thus causing no disease, and only few cases of grade III neoplasia, which causes histopathological damage, were detected. However, if the host organisms are submitted to stress, such as changes in salinity, oxygen concentration, temperature or pH, reduced food (in quantity or quality), their immune surveillance becomes compromised, and their disease resistance diminished (Akaishi et al., 2007; Ellis et al., 2011; Lacoste et al., 2002; Malagoli et al., 2007). As a result, a harmless symbiont, such as the gill IMC could become a pathogen in a severely stressed population.

Other possible causes of mortalities are microalgae that are toxic for them. The Los Lagos Region has frequent algal blooms harmful for humans. The last massive bloom corresponded to *Alexandrium catenella* in 2016, it also affected some molluscs, such as the yellow clam *Mesodesma donacium* (Álvarez et al., 2017). There are several studies on the effect of this dinoflagellate on the physiology (Navarro and Contreras, 2010; Navarro et al., 2008; Velasquez and Navarro, 2014); and immunity (Detree et al., 2016) of *Mytilus chilensis*. It appears that *A. catenella* provokes limited, non lethal effects in *M.chilensis*. It is not known if microalgae that could kill *M. chilensis* are present in this region, there are some studies of the effect of challenges with microalgae on mytilids worldwide. For *M. edulis*, the pathology and immune response was evaluated after an exposure to the dinoflagellate *Prorocentrum minimum* (Galimany et al., 2008). For *M. galloprovincialis* the histopathological effect of exposure to at least two dinoflagellates has been studied: *Ostreopsis ovata*, which causes massive blooms in the Mediterranean basin (Gorbi et al., 2013; Carella et al., 2015); and *Heterocapsa circularisquama*, which causes mortalities in different molluscs in Japan (Basti et al., 2015).

Another possible source of pathogens is due to transplantation of mussels (Bower and Figueras, 1989), as would be the case of the import of juvenile mussels for ongrowth. Until now, enough *M. chilensis* seeds are collected from the natural beds in the same culture region, there has been no need to import mussels from other regions. Involuntary introduction of infectious agents could be associated to the increasing touristic activity, with about 60 cruises landing each year in the Los Lagos Region; new pathogens could arrive within mytilids attached to the ship hulls.

4.3. Concluding remarks

It can be said that the group of *M. chilensis* that was histologically evaluated was quite healthy, none of the symbionts or parasites found in this study is listed by the World Organisation for Animal Health (OIE, 2017) nor in the lists of high risk diseases in Chile (SUBPESCA, Resolución 1741/2013). The findings that could be of some concern are the intracellular microcolonies of bacteria (IMC), the microsporidian resembling *Steinhausia mytilovum* and disseminated neoplasia. All of them were present at low prevalences, and at low to very low intensities, not appearing to cause any problem. Although no immediate risk of disease for *M. chilensis* can be visualized with the findings of the present study, it is necessary to continue monitoring in different seasons, to protect the massive mussel culture industry of the Los Lagos Region in Chile.

Conflict of interest

We declare no conflicts of interest.

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