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Parasites in two coexisting bivalves of the Patagonia coast, southwestern Atlantic Ocean: The Puelche oyster (*Ostrea puelchana*) and false oyster (*Pododesmus rudis*)

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ABSTRACT

The purpose of this study was to compare the parasites of two coexisting bivalves, the edible Puelche oyster (*Ostrea puelchana*) and the false oyster (*Pododesmus rudis*) that lives attached to *O. puelchana* shells, and to investigate their host specificity. Samples from wild populations, 465 *O. puelchana* and 131 *P. rudis,* were collected seasonally during two years in the San José Gulf (northern Patagonia, Argentina) and were processed using standard histological techniques. To increase the natural low prevalences of *Bonamia* spp. and *Perkinsus* spp. that are present in wild populations, an *in situ* experiment was performed by maintaining captive sentinel bivalves at high densities inside a plastic mesh bag to enhance parasite transmission. Polymerase chain reaction (PCR) assays were used to test for apparent *Bonamia* sp. infections among captive sentinel *O. puelchana* specimens (n = 80), and Ray's fluid thioglycollate medium (RFTM) assays and histological immunoassays tested for apparent *Perkinsus* sp. infections among captive sentinel *P. rudis* specimens (n = 100). Despite histological observations that revealed the presence of microcells resembling *Bonamia* sp. infecting hemocytes of some Puelche oysters, PCR assays did not confirm that parasite identification. Among captive sentinel *P. rudis* that showed histological evidence of *Perkinsus* sp. infections, neither RFTM nor immunoassays confirmed such parasites. *Ostrea puelchana* from wild populations were occasional hosts for both *Rickettsia*-like organism (RLOs) and *Urastoma*-like turbellarians. In contrast, six parasite taxa infected *P. rudis* from coexisting populations, including RLOs, *Urastoma*-like turbellarians, an intracellular gregarine species, *Nematopsis*-like oocysts, an unidentified coccidian and a *Perkinsus qugwadi*-like protozoan. These results demonstrated specific infection patterns of the identified parasites in relation to their hosts.

1. Introduction

The study of parasites and diseases affecting molluscs of economic interest is important both for the management of natural stocks and for aquaculture. Furthermore, non-commercial bivalves may play important roles as reservoirs of parasites that affect bivalves of commercial interest (Pichot et al., 1980).

The Puelche oyster, *Ostrea puelchana* d'Orbigny, (Ostreidae) inhabits waters of the San José Gulf on the Patagonia coast of Argentina, and in the northern San Matías Gulf this species forms economically important beds (Orensanz et al., 2006). The primary interest in the Puelche oyster lies in its culture because its flesh is similar to that of the European flat oyster, *O. edulis*. The wild fishery has been closed in the San Matías Gulf for the last several years (Lasta et al., 1998), but oyster culture, including seed production, was successful until the appearance of *Bonamia exitiosa* infections that caused mass mortalities of both cultured and wild oysters (Kroeck and Montes, 2005; Hill et al., 2014).

Pododesmus rudis Broderip (Anomiidae), also called false oyster, lives attached to *O. puelchana* shells and it is considered a by-catch species without economic potential due to its small size (Lasta et al., 1998). Because it is non-commercial, no attention has been accorded the parasites of *P. rudis*, even though it inhabits waters in which economically significant parasites are enzootic (Cremonte et al., 2005a). Thus, its role in the epizootiology of potential parasites of economically important bivalves in northern Patagonian coastal environments is unknown.

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Most investigations of host-parasite systems focus on a single host and a single parasite. Relatively few studies have reported on co-occurrences of different parasite groups among coexisting hosts of the same phylum, especially including both macro- and micro-parasites (Ngo and Choi, 2004). The aims of this study were to describe the parasites of the two coexisting bivalve hosts, *O. puelchana* and *P. rudis* in the San José Gulf and to determine the host specificity for each parasite. Moreover, we investigated how the prevalences and intensities of infections are influenced by environmental and biological variables (water temperature, sex, gonad developmental stages and shell height) using generalized linear models (GLMs) analyses.

2. Materials and methods

2.1. Study area and sampling

Playa Fracasso (42°25′S, 64°07′W) of the San José Gulf is located in the north of the Península Valdés (Protected Provincial Area and Natural Human Patrimony by UNESCO) of Chubut Province in northern Patagonia (Argentina). Approximately 40 wild Puelche oysters were randomly collected from January 2006 to October 2007 (n = 465) at 15 m of depth. Specimens of wild *P. rudis* (n = 131) that were attached to the shells of the tested Puelche oysters were removed, and both were processed as follows. Data on surface water temperatures corresponding to each season were downloaded from an internet archive (Acker and Leptoukh, 2007).

2.2. Histological processing

The bivalves were transported to the laboratory and maintained in an aquarium with aerated seawater at 13 °C for 24 h until processing. Shell height of each specimen was measured with a caliper from the umbo to the ventral edge. The soft parts of both bivalve species were carefully removed from their shells and were then fixed in Davidson's solution (Shaw and Battle, 1957) for 24 h before transfer to 70% ethanol. A transverse, 5 mm-thick slice containing gill, digestive gland, intestine, mantle, nephridia, and gonad tissues was taken from each specimen. Tissues were embedded in paraffin, sectioned at 5–7 μm, and sections were stained with haematoxylin and eosin by the formulations of Harris (HHE) or Mayer (MHE) (Howard et al., 2004). Histological sections were examined by light microscopy to identify and describe parasites and pathological alterations. For each individual, sex and gonad development stages were recorded. Particularly in the case of protandric *O. puelchana,* the sex was determined according to the dominant type of gamete present (male or female) and gonad stages were determined according to Morriconi and Calvo (1979) (a: proliferation, b: early maturation, c: maturation, d: total maturation, e: regression, f: post spawn masculinization, g: male predominant). For *P. rudis* a 6-stage gametogenic scale was determined (1: proliferation, 2: early maturation, 3: maturation, 4: total maturation, 5: spawning, 6: post spawned).

2.3. Testing for Bonamia spp. in captive sentinel Ostrea puelchana

In addition to regular samples, 80 Puelche oysters of Playa Fracasso were maintained inside a plastic mesh bag that was secured to a metal table submerged at 0.5 m above the bottom at 15 m of depth for one year (September 2007 to October 2008), to simulate high-density culture conditions and to enhance transmission of *Bonamia* spp. Twenty oysters from the mesh bag (captive sentinel specimens) were seasonally sampled and were processed and analyzed by both standard histological and molecular methods.

2.3.1. DNA extraction and diagnostic PCR

Small samples of gill and digestive gland tissues from each captive sentinel Puelche oyster (n = 80 from the plastic mesh bag) were preserved in 100% ethanol until processing. DNA from the oyster tissues was extracted with phenol-chloroform. Briefly, 25 mg of oyster tissue samples were manually homogenized in 300 μl of homogenization buffer (NaCl 100 mM, EDTA 25 mM, SDS 0.5%, pH 8). After homogenization, 20 μl of Proteinase K was added and samples were incubated at 37 °C overnight. After incubation, 300 μl of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added, mixed vigorously by hand, and cooled on ice for 10 min. Samples were centrifuged10 min. at 12,000*g* and the top layer was transferred to a new tube. Sodium acetate 3 M 1/10 vol was added to each sample. After mixing, 2 volumes of cold 100% ethanol were added and samples were cooled at −20 °C for DNA precipitation overnight. Samples were centrifuged and washed twice with 70% ethanol. Supernatants were aspirated and discarded before DNA pellets were air dried and then re-suspended in sterile distilled water. DNA quality and quantity were checked with a Nanodrop spectrophotometer, and template DNA concentrations were adjusted to 100 ng/μl.

PCR analyses for *Bonamia* spp. were performed with the genus-specific primers for rRNA gene sequences Bo and BoAs (Cochennec et al., 2000). Reactions were carried out in volumes of 25 μl containing 1 μl of DNA (100 ng/μl), 10 mM of Tris, 50 mM KCl (pH 8.3), 2 mM of $MgCl_2$, 0.4 mM of dNTPs, 0.4 μM of each primer and 1.25 U of Taq polymerase. PCR reactions were heated for 5 min at 95 °C for denaturation; followed by 30 cycles of 1 min at 94 °C for denaturation, 1 min at 55 °C for annealing, 1 min at 72 °C for elongation; and concluded with a final extension step of 10 min at 72 °C. Reactions were carried out in a PCR 9700 thermocycler (Applied Biosystems). All PCR assays included a positive control of DNA from an oyster (*Ostrea edulis*) with a heavy *Bonamia ostreae* infection, and a negative control without DNA. PCR products were resolved in 1% agarose gel (w/v) in TAE buffer gel stained with ethidium bromide, and scanned in a GelDoc XRS + documentation system (Bio Rad).

Samples were also tested by a multiplex qPCR to detect *B. ostreae* as well as *B. exitiosa* (Ramilo et al., 2013). Reactions were carried out in a total volume of 25 μl containing 2 μl of DNA (100 ng/μl), 12.5 μl of Sybrgreen PCR Master Mix (Applied Biosystems), 0.3 μm of each primer (BOSTRE-R and BEXIT-R), 0.6 μm of BOSTRE-F, and 10 μl of distilled water. Assays were performed in a 7300 Real Time PCR system (Applied Biosystems). Samples were analyzed in triplicate. All qPCR assays included positive controls for *B. ostreae* and *B. exitiosa*, as well as a negative control without DNA.

2.4. Testing for Perkinsus spp. in Pododesmus rudis

The presence of *Perkinsus* (Alveolata) parasites was investigated because one wild specimen of *P. rudis* that was suspected, from histopathological observations, to be infected by a *Perkinsus* sp. Similar samples of a captive sentinel *P. rudis* were also tested as described above for *O. puelchana*. One hundred specimens collected at Playa Fracasso were placed inside a plastic mesh bag (secured to a metal table and submerged to 15 m at 0.5 m above the bottom for one year (September 2007 to October 2008) to simulate high-density culture conditions. Twenty-five captive sentinel false oysters (in the plastic mesh bag) were sampled seasonally for processing and analyses by standard histological methods, by Ray's fluid thioglycollate medium assays, and by immunoassays on selected histological samples.

2.4.1. Ray's fluid thioglycollate medium (RFTM) assays

Tissue samples from both gill and labial palps (n = 100 captive sentinel false oysters) were excised and incubated in RFTM (Ray, 1996) for 7 days (24 h dark, 21 °C). Following incubation, the tissue fragments from each tube were placed on a glass slide and macerated in a drop of Lugol's iodine solution. The preparation was covered with a coverslip, allowed to sit for 10 min and examined by light microscopy for dark-stained, spherical *Perkinsus* sp. hypnospores.

2.4.2. Immunoassays

Sections from a block of one wild *P. rudis* collected in November 2006 were suspected to show *Perkinsus* sp. trophozoites. Sections from another block of a *P. rudis* specimen collected from the captive sentinel group in February 2008 were suspected to show *Perkinsus qugwadi*-like trophozoites (Blackbourn et al., 1998; Itoh et al., 2013). Histological sections from both blocks and from paired negative control blocks of unaffected *P. rudis* of the same respective samples were tested for labeling by genus *Perkinsus*-specific antibodies that bind to all tested *Perkinsus* spp. including *P. qugwadi* (Dungan and Roberson, 1993; Blackbourn et al., 1998). Fluorescence immunoassays were performed by described methods for histological samples (Bushek et al., 2002), with the following differences. Deparaffinized sections were incubated in blocking buffer for 90 min, and affinity-purified goat anti-rabbit IgG secondary antibodies were used at 5 μg/ml and were labeled with Alexa Fluor 488. Control assays for non-specific antibody binding were performed on replicate sections from each of the four tested histological blocks, with normal rabbit IgG substituted as the primary antibody. Autofluorescence controls were replicate sections from each tested histological sample that were incubated only in blocking buffer without antibodies. Immunostained and control sections were all exhaustively examined microscopically for cells showing green fluorescence under excitation by blue light (490 nm).

2.5. Statistical analyses

Prevalence (P) and mean intensity (MI) of the different parasites were calculated. Prevalence was calculated as the total number of bivalves parasitized divided by the number of bivalves examined, and mean intensity as the number of parasites per histological section divided by the number of parasitized sample bivalves (Bush et al., 1997). To evaluate the variables (water temperature, sex, developmental gonad stages and shell height) affecting the presence and abundance of parasites in wild *P. rudis* population, Generalized Linear Models (GLMs) were applied as described in Vázquez et al. (2013). All statistical analyses were performed in R (R Development Core Team, 2011). Results were expressed in terms of odds ratios. Odds were calculated as the exponential of the coefficient of each parameter corresponding to the averaging model.

3. Results

When all wild individuals (465 *Ostrea puelchana* and 131 *Pododesmus rudis* specimens) were examined, it was found that *O. puelchana* was parasitized at very low prevalence levels (P) and mean intensity of infections (MI) by only two parasites, *Rickettsia*-like organisms (RLOs) (P = 1.6%, MI = 1.5) and a *Urastoma*-like turbellarian (P = 0.3%, MI = 1). In contrast, *P. rudis* hosted parasites belonging to six taxa: RLOs (P = 37%, MI = 50); apicomplexan protozoans including intracellular gregarines (P = 6%, MI = 100), *Nematopsis*-like oocysts (P = 25%, MI = 10), an unidentified coccidian (P = 4%, MI = 37); a *Perkinsus*-like protozoan (P = 1.5%) and *Urastoma*–like turbellarian (P = 3.5%, MI = 1). A summary of the seasonal main host characteristics and the parasites observed by histological examinations (shell height, sex ratio, main gonad stage, parasites, prevalence and mean intensity) of wild *O. puelchana* and *P. rudis* are listed in Table 1.

Histological sections of both *O. puelchana* and *P. rudis* from the captive sentinel groups revealed the presence of microcells resembling *Bonamia* sp. (P = 3.7) and protozoans resembling *Perkinsus qugwadi* (P = 3) along with other parasites (Table 2). Differences were not observed in prevalences and mean infection intensities for the other parasites of either bivalve host, or between members of the captive sentinel groups and conspecific wild populations.

Table 1
Morphological characteristics and histological results of wild Puelche oysters *Ostrea puelchana* and false oysters *Pododesmus rudis* of the San José Gulf, Argentina. Mean shell height (mm) ± standard deviation; sex ratio (M: male; F: female) followed by percentage of predominant gonad stage in parenthesis; parasite prevalence (P) (%) and mean intensity (MI). References: *O. puelchana* development gonad stages = a: proliferation, b: early maturation, c: maturation, d: total maturation, e: regression, f: post spawn masculinization, g: male predominant; for *P. rudis* = 1: proliferation, 2: early maturation, 3: maturation, 4: total maturation, 5: spawning, 6: post spawned; RLOs = *Rickettsia*-like organisms; ~ = approximately.

Season	Host bivalve species																									
	Ostrea puelchana n = 465							*Pododesmus rudis* n = 131																		
	Mean height ± SD: 93 ± 3 mm							Mean height ± SD: 63 ± 5 mm																		
	N	Shell height	% Sex M:F (gonad stage)	RLOs		*Urastoma* -like		N	Shell height	% Sex M:F (gonad stage)	RLOs		Gregarine-like		*Nematopsis*-like		Coccidean		*Perkinsus*-like		*Urastoma*-like	
				P	MI	P	MI				P	MI	P	MI	P	MI	P	MI	P	MI	P	MI		
2006																										
Summer	72	94 ± 10	50:49 (B,F)	6	3	0	–	12	67 ± 8	40:60 (4)	42	43	17	100	8	5	17	53	0		0	–		
Autumn	57	91 ± 8	43:54 (B)	2	1	2	1	13	59 ± 3	78:22 (5)	38	44	0	–	15	15	8	35	0		15	1		
Winter	87	90 ± 8	50:49 (C,F)	3	1	1	1	3	53 ± 1	100 M (2)	33	36	0	–	33	1	0	–	0		0	–		
Spring	56	93 ± 9	60:33 (D,F)	2	1	0	–	18	64 ± 8	56:44 (4)	39	26	25	100	22	4	0	–	5		0	–		
2007																										
Summer	43	97 ± 9	35:59 (B,F)	0	–	0	–	26	66 ± 7	42:58 (4)	58	63	8	100	38	11	8	22	0		0	–		
Autumn	60	95 ± 9	40:57(B)	0	–	0	–	10	63 ± 4	30:70 (5)	40	8	0	–	60	17	0	–	0		10	1		
Winter	30	87 ± 12	47:53 (C,F)	0	–	0	–	20	59 ± 5	26:74 (2)	15	193	0	–	15	4	0	–	0		3	1		
Spring	60	97 ± 9	30:67 (F)	0	–	0	–	29	72 ± 8	31:69 (4)	28	23	0	–	7	8	0	–	0		0	–		
Mean Prevalence				1.6		0.3					37		6		25		4		1.5		3.5			

Table 2
Morphological characteristics and histological results of captive sentinel Puelche oysters *Ostrea puelchana* and false oysters *Pododesmus rudis* of the San José Gulf, Argentina. Mean shell height (mm) ± standard deviation; sex ratio (M: male; F: female) followed by percentage of predominant gonad stage in parenthesis; parasite prevalence (P) (%) and mean intensity (MI). References: *O. puelchana* development gonad stages = a: proliferation, b: early maturation, c: maturation, d: total maturation, e: regression, f: post spawn masculinization, g: male predominant; for *P. rudis* = 1: proliferation, 2: early maturation, 3: maturation, 4: total maturation, 5: spawning, 6: post spawned; RLOs = *Rickettsia*-like organisms; ~ = approximately.

Season	Captive bivalve species																			
	Ostrea puelchana n = 80 Mean height ± SD: 97 ± 9 mm			*Bonamia*-like		*Pododesmus rudis* n = 100 Mean height ± SD: 63 ± 6 mm			RLOs		Gregarine like		*Nematopsis*-like		Coccidean		*Perkinsus*-like		*Urastoma* like	
	N	Shell height	% Sex M:F (gonad stage)	P	MI	N	Shell height	% Sex M:F (gonad stage)	P	MI	P	MI	P	MI	P	MI	P		P	MI
Summer	20	95 ± 8	50:49 (B,F)	0	–	25	65 ± 5	40:60 (4)	48	22	4	100	24	4	0	–	12		0	–
Autumn	20	93 ± 9	43:54 (B)	0	–	25	61 ± 5	78:22 (5)	20	8	0	–	44	4	0	–	0		8	1
Winter	20	99 ± 10	50:49 (C,F)	15		25	60 ± 6	100 M (2)	28	54	0	–	20	5	0	–	0		4	1
Spring	20	98 ± 8	60:33 (D,F)	0	–	25	65 ± 8	56:44 (4)	24	16	8	100	20	6	0	–	0		8	1
Mean Prevalence				3.7					30		3		27				3		4	

Fig. 1. Severe intensity of infection by intracellular colonies of *Rickettsia*-like organisms (arrows) in the epithelium of a digestive gland tubule of *Pododesmus rudis*. Scale bar = 100 µm.

3.1. Prokaryotic organisms

Rounded intracellular basophilic colonies of *Rickettsia*-like organisms were observed in the epithelial cells of the digestive gland of both bivalve species. The mean diameter of the intracellular colonies in *P. rudis* was 15.74 µm ± 1.6 (n = 20). These colonies occupied the host cell cytoplasm, causing hypertrophy and lysis of infected host cells at severe infection intensities (Fig. 1). Nevertheless, there was no apparent host reaction. The intensity of infection was occasionally high, showing up to 439 colonies per histological section and 45 colonies in a single digestive gland tubule of *P. rudis*.

3.2. Protozoan organisms

Histological sections revealed the presence of *Bonamia*-like cells in three Puelche oyster specimens collected in June 2008 from the captive sentinel group, 9 months after their deployment. Usually, 1–2 microcells were observed in the cytoplasm of hemocytes in a section (Fig. 2), at infection intensities that ranged from 1 to 10 microcells per histological section from tissue blocks of infected oysters. Among infected oysters, a severe hemocyte infiltration response was observed. Infected hemocytes reached diameters of 8–9 µm (8.85 µm ± 0.55; n = 5) and showed an eccentric nucleus. The parasite was basophilic with a spherical shape of 3–5 µm in diameter (5.14 µm ± 0.93; n = 5). No *Bonamia*-like parasite cells were observed free among connective tissues.

Gregarine-like protists of rounded shape and diameter mean of 5.36 µm were surrounded by halos of intracellular space within infected intestine epithelial cells of *P. rudis*. Although the mean prevalence was low (6%), infections of moderate intensity were recorded (approximately 100 cells per histological section). A local hemocyte infiltration was associated with gregarine infections in the connective tissues that surrounded infected intestinal epithelia (Fig. 3A, B).

Oocysts of gregarines similar to those of the genus *Nematopsis* (Apicomplexa: Eugregarinida) enclosing a single oval basophilic sporozoite, were observed in connective tissues of the visceral mass, gill, mantle, muscle, gonad, and nephridia of *P. rudis*. Oocysts measured

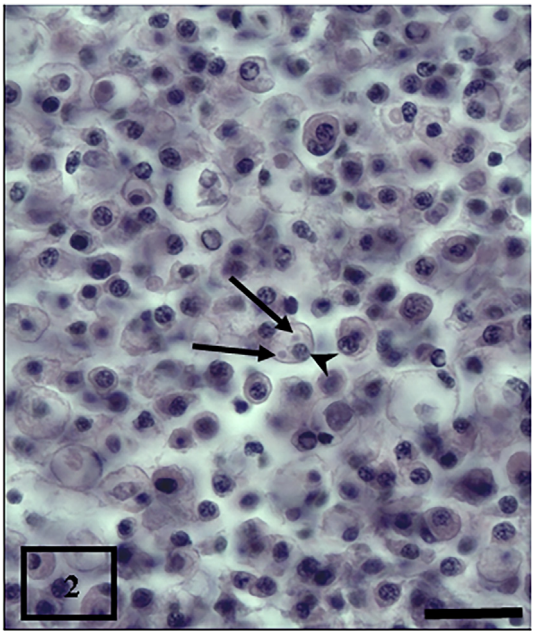

Fig. 2. Microcells resembling *Bonamia* sp. (arrows) infecting hemocytes of *Ostrea puelchana*. The nucleus of the host hemocyte is shown by the arrowhead. Scale bar = 20 μm.

mean length 11.2 μm × width 7.3 μm (Fig. 4). The connective tissues of the digestive gland were most frequently infected (41% prevalence), with the maximum intensity of infection of 69 oocysts per histological section. Oocysts occurred within phagocytic hemocytes, where two oocysts per host cell were most common and four oocysts were also observed. A slight and focal infiltration by host hemocytes induced by the parasites was observed.

Unidentified coccidians were primarily observed in nephridial epithelia (prevalence 40%) and less frequently in the epithelial cells of the intestine and digestive gland of *P. rudis*. This apicomplexan parasite was only observed during summer and autumn, at a maximum infection

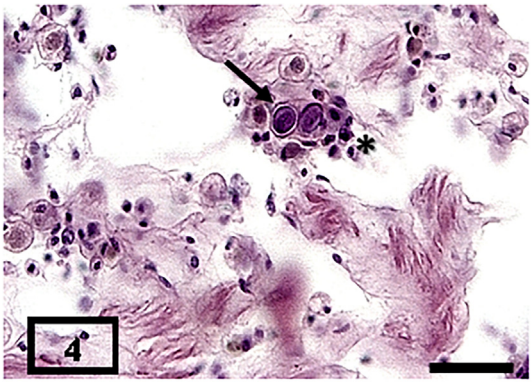

Fig. 4. Oocysts of a *Nematopsis*-like gregarine (arrow) in connective tissue of the digestive gland of *Pododesmus rudis*. Infiltrating hemocytes aggregate around the oocysts (*). Scale bar = 100 μm.

intensity of 56 coccidians per histological section. Merogonic and gamontocyte stages were identified. Immature meronts with peripherally arranged nuclei (Fig. 5A) were observed in lumens of infected nephridial tubules, and free forms resembling merozoites (mean length 9.24 μm ± 1.56; n = 8) were observed in connective tissues of the digestive gland. Microgamonts packed with numerous microgametes budding from the central mass of the cytoplasm (Fig. 5B) and intracellular trophozoites (mean length 35.2 μm × width 13.8 μm) extended into the lumens of the nephridial tubules (Fig. 5B). Macrogamonts with a general spherical shape (mean diameter 19.18 μm; n = 12), a granular cytoplasm containing a prominent central nucleus (mean diameter 7.18 μm, n = 12) and a prominent nucleolus were observed inside cells of nephridial tubules (Fig. 5C). These infections induced light to moderate lesions. Infected nephridial cells were hypertrophied, causing lysis of the host cells and release of the parasites into the lumens of nephridial tubules (Fig. 5D).

Two described *P. qugwadi* development stages (Blackbourn et al., 1998) were present, including trophozoites, measuring up to 13 μm in diameter with prominent central nuclei and nucleoli, and a tomont with 3 developing trophozoites (Fig. 6A). *Perkinsus qugwadi*-like cells were

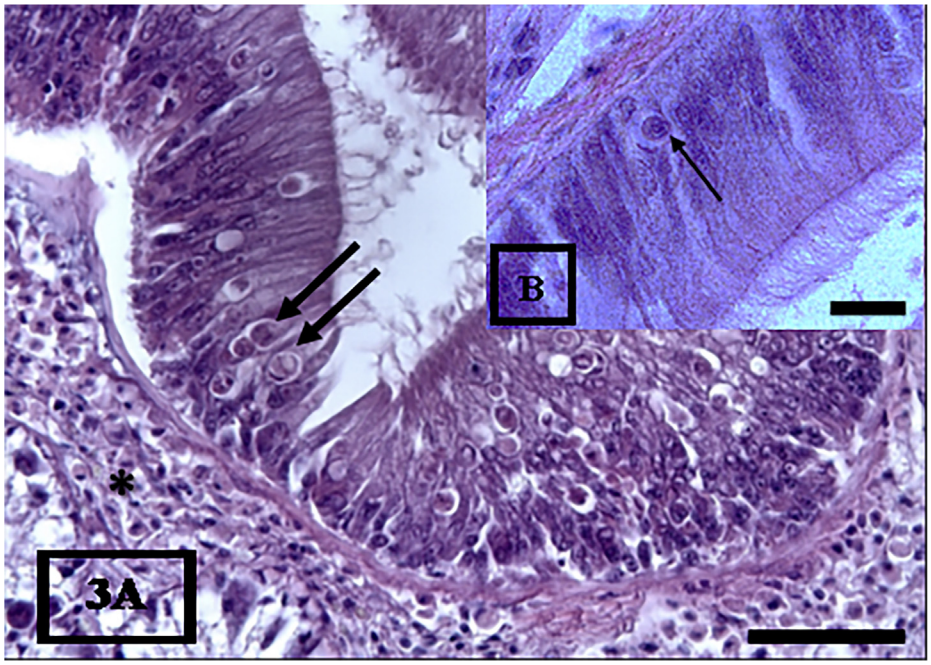

Fig. 3. Gregarine-like apicomplexan in the intestinal epithelium of *Pododesmus rudis*. (A) Rounded gregarine-like cells (arrows) evoking local hemocyte infiltration of supporting connective tissues (*). Scale bar = 50 μm. (B) Details of a gregarine protist (arrow). Scale bar = 20 μm.

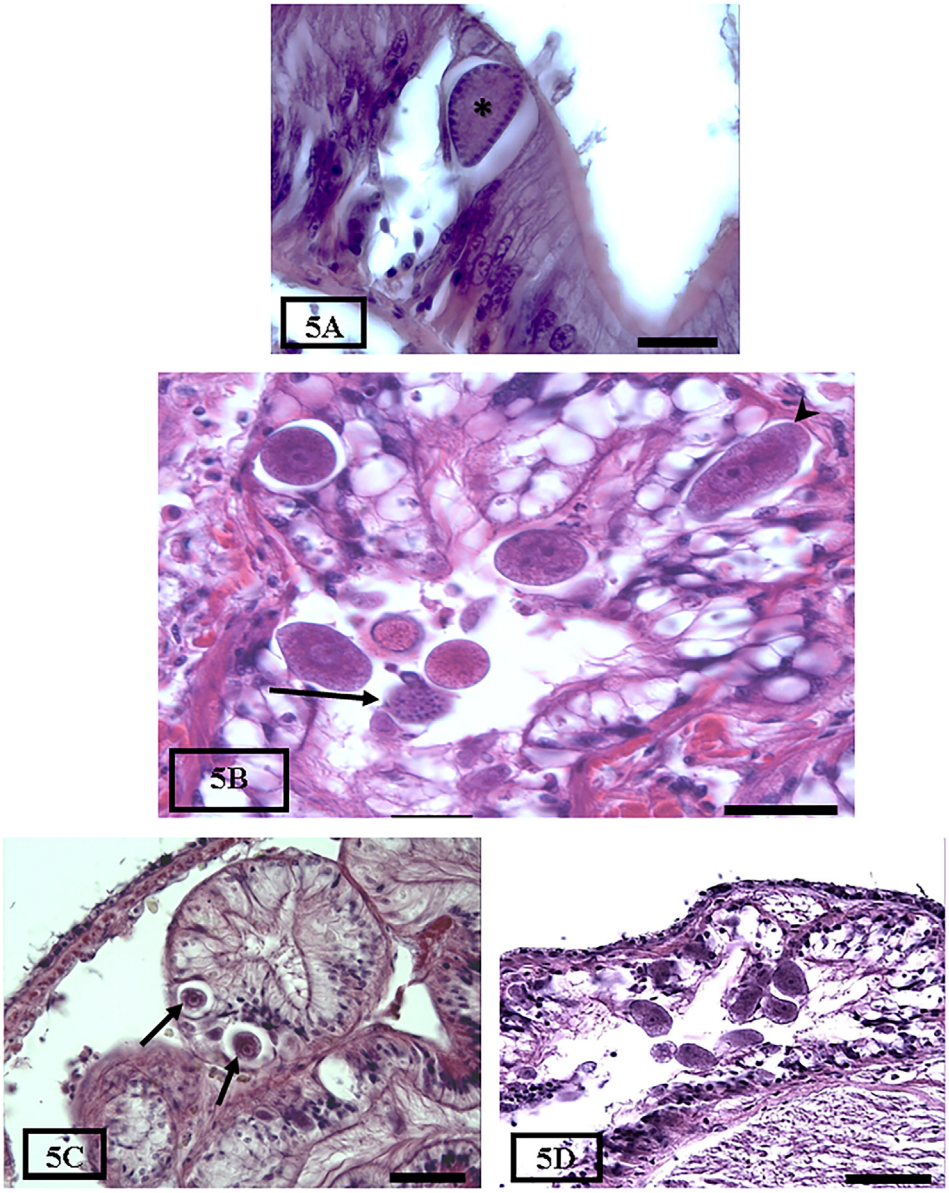

Fig. 5. Coccidean parasites in the tissues of *Pododesmus rudis*. (A) Immature meront with peripherally arranged nuclei (*). (B) Microgamont with microgametes budding from the central mass of the cytoplasm (arrow) and intracellular trophozoite (arrowhead). (C) Mature macrogamonts with central nucleus (arrows) in a nephridial epithelium. (D) Lysis of the host cells and release of the parasites into the lumen of nephridial tubule. Scale bars = 50 μm.

often phagocytized by hemocytes (Fig. 6B), indicating that the protozoan generates a host defensive response. Additionally, extensive proliferation of roundish cells resembling *P. qugwadi* was observed throughout the visceral connective tissue of digestive glands in three *P. rudis* individuals of the captive sentinel group that were collected in February 2008 (Fig. 6C). Additionally, some large cells clustered within the lumens of atrophied or necrotic digestive diverticula showed vacuolated, signet ring shapes characteristic of *Perkinsus* spp., while others resembled hemocytes like those that heavily infiltrated surrounding visceral connective tissues (Fig. 7).

3.3. Turbellaria

Platyhelminths similar to those of the genus *Urastoma* were found within the pallial cavities of both host bivalves. They were characterized by their ciliated epidermis, two pigmented anterior eyes, and a pharynx at the posterior end of the body. Both the prevalences and the intensities of *Urastoma* sp. infestations were low for all individuals of each bivalve host (Tables 1 and 2).

3.4. Specific tests for Bonamia sp. and Perkinsus sp. Parasites

In contrast to histological observations, PCR assays performed on tissue DNA of apparent *Bonamia* sp.-infected *O. puelchana* from the captive sentinel group gave negative results, as did species-specific qPCR assays for *B. ostreae* and *B. exitiosa*. Neither RFTM assays of fresh gill and labial palp tissues, nor immunoassays of histological sections with genus *Perkinsus*-specific antibodies, confirmed the presence of *Perkinsus* sp. cells in *P. rudis* collected from either wild populations or from the captive sentinel group. *Perkinsus marinus* cells were strongly and specifically labeled in positive control sections from an infected oyster, *Crassostrea virginica*, but no *Perkinsus* sp. cells were labeled in sections from either a negative control (uninfected) *P. rudis* or from two *P. rudis* that showed *Perkinsus*-like trophozoites in histological sections.

The presence of parasites was studied as a function of site, water temperature, sex, gonad stages and shell height of *P. rudis*. Model

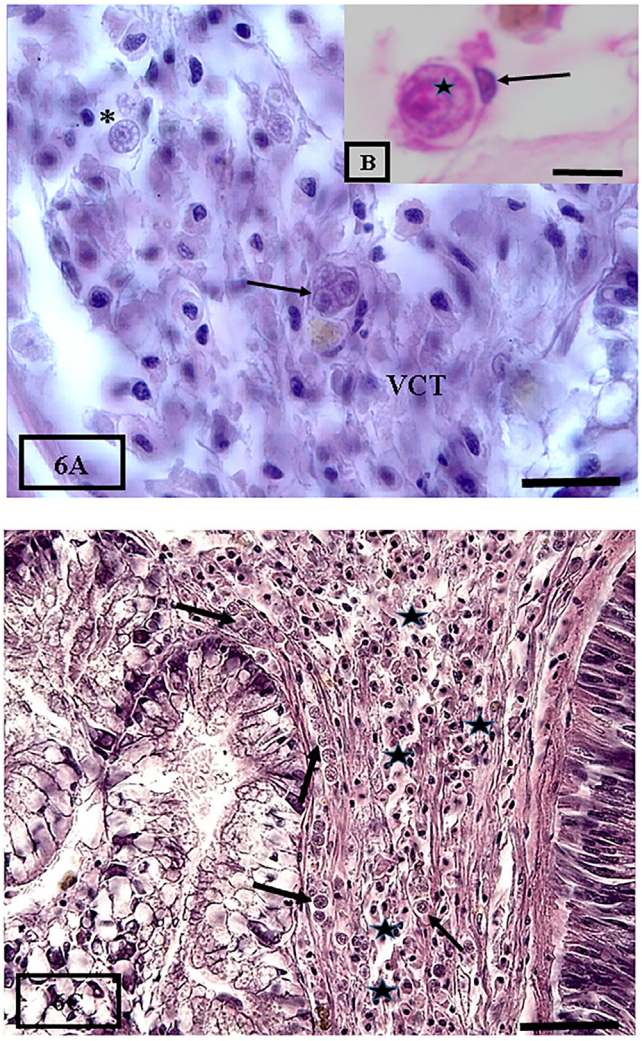

Fig. 6. *Perkinsus qugwadi*-like cells in tissues of *Pododesmus rudis*. (A) Trophozoites (*) with a prominent central nucleus and an immature tomont (arrow) containing 3 developing trophozoites in visceral connective tissue of the digestive gland (VCT). Scale bar = 50 μm. (B) Detail of a single *P. qugwadi*-like cell (*) with its prominent nucleus and nucleolus that is phagocytized by a distended *Pododesmus rudis* hemocyte with an emarginate nucleus (arrow). Scale bar = 5 μm. (C) Abundant apparent proliferation of round cells resembling *Perkinsus qugwadi* trophozoites (arrowheads) that are surrounded by intensive hemocyte infiltration (*) in the visceral connective tissue (VCT) of the digestive gland of *Pododesmus rudis*. Scale bar = 100 μm.

analysis on the presence of the parasites resulted in three candidate models with ΔQAIC < 2 of the best model. The presence of parasites was mainly correlated with water temperature, showing a negative relationship (relative importance weight of 1.00 and 95% confidence interval bounded away from zero) (Table 3). The probability of finding a false oyster parasitized in winter and spring seasons were 8.5 ± 1.9 and 2.8 ± 1.6 times lower respectively, than in summer season. The intensity of parasites was also evaluated by the same variables. Model analysis resulted in models of nine candidates with ΔQAIC < 2 of the best model. All variables showed very low relative importance weights, indicating no relationship with infection intensity (Table 3).

4. Discussion

Relationships of all species in a community are close and, among them, parasites play an important role in the health status of community members. Parasites may be specific for the host or have multiple species as hosts (broad host range) (Rohde, 2005). Remarkable differences were found in the species parasitizing the two coexisting bivalve species. Extremely low prevalences and intensities of both *Rickettsia*-like organisms (RLOs) and *Urastoma*-like turbellarians were observed in wild Puelche oysters, which appears to act as an occasional host. In contrast, *Pododesmus rudis* hosts diverse parasites including RLOs and *Urastoma*-like turbellarians, as well as intracellular gregarines, a *Nematopsis*-like apicomplexan, coccidians and a *Perkinsus qugwadi*-like. protozoan. Such differences suggest host-specific requirements or preferences among some of these parasites.

Rickettsia-like organisms were the most prevalent parasite infecting *P. rudis*. The occurrence of RLOs infecting epithelial cells of gills and digestive organs is widespread among molluscs (Bower et al., 1994). On the Patagonian coast of Argentina, these prokaryotic organisms infect the Tehuelche scallop (*Aequipecten tehuelchus*), the Puelche oyster, mytilids (*Mytilus edulis, Aulacomya atra)* and clams (*Panopea abbreviata, Ensis macha*) (Vázquez and Cremonte, 2017). Some effects reported from histological examination include disruption of the epithelia of digestive gland tubules that contain large RLO colonies in the venerid clam *Pitar rostrata* (Cremonte et al., 2005b), and the hypertrophy and lysis of host epithelial cells associated with mass mortalities in scallops (Gulka et al., 1983; Le Gall et al., 1988), clams (Villalba et al., 1999; Carvalho et al., 2013) and abalones (Friedman et al., 1997). Damage to the infected digestive gland tubules containing large colonies included hypertrophy and lysis of the host cells with no obvious host defense responses.

Nematopsis-like gregarines infected connective tissues supporting most organs of *P. rudis*, although the most frequent location was the digestive gland. In the same study area, similar gregarines have been previously found in the gonad of *A. tehuelchus* and in the labial palps of *P. rudis*, with no notable defensive responses by either host (Cremonte et al., 2005a). These gregarines induced slight, focal hemocyte responses without severe pathogenic effects. Similar results were reported by Bower et al. (1992), who suggest that no indication of pathogenicity was evident, except a focal inflammatory reaction. However, Azevedo and Cachola (1992) attributed mass mortalities of wild cockles *Cerastoderma edule* in southern Portugal to *Nematopsis* sp. infections. Their ultrastructural analysis revealed complete destruction of the gill cells in the area immediately in contact with the oocyst, apparently from parasite activity. Although pathogenicity of *Nematopsis* sp. in bivalves is inconclusive, most apicomplexan parasites are found to be pathogenic to their hosts, causing disintegration of cellular organization (Lauckner, 1983). These protozoans utilize bivalves as intermediate hosts and marine arthropods as final hosts (Lauckner, 1983). The crab *Platyxanthus patagonicus* could act as the final host for these gregarines in Patagonia, because they are frequently observed feeding on bivalves (R. Vera, pers. comm.). Further studies of the parasites of crustaceans in the study area should be performed to resolve details of their life cycles.

Although an unidentified coccidian infected the nephridial tubules of *P. rudis* at low prevalences, relatively high infection intensities commonly induced light to moderate damage of infected host epithelia. Carballal et al. (2001) considered that heavy infections by these parasites have the potential to cause renal dysfunction. High specificity for the host seems to be characteristic of these apicomplexan protozoans, since none of several bivalves previously studied from the same area (*A. tehuelchus, M. edulis, A. atra, P. abbreviata, E. macha, Leukoma antiqua*) or *O. puelchana* of the current study, were infected by these coccidians (Vázquez and Cremonte, 2017). The life cycle of this parasite appears to be monoxenus, since observed developmental stages included merogony and gamogony (inmature meront, likely merozoites, microgamonts and macrogamonts). Sporogony (mature oocysts) was not observed, however, perhaps due to the small number of specimens examined over a relatively short period; life cycles of some apicomplexans in molluscs have been shown to take an entire year.

Species of the genus *Bonamia* are haplosporidian protozoan parasites that colonize the hemocytes of several oyster species, inducing

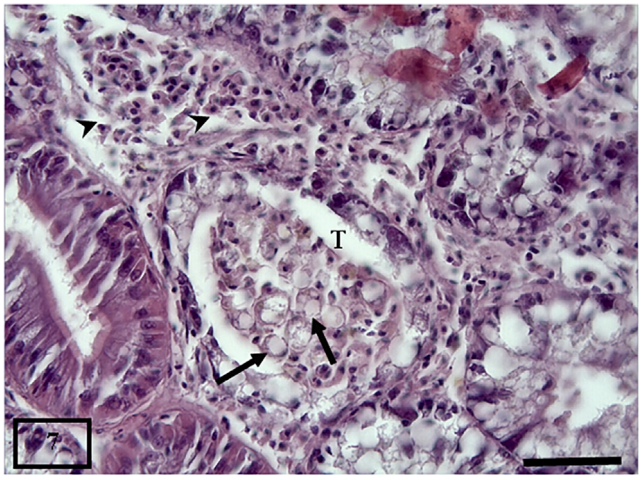

Fig. 7. A clump of cells of different sizes and shapes in the lumen of an atrophied or necrotic digestive gland tubule (T). Large cells show signet ring morphologies typical of *Perkinsus* spp. (arrow), while smaller cells resemble host hemocytes that also infiltrate surrounding visceral connective tissues (arrowheads). Scale bar = 50 μm.

physiological disorders and eventually causing mortality of infected hosts (Cranfield et al., 2005; Engelsma et al., 2014). Three *Bonamia* species have been identified worldwide, *B. ostreae* in Europe and North America, *Bonamia perspora* from North America and *Bonamia exitiosa* from North and South America, Australia, New Zealand, and the Mediterranean Sea (Hill et al., 2014). *Bonamia exitiosa* has been blamed for mass mortality among wild and cultured *O. puelchana* in San Antonio Bay (San Matías Gulf) (Kroeck and Montes, 2005; Hill et al., 2014). Most of the microscopic signs of *Bonamia* sp. infection observed in our study agreed with the histological descriptions of Kroeck and Montes (2005), including hemocyte infiltration in all connective tissues (systemic infiltration), a dissociated appearance of connective tissues containing hemocytes with pycnotic nuclei, and the presence of intracellular microcells within hemocytes. The negative results from our PCR assays were inconsistent with histological examinations. It is possible that levels of target DNA were below detection thresholds due to extremely low intensity of histologically detected infections, as well as to the small volumes of gill tissues from which template DNAs for PCR assays were extracted and amplified (Burreson, 2008).

In South America, several species of *Perkinsus* have been reported infecting bivalve species. *Perkinsus olseni* was found infecting wild *Pitar rostrata* on natural beds in Uruguay (average sea temperature = 23 °C) (Cremonte et al., 2005b); *Perkinsus beihaiensis* infected wild clams *Anomalocardia brasiliana* (Ferreira et al., 2014) and *Perkinsus marinus* infected *Crassostrea rhizophorae* (da Silva et al., 2013), both from Brazil (sea temperature > 27 °C). Proliferation of *Perkinsus* species other than *P. qugwadi* is enhanced by environmental water temperatures above 20 °C, which increase parasite proliferation and pathogenesis within the host (Andrews, 1988; Burreson and Ragone Calvo, 1996; Soniat, 1996; Villalba et al., 2005). Large foci of hemocyte infiltration were observed in histological sections of the connective tissues of *P. rudis* digestive gland where phagocytized cells resembled *P. qugwadi,* suggesting a strong host defensive response to local pathological conditions. Nevertheless, the use of special diagnostic techniques such as Ray's fluid thioglycollate medium assays and the immunoassays to detect *Perkinsus* spp. pathogens did not detect or confirm *Perkinsus* sp. infections. The absence of *Perkinsus* sp. cells in the bivalves studied here may reflect unfavorable conditions in the colder waters of the study area for thermophilic *Perkinsus* spp. Despite the use of an immunoassay that can detect *Perkinsus* spp., including *P. quqwadi,* (Dungan and Roberson, 1993; Blackbourn et al., 1998), it is possible that the assay is inadequate to detect or identify an unknown or emergent *Perkinsus* sp. pathogen of Patagonian *P. rudis*. Alternatively, the *Perkinsus*-like cells that we detected histologically in *P. rudis* may have a different protistan affiliation.

The turbellarian *Urastoma cyprinae* is reported worldwide as a parasite inhabiting the gills of clams, mussels, and oysters (Cremonte, 2011). The effect of its presence within the host remains controversial. Robledo et al. (1994) blamed *U. cypriane* for the disarrangement of the gill filaments that induced inflammatory hemocyte infiltration in the mussel *Mytilus galloprovincialis*. On the other hand, Fleming et al. (1981) considered *U. cyprinae* to be a commensal in oysters, and therefore, not harmful to its host. In the present study, no apparent defense response was observed by either mollusc host to *Urastoma*-like associates of their pallial cavities and gills. The lack of observed pathological consequences may reflect the low prevalence and intensity of such infestations among our samples. There is evidence that the prevalence and mean intensity of the turbellarians depend on the examination method, revealing that histological methods underestimate the real values of these parameters (Brusa et al., 2011); therefore, the true

Table 3
Predictor variables from top models for each response variable in wild *Pododesmus rudis* population from San José gulf. Coefficient estimates, their unconditional standard error (SE), 95% confidence interval (CI) and relative importance weights (w(i)) after model averaging are shown for each variable. Variables in bold have a 95% confidence interval bounded away from zero (significant results).

Response	Parameter	Coefficient	Adjusted SE	Confidencial Interval		Relative importance
Presence of parasites						
	Intercept	0.691	0.346	0.006	1.380	
	Sex	0.020	0.193	−0.362	0.403	0.22
	Temp 15 °C (autumn)	−0.429	0.553	−1.520	0.667	1
	Temp 11 °C (winter)	−2.140	0.671	−3.470	−0.811	**1**
	Temp 13 °C (spring)	−1.020	0.471	−1.950	−0.086	**1**
	Shell height	0.001	0.206	−0.407	0.410	0.21
Intensity of parasites						
	Intercept	3.430	0.585	2.270	4.580	
	Sex	−0.252	0.390	−1.020	0.517	0.49
	Stage 2 (early maturation)	−2.300	675	−1340	1330	0.15
	Stage 3 (maturation)	0.081	0.467	−0.840	1.000	0.15
	Stage 4 (total maturation)	0.111	0.548	−0.971	1.190	0.15
	Stage 5 (spawning)	0.213	0.667	−1.100	1.530	0.15
	Stage 6 (post spawned)	0.028	0.485	−0.931	0.988	0.15
	Temp 15 °C (autumn)	−0.428	0.624	−1.660	0.800	0.48
	Temp 11 °C (winter)	−0.170	0.429	−1.020	0.677	0.48
	Temp 13 °C (spring)	−0.447	0.576	−1.580	0.686	0.48
	Shell height	0.011	0.197	−0.379	0.402	0.23

prevalence and the intensity of such infestations may be higher than we observed in this study.

The present study found differences in parasite species associated with the coexisting mollusc hosts. Studies on parasites in coexisting bivalves are scarce and mostly consider metazoan parasites (Bagnato et al., 2015). The host range of a parasite may be due to phylogenetic, ecological, physiological and immunological factors (Detwiler and Janovy, 2008). The size of the host plays an important role in the intensity of infection since parasite intensity can be increased in larger bivalves with high filtration capacities, and by longer exposure to infective parasite stages (Villalba et al., 2005). A positive correlation between size of *Mytilus californianus* and metazoan parasite intensity was found when compared to two smaller coexisting mytilds (Cáceres-Martínez and Vásquez-Yeomans, 1999). In the sympatric infaunal clams *Panopea abbreviata* and *Ensis macha* in northern Patagonia (Argentina), host specificity of the green alga *Coccomyxa parasitica* and *P. abbreviata* was observed, suggesting that the degree of the exposure of tissues to light is a determinant factor (Vázquez et al., 2010). In the present study where Puelche oysters were larger (93 mm) than false oysters (63 mm), animal size did not appear to play a role in the host specificity. There is obviously a host phylogeny-dependent factor for the parasites. Future comparative immunology investigations on these host species may reveal characteristics and mechanisms that foster their different parasite communities. Although the current survey did not identify any pathogens or diseases of concern, with the possible exception of a *Bonamia*-like haplosporidian found for the first time in *O. puelchana* in the San José Gulf and a *Perkinsus qugwadi*-like parasite in *P. rudis*, it provides baseline health assessment data for these species, against which future disease developments or significant changes in population health can be compared. These data are also valuable to the development and implementation of informed public policies promoting to aquatic animal health.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jip.2018.08.011.

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