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A histopathological survey of the razor clam *Ensis macha* (Pharidae) along the Patagonian Argentina coast

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ABSTRACT

This is the first study performed to determine the health status of the razor clam, *Ensis macha*, including six different populations along Argentina Patagonian coast and one of Chile. The parasites and pathologies affecting *E. macha* were analyzed and their prevalence and mean intensity values were calculated. To establish which factors affect the presence and intensity of infection, Generalized Linear Models (GLMs) were applied. Basophilic inclusions, ciliates, coccidians protozoans and turbellarians were found. We report an Aporocotylidae digenean and hemocyte infiltrations. None of the parasites is OIE (World Organisation for Animal Health) notifiable, and none seemed to be pathogenic, with the exception of the digenean. The prevalence of the parasites was affected mainly by environmental factors (such as site of sampling and season) instead of intrinsic conditions of the clam (such as size, condition index, sex and gonadal stage). On the other hand, the maximum intensity of parasites was not only related with cold seasons but also with the partially spawned gonadal stage of *E. macha*. During this stage, the clams would need to store energy for the next gametogenesis cycle, might be more susceptible to infection by the parasites.

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1. Introduction

The razor clam *Ensis macha* (Molina, 1782) (Pharidae) is distributed along the coast of Southwest Atlantic from the Beagle Chanel (55°S) to north of San Matías Gulf (40°S) (Lasta et al., 1998; Márquez and Van der Molen, 2011), and on the Pacific coast from Caldera (27°S) to the Magellan Strait (52°S) (Osorio, 2002). It is one of the razor clam species that belong to soft bottom marine fauna, and lives burrowed in sand, silt, or mud substrates. Recent work has demonstrated that the beds from the northern Patagonian gulfs (Argentina, 42–43°S) have a potential for exploitation. The interest in the exploitation has increased after samples sent to Chile, Asia and domestic markets were found acceptable for marketing (Lasta et al., 1998). In Spain, during the last decade, the exploitation of the razor clams (*Ensis siliqua*, *Ensis arcatus* and *Solen marginatus*) has shown a significant increase (Montes Pérez, 2008). In Chile, *E. macha* is one of the most important razor clam species in volume of captures (Sernapesca, 2000), where beds have begun to show clear signs of overexploitation (Bustos et al., 1999).

Although in Chile and Europe there are studies regarding different aspects of the razor clams' biology, little is known about its health status. Montes Pérez (2008) and López Gómez et al. (2008) described the presence of parasites and pathologies in *E. siliqua*, *E. arcatus* and *S. marginatus*; López and Darriba (2006) and Ceschia et al. (2001) reported infection of *Marteilia* sp. in *S. marginatus* from Galicia, Spain and in *Ensis minor* from Italy, respectively. The disease caused by *Marteilia refringens* is currently listed by the World Organisation for Animal Health (OIE, 2011) as a notifiable disease. In Argentina, there are only two studies regarding morphometry, growth and reproduction aspects of *E. macha* (Barón et al., 2004; Robledo, 2009) and nothing about its health status. Therefore, this study was performed to determine a histological health status of the razor clam *E. macha* from Patagonian Argentina coast. Furthermore, to evaluate the factors affecting the presence and intensity of infection, the seasonal and geographical variations of prevalence values as well as their relationship with the condition index, size, sex and gonadal stages were analyzed in two populations in the northern Patagonian gulfs.

2. Materials and methods

2.1. Sample collection

During 2007, 480 razor clams of 124.42 ± 11.87 mm (mean ± SD) were seasonally collected at 10 m depth at Puerto Lobos (42°00′S, 65°05′W – San Matías gulf) and at Fracasso Beach (42°25′S, 64°07′W – San José gulf) (Fig. 1) (60 clams collected

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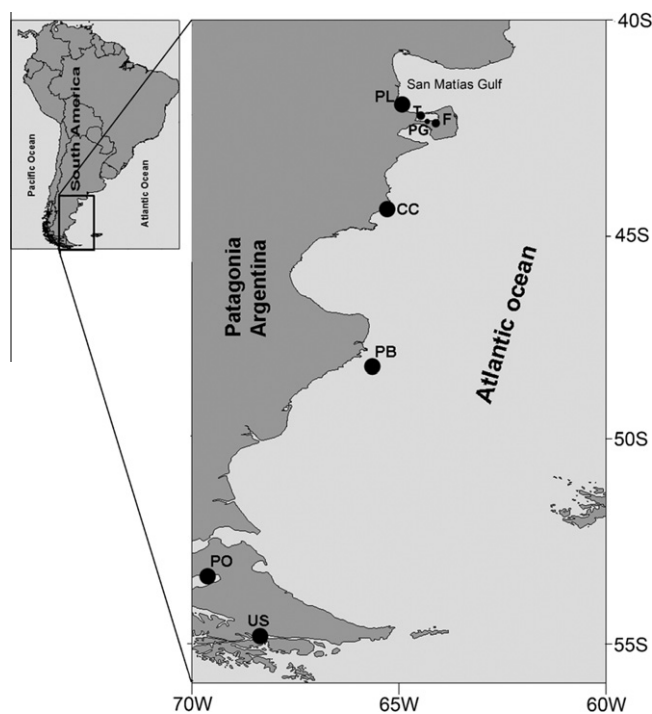*E-mail address:* fcremont@cenpat.edu.ar (F. Cremonte).

Fig. 1. *Ensis macha* sampling populations: Puerto Lobos (PL), La Tapera (T), Punta Gales (PG), Fracasso Beach (F), Caleta Carolina (CC), Punta Buque (PB), El Porvenir (PO), Ushuaia (US).

during each season and at each population). The clams were collected by scuba diving using a water jet pump and transported to aquaria with aerated seawater, maintained for 24 h until processing. Moreover, from 2006 to 2009, one sample of approximately 30 clams were taken of each of the following populations: La Tapera (42°33′S, 64°55′W) ($n = 30$), Caleta Carolina (44°54′S, 65°35′W) ($n = 30$), Punta Buque (48°2′S, 65°55′W) ($n = 28$), El Porvenir (53°24′S, 69°54′W) (Chile) ($n = 21$) and Ushuaia (54°48′S, 68°15′W) ($n = 28$) (Fig. 1).

2.2. Histological processing

Maximum shell length of each specimen was measured; shell and flesh were weighed separately to calculate the condition index, as the ratio of the wet flesh weight to shell weight × 100 (Lucas and Benninger, 1985). Soft parts were fixed in Davidson's fixative (Shaw and Battle, 1957) for 24 h. Two oblique transverse 5 mm thick sections, containing gill, digestive gland, mantle, nephridia and gonad (Fig. 2) were taken from each clam. Tissue samples were embedded in paraffin and then 5 μm sections were stained with haematoxylin and eosin. Histological sections were examined un-
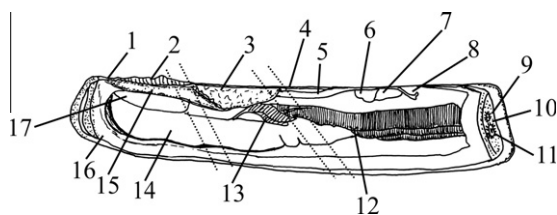

Fig. 2. Diagram of the two oblique cuts of 3–5 mm thick (dotted line) through the visceral mass of *Ensis macha*, which were included in the biopsy cassette. Abbreviations: 1: hinge; 2: ligament; 3: digestive gland; 4: pericardial cavity; 5: rectum; 6: posterior adductor muscle; 7: foot retractor muscle; 8: anus; 9: pallial tentacles; 10: inhalant siphon; 11: exhalant siphon; 12: gills; 13: labial palps; 14: foot; 15: gonad; 16: robe; 17: anterior adductor muscle.

der a light microscope for presence of parasites and pathological alterations. For each tissue section, sex and gonadal stages were recorded. A 6 stage gametogenic scale was determined after examining the oocytes and espermatocytes developmental pattern, following Robledo (2009): (1: early active, 2: late active, 3: ripe, 4: partially spawned, 5: spawned, 6: restoration). For the hemocyte infiltration a qualitative scale was established based on the connective tissues area covered by hemocytes: low (1), moderate (2) and heavy (3).

2.3. Statistical analyses

Clams collected from all populations were examined for parasites and pathologies, and Prevalence (P) and Mean Intensity (I) of the different parasites were calculated. Mean intensity was calculated as the number of parasites per total parasitized hosts (Bush et al., 1997). The intensity was estimated by counting the number of parasites in each histological section of 5 μm using a magnification of 400×. Only the samples from Puerto Lobos and Fracasso Beach were included in the following statistical analyses.

To evaluate different factors affecting the presence and intensity of parasites, Generalized Linear Models (GLMs) were applied. Presence-absence of parasites (binary response) was evaluated by GLMs with binomial distribution with a logit link function, and intensity of parasites (count data) was evaluated with Poisson distribution of response variable with a log link function (Agresti, 2007). Different models were used to test these variables with regard to the following explanatory variables: site (Puerto Lobos, Fracasso Beach), season (1: summer, 4: autumn, 7: winter, 10: spring), sex (1: male, 2: female), gonadal stages (1 to 6), shell size and condition index.

The Akaike information criterion (AIC) was used to determine the best model for the analyzed data set. Model selection was performed with an Information Theory (IT) approach using Akaike's information criterion (AIC) and Model averaging (Burnham and Anderson, 2002; Grueber et al., 2011). The AIC values (Akaike, 1973) and the AIC for small samples (AICc) (Hurvich and Tsai, 1989) were calculated for each model. From the AICc differences (Δi), where $\Delta i = AICC (i) – AICC (min)$, Akaike weights (wi) (Akaike, 1978) were obtained for all candidate models. For each data set, the models were ranked by their wi values; the model with the highest wi was considered the one with the best supporting data (Burnham and Anderson, 2002). Model averaging was calculated using candidate models, which together account for 95% confidence interval. The top model set was averaged using the zero method (Symonds and Moussalli, 2011), where the best AIC model was not strongly weighted. The results are expressed in terms of odd ratio. The odds are calculated as the exponential of the coefficient of each parameter corresponding to the averaging model.

All statistical analyses were performed in R (R Development Core Team 2011). The standardized function to input variables is available within the arm package (Gelman et al., 2009). Model selection and averaging were calculated with the MuMIn package (Barton, 2009).

3. Results

A summary of the main characteristics (sex ratio, shell size and condition index) of *E. macha* from Puerto Lobos and Fracasso Beach, and the results of the histological examinations (parasites, pathologies, mean prevalence and mean intensity) from all sampling populations are presented in Tables 1 and 2 respectively.

Intracellular inclusions caused by prokaryote-like organisms in the digestive gland epithelium, ciliates in gills, turbellarians of the genus *Paravortex* Wahl, 1906 in the intestine lumen and different

Table 1
Main characteristics of *Ensis macha* (sex ratio, shell length in mm and condition index) from Puerto Lobos and Fracasso Beach during 2007.

Population	Summer	Autumn	Winter	Spring
Puerto Lobos				
% Sex F:M	40:60	50:50	47:53	50:50
Range shell size	108.8–221.63	83.32–151.6	95.18–152.66	79.05–221.63
Condition index	154 ± 17.1	157.43 ± 37.2	129.09 ± 24.4	160.8 ± 26.01
Fracasso Beach				
% Sex F:M	42:58	55:45	40:60	40:60
Range shell size	85.2–139.77	96.32–138.38	98.01–145.09	85.2–147.98
Condition index	200.73 ± 23.93	201.36 ± 32.95	174.09 ± 25.25	190.57 ± 23.47

Table 2
Mean prevalences (*P*) and mean intensities (*I*) of parasites and pathologies affecting *Ensis macha* from all sampling populations; *n* = number of specimens analyzed.

Population	*n*	Date of collection	Mean maximum shell (mm)	Prokaryote-like colonies		Ciliate		Coccidian	*Paravortex* sp.		Trematode	Hemocyte infiltration	
				P	*I*	*P*	*I*	*P*	*P*	*I*	*P*	*P*	*I*
Puerto Lobos	240	January–November 2007	125.2	3.8	8.8	54.6	4	0 (*n* = 50)[a]	4.2	1	0	81.9	2.8
Fracasso Beach	240	January–November 2007	123.4	3.8	19.3	39.2	3.9	0 (*n* = 46)	8.3	1.4	0	82.5	2.9
La Tapera	30	November 2006	117.9	0	–	60	1.9	–	6.7	1	3.3	43.3	2.2
Caleta Carolina	30	September 2008	114	16.7	8.2	36.7	2.1	50 (*n* = 10)	0	–	0	73.3	1.6
Punta Buque	28	February 2009	124.1	57.1	52.6	0	–	55.5 (*n* = 9)	0	–	0	82.1	2.1
El Porvenir	21	April 2009	140.2	38.1	2.1	14.3	1.3	–	4.8	1	0	76.2	3.8
Ushuaia	28	April 2009	139.3	85.7	37.1	7.1	9.5	20 (*n* = 5)	0	–	0	85.7	1.9

[a] The nephridium, where the coccidians were observed, was not included in all histological sections.

levels of hemocyte infiltration in connective tissue of several organs were recorded. Renal coccidian protozoans were observed at the 3 southern populations (Caleta Carolina, Punta Buque and Ushuaia), and La Tapera was the only population where a digenean of the family Aporocotylidae Odhner 1912 was found in the gonad of clams.

Rounded intracellular inclusions of prokaryote-like colonies, with 6.65 ± 1.79 μm of length were found in the epithelium of the digestive gland. These colonies occupied the host cell cytoplasm, causing in some cases the hypertrophy of them (Fig. 3a). Nevertheless, there was no apparent host reaction. The maximum values of prevalence and mean intensity were 6.7% and 18 colonies per histological section respectively, for clams from Puerto Lobos, and 8.3% and 27 colonies per histological section respectively for clams from Fracasso Beach; no prokaryote-like colonies were observed in summer at Puerto Lobos and in spring at Fracasso Beach (Fig. 5a).

Unidentified ciliates were recorded mainly in gills and often in labial palps (Fig. 3b). These protozoans were oval-shaped, with dense ciliature, and measured 31.02 ± 6.21 μm in length. These ciliates do not appear to cause any specific host response. The maximum values of prevalence and mean intensity were 88% and 4.8 ciliates per histological section respectively for clams from Puerto Lobos, and 80% and 4.6 ciliates per histological section respectively for clams from Fracasso Beach. The lowest prevalences were recorded during summer at both Puerto Lobos and Fracasso Beach (Fig. 5b).

Coccidians were observed filling the nephridial tubules at three populations (Caleta Carolina, Punta Buque and Ushuaia), associated with the presence of "brown cells" in the tissues of infected clams. Different stages of the coccidian (trophozoites, large and crescent-shaped macrogamonts, and both mature and immature meronts containing merozoites) were observed in the nephridian lumen (Fig. 3c). No hemocyte response was elicited by infection of this parasite. The maximum prevalence was 55.5% at Punta Buque (Table 2).

Turbellarians of genus *Paravortex* (Rhabdocoela) were observed in the intestinal lumen and were characterized by their ciliated epidermis, ocelli, muscular pharynx, short esophagus and paired embryos in the parenchyma (Fig. 3d). No histopathological damage was observed. The maximum values of prevalence and mean intensity were 10% and 1 turbellarian per histological section respectively for clams from Puerto Lobos, and 25% and 1.5 turbellarian per histological section respectively for clams from Fracasso Beach. The lowest prevalences were recorded during summer at both Puerto Lobos and Fracasso Beach.

Sporocysts of a digenean of the family Aporocotylidae were found in one clam from La Tapera population (Table 2). Sporocysts were small and oval shaped, with a thick wall and containing germ cells and cercariae at different developmental stages. Cercariae had no suckers and presented penetration glands and a long and forked tail (Fig. 3e). The razor clam infected by this parasite was completely castrated, and digestive gland, foot muscle and gills were also parasitized.

Heavy hemocyte infiltration was found in the connective tissues of the gonad, gills, digestive gland (Fig. 3f), intestine and nephridia of most clams from all studied populations. This condition could not be linked to any detectable pathogenic organism. The maximum prevalences of infiltration were 95% and of 96.7% in winter at Puerto Lobos and Fracasso Beach, respectively (Fig. 5d). In some specific cases, encapsulations were observed in the foot as groups of hemocytes surrounding the strange particle (hemocytosis); calcareous concretions in several organs and granulocytomas in the gills. None of these abnormalities were found to be associated with any pathogenic agent that could be detected by histological methods (Fig. 4).

The presence of parasites was studied as a function of site, season, sex, gonadal stage, shell size and condition index. Sixty-four candidate models were compared. The presence of parasites was mainly affected by site and season (variables with 100% of relative importance) (Table 3). The probability of finding a clam parasitized in Puerto Lobos was 2.23 ± 1.31 times higher than in Fracasso Beach. The prevalence of parasites was highest in winter and lowest in summer (Fig. 6). Taking the summer (1) as reference point, the probability of finding a clam parasitized was 181.27 ± 1.79 times higher in winter (7), 68.03 ± 1.75 times higher in autumn (4) and 24.78 ± 1.73 times higher in spring (10).
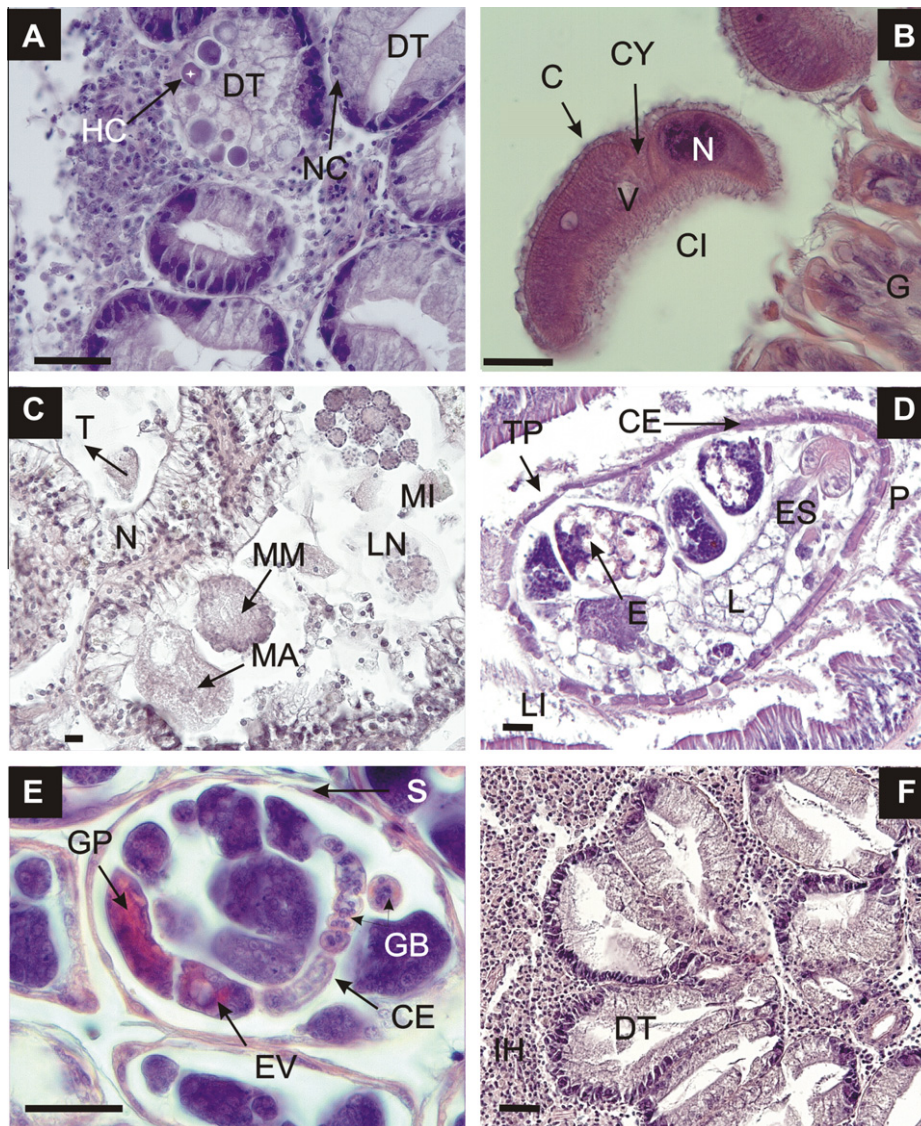
Fig. 3. Photomicrographs of *Ensis macha* histological sections (H and E). Scale bars: 20 μm. (A) Hypertrophy epithelial cells (HC) containing Prokaryote-like colony within digestive tubule (DT) and normal epithelial cells (NC). (B) Ciliate (C) in the gills (G) showing the dense ciliatura (CI), the macronucleus (N), vacuoles (V) and the cytostome (CY). (C) Different stages of coccidian in lumen (LN) of nephridia (N): trophozoits (T), macrogamonts (MA), and both mature (MM) and immature (MI) meronts containing merozoites. (D) Turbellaria *Paravortex panopea* (TP) within the lumen of the intestine (Li), showing ciliated epithelium (CE), pharynx (P), esophagus (ES), the saccular intestine (I) and embryos (E). (E) Digenean trematode of the family Aporocotylidae, oval sporocysts (S) and cercaria (CE) with penetration glands (GP) excretory vesicle (EV), a long brevifurcate tail (T) and germ balls (GB). (F) Hemocyte infiltration (IH) in connective tissue of the digestive gland (DT).

The intensity of parasites was also evaluated by the same variables. Sixty-four candidate models were compared. Intensity of parasites was mainly affected also by season and by gonadal stages (variables with 100% and 98% of relative importance, respectively) (Table 4). It was higher in cold seasons and decreased in summer (Fig. 7). Taking the summer (1) as reference point, the number of parasites was 33.97 ± 1.99 times higher in winter (7), 26.05 ± 2.00 times higher in autumn (4) and 14.44 ± 2.02 times higher in spring (10). Although not being able to reach a conclusion regarding the gonadal stages because the standard error of this variable was very high (some categories have few observations and others have many observations), there is a tendency that clams hosted more parasites during the partially spawned stage (Fig. 8).

4. Discussion

This work presents the first histopathological study of the razor clam *E. macha*. All parasite taxa (prokaryote-like colonies, ciliates, coccidian, turbellarian and digenean) have been previously reported in other members of the superfamilia Solenacea (Darriba et al., 2010; López Gómez et al., 2008).

Prokaryote-like colonies were reported from different tissues of diverse bivalve species (Lauckner, 1983). Nevertheless, heavy infections of intracellular colonies of these organisms could be very pathogenic since infections by prokaryote-like colonies have been associated with mass mortality in some bivalves such as scallops (Gulka et al., 1983; Le Gall et al., 1988), clams (Norton et al., 1993; Villalba et al., 1999) and abalones (Friedman et al., 1997). In *E. siliqua* and *S. marginatus* from natural Spanish beds, prokaryote-like colonies have been reported with no pathologies associated (Darriba et al., 2010; López Gómez et al., 2008; Montes Pérez, 2008). In the present study, only a few cells were affected, causing hypertrophy; however, the intensities of infection observed were low in all cases.

Ciliates are common inhabitants of the bivalve gills (Bower et al., 1994; Lauckner, 1983) but they are usually considered harmless commensals (Boussaïd et al., 1999). In this study, ciliates were
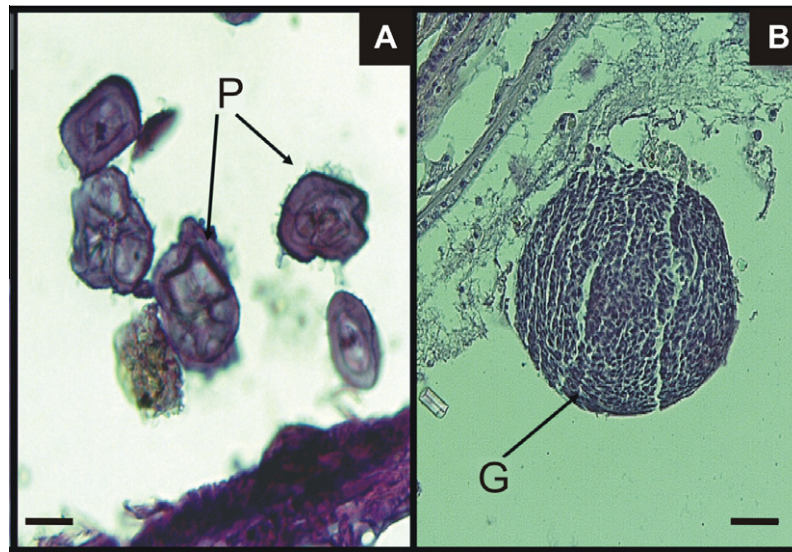
Fig. 4. Photomicrographs of *Ensis macha* histological sections (H & E). Scale bars: 20 μm. (A) Calcareous concretions (P) in the gill. (B) Cytoma-granule (G) in the gill.

observed predominantly in the gills, without causing any apparent pathology, probably due to the low intensity of infection recorded.

Fig. 5. Prevalence (%) of parasites and hemocyte infiltration in *Ensis macha* for populations of Puerto Lobos (PL) and Fracasso Beach (F) in different seasons .

In *E. arcuatus*, *E. siliqua* and *S. marginatus* from Spain, ciliates similar to *Trichodina* have been reported in gills and labial palps, also, without pathological reaction (Darriba et al., 2010; López Gómez et al., 2008).

Renal coccidians are commonly reported inhabiting bivalves (Lauckner, 1983), usually without severe pathologies associated (Bower et al., 1994), although some reports describe heavy infections associated with nephridia damage (Carballal et al., 2001; Cremonte et al., 2005; Morado et al., 1984) and related with the presence of numerous brown cells. In the present study, although the coccidians were confined to the nephridia, there was an increment of brown cells throughout the bodies of the heavily infected clam and they appeared to be migrating across the epithelium by diapedesis (Morado et al., 1984). Similar results have been reported for *E. siliqua*, *E. arcuatus* and *S. marginatus* from Spain (Conchas et al., 2001; Darriba et al., 2010; Montes Pérez, 2008), without pathological alterations.

Turbellarian members of *Paravortex* sp. are commonly reported obstructing the intestinal lumen without causing lethal effects in bivalves (Jennings, 1971). Brusa et al. (2011) reported specimens of *E. macha* from Punta Gales (San José gulf) infected with *Paravortex panopea* Brusa, Vázquez and Cremonte (2011), obstructing the intestinal lumen. In this study, mature turbellarians were recorded in the intestinal lumen without any apparent damage and these probably belong to the same species reported by Brusa et al. (2011) from Punta Gales. Prevalence and mean intensity recorded in this study were lower. They were even lower than those recorded for the geoduck clam *Panopea abbreviata*, an infaunal clam that cohabits with *E. macha*, and that showed prevalences from 23.96% to 30.33% and mean intensity from 1 to 15 per histological section (Brusa et al., 2011). Nevertheless, there is evidence that the prevalence and mean intensity of the turbellarians depend on the examination method, since the histological method underestimates the real value of these parameters (Brusa et al., 2011); therefore the true prevalence and the intensity of infection would likely be higher than what we observed in the present study. In *S. marginatus* and *E. siliqua* from Spain, another member of a *Paravortex* sp. was reported in the lumen of the intestine, with up to 26% prevalence and low intensity of infection (López Gómez et al., 2008).

Digeneans typically have a dramatic effect on the health of their molluscan first intermediate host. Intramolluscan stages live within the hemocoel and occupy the gonadal space causing castration
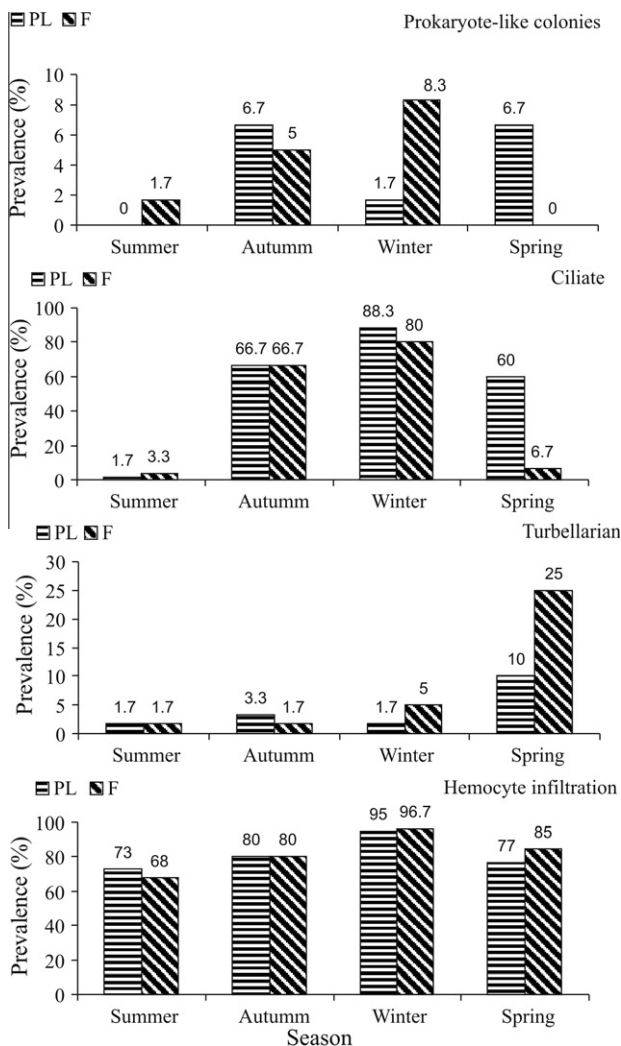
Table 3
Summary results after model averaging: effect of each variable on presence of parasites of *Ensis macha*.

Parameter	Coefficient	Adjusted SE	Confidential interval		Relative importance
			Lower	Upper	
(Intercept)	−3.77	103	−205	197	
Sex	−0.0538	0.16	−0.368	0.26	0.31
Site (Puerto Lobos)[a]	0.801	0.269	0.275	1.33	1
Gonadal stage2[a]	0.446	103	−201	201	0.03
Gonadal stage3[a]	0.429	103	−201	201	0.03
Gonadal stage4[a]	0.428	103	−201	201	0.03
Gonadal stage5[a]	0.425	103	−201	201	0.03
Gonadal stage6[a]	0.417	103	−201	201	0.03
Season10[a]	3.21	0.55	2.14	4.29	1
Season4[a]	4.22	0.558	3.12	5.31	1
Season7[a]	5.2	0.585	4.05	6.35	1
Conditional index	0.027	0.178	−0.322	0.376	0.26
Size	0.00902	0.138	−0.262	0.28	0.26

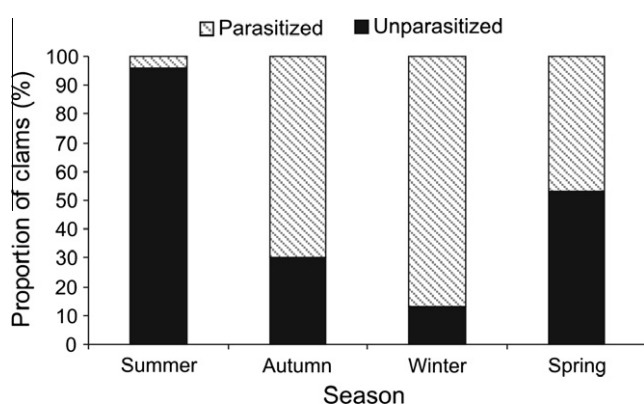[a] Fracasso Beach. Gonadal stage 1 and season 1 were the reference categories.

Fig. 6. Proportion of clams *Ensis macha* (%) parasitized and unparasitized in different seasons.

(Cribb et al., 2003). Their larvae have been reported in almost all bivalves (Cremonte, 2011; Lauckner, 1983). In this paper, we report *E. macha* as first intermediate host of an aporocotylid digenean. This is the second record of the larval member of Aporocotylidae in South America, since it was recently reported in the clam *Amiantis purpurata* (Gilardoni et al., 2011). The infected razor clam showed complete castration (i.e. the gonad was not developed),

even though developed gonads were observed in other clams from La Tapera.

Unusually high levels of hemocyte infiltration were observed in connective tissue of several organs (gonad, gills, digestive gland, intestine and nephridia) of *E. macha*. Histological techniques did not reveal the causes of this infiltration, and taking into account that most of the specimens studied exhibited hemocyte infiltration, it could be attributed to the stress generated by the sampling method (water pump).

The presence of the parasites seemed to be affected mainly by environmental factors, as site and season, instead of intrinsic conditions of the clam. The higher density of the natural beds of *E. macha* at Puerto Lobos (Ciocco et al., 2001) could explain the higher prevalence of parasites recorded in this population, since the close contact among the clams, favors the transmission of the parasites. The highest intensity was recorded during cold seasons, corresponding with the poorest condition index and with the partially spawned stage of *E. macha*. During this stage, the clams would need to store energy for the next gametogenic cycle and might be more susceptible to infection.

The populations of *E. macha* studied seem to be devoid of serious pathogens. None of the parasites found in this study appears to be a problem to the fishery as well as to future farming, based on either low infestation levels or low pathological effects. Moreover, none of these parasites is OIE notifiable.
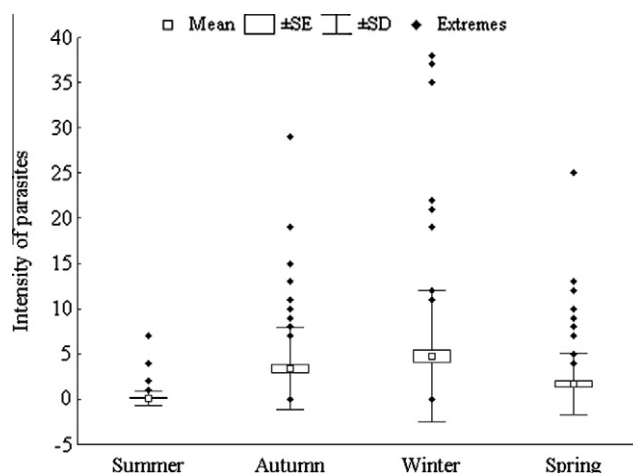
Table 4
Summary results after model averaging: effect of each variable on intensity of parasites of *Ensis macha*.

Parameter	Coefficient	Adjusted SE	Confidence interval		Relative importance
			Lower	Upper	
(Intercept)	−16	975	−1930	1900	
Sex	0.0161	0.0847	−0.15	0.182	0.27
Site (Puerto Lobos)[a]	0.044	0.127	−0.205	0.294	0.32
Gonadal stage2[a]	14.3	975	−1900	1930	0.98
Gonadal stage3[a]	13.9	975	−1900	1930	0.98
Gonadal stage4[a]	14.2	975	−1900	1930	0.98
Gonadal stage5[a]	13.4	975	−1900	1920	0.98
Gonadal stage6[a]	12.5	975	−1900	1920	0.98
Season10[a]	2.67	0.703	1.29	4.04	1
Season4[a]	3.26	0.691	1.91	4.62	1
Season7[a]	3.61	0.687	2.27	4.96	1
Conditional index	0.0527	0.136	−0.214	0.32	0.33
Size	0.018	0.0991	−0.176	0.212	0.28

[a] Fracasso Beach. Gonadal stage 1 and season 1 were the reference categories.

Fig. 7. Intensity of parasites of *Ensis macha* (box plot) in different seasons.
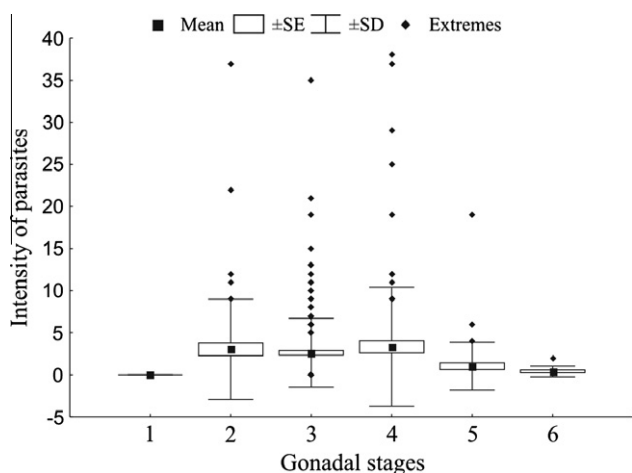
Fig. 8. Intensity of parasites of *Ensis macha* (box plot) at different gonadal stages (1: early active, 2: late active, 3: ripe, 4: partially spawned, 5: spawned, 6: restoration).

Acknowledgments

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