

Detection of a parasitic amoeba (Order Dactylopodida) in the female gonads of oysters in Brazil

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ABSTRACT: The impacts of oocyte parasites on the reproductive success of molluscs are largely unknown. In this study, we evaluated the presence of gonad parasites in 6 species of marine bivalve molluscs native to southern Brazil. Cultured bivalves included the mangrove oyster *Crassostrea gasar* (sometimes called *C. brasiliiana*), the brown mussel *Perna perna*, the lion's paw scallop *Nodipecten nodosus* and the wing pearl oyster *Pteria hirundo*. Another species of mangrove oyster, *C. rhizophorae*, and the carib pointed venus clam *Anomalocardia brasiliiana* (syn. *A. flexuosa*) were collected from the wild. Molluscs were collected in winter 2009 and summer 2010 for histopathological and molecular evaluation. An unknown ovarian parasite (UOP) was observed in histopathological sections of female gonads of *C. gasar* and *C. rhizophorae*. The UOP possessed features suggestive of amoebae, including an irregular outer membrane, frothy cytoplasm, a nucleus with a prominent central nucleolus and a closely associated basophilic parasome. PCR analysis was negative for *Marteilioides chungmuensis*, *Perkinsus* spp. and *Paramoeba perurans*. However, real-time PCR successfully amplified DNA from oyster gonads when using universal *Paramoeba* spp. primers. Also, conventional PCR amplified DNA using primers specific for *Perkinsella amoebae*-like organisms (syn. *Perkinsiella*), which are considered as endosymbionts of *Paramoeba* spp., previously thought to be the parasome. Our results suggest that this UOP is a species of amoeba belonging to 1 of the 2 families of the order Dactylopodida, possibly related to *Paramoeba* spp. This study represents the first report of this type of organism in oysters. We found that *C. gasar* and *C. rhizophorae* were the most susceptible molluscs to these UOPs.

KEY WORDS: Oocytes · Ovarian parasite · Marine bivalve · Amoeba

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INTRODUCTION

Harvesting bivalve molluscs is important for the economy and quality of life in coastal communities of Brazil. Currently, 1 introduced species (the Pacific oyster *Crassostrea gigas*), and 2 native species (the

brown mussel *Perna perna* and the lion's paw scallop *Nodipecten nodosus*) are farmed commercially in Brazil. Moreover, 2 native species are targeted for future cultivation: the mangrove oyster *C. gasar* and wing pearl oyster *Pteria hirundo*. In addition, native species of importance to commercial fisheries include

the carib pointed venus clam *Anomalocardia brasiliiana* and another species of mangrove oyster, *C. rhizophorae*. During surveys of these and other bivalves for pathogens and parasites, ovarian parasites were detected in some bivalves. Matos et al. (2005) reported *Steinhausia mytilovum* in oocytes of the mussel *Mytella guyanensis* from the Amazon River estuary, and Da Silva et al. (2012) found *Steinhausia*-like microsporidia in the oocytes of the clam *A. brasiliiana* from Santa Catarina State. In oysters, *Steinhausia*-like parasites were reported in the oocytes of *C. rhizophorae* from Todos os Santos Bay (Nascimento et al. 1986) and from Ceará (Sabry et al. 2013) and in *C. rhizophorae* and *C. gigas* from Santa Catarina Island, Brazil (Pontinha 2009, Sabry et al. 2011, Da Silva et al. 2012). An unusual protistan parasite was reported in oocytes of some oysters from southern Brazil (Da Silva et al. 2012, Ivachuk 2013).

Steinhausia spp. (Kingdom Fungi, Phylum Microsporidia) are characterized by spheroid sporocysts (9–18 µm in diameter) that contain numerous spherical developing spores (about 1–3 µm in diameter). Sporocysts occur in parasitophorous vacuoles, delimited by a thin membrane, within the cytoplasm or nucleus of the host oocyte (Sagrìstà et al. 1998). These parasites have a global distribution and have been reported from the oocytes of mussels, cockles, clams and oysters (Comtet et al. 2003, Green et al. 2008).

Another parasite that infects the oocytes of bivalves is *Marteilioides chungmuensis* (Kingdom Chromista, Phylum Cercozoa but initially placed in the Phylum Paramyxia; Berthe et al. 2000). *M. chungmuensis* is characterized by 1 to 3 (usually 2) secondary cells (sporonts) in the remnants of the stem cell within the oocyte of its host. Each sporont contains 1 tertiary cell that develops into a tricellular spore by internal cleavage (Itoh et al. 2004a). This parasite can affect oocytes in large areas of the reproductive follicles, which causes irregular enlargement of the infected gonadal tissues, giving the infected oyster a nodular appearance suggestive of multiple tumours. Originally described in *C. gigas* from Korea (Comps et al. 1987), it also occurs in *C. gigas* and *C. nippona* from Japan (Itoh et al. 2004b), and a similar parasite was reported from the ova of *C. echinata* from Australia (Hine & Thorne 2000). A *Marteilioides*-like parasite was also reported at low prevalence (1.6%) from the oocytes of Manila clams *Venerupis (=Tapes) philippinarum*, in coastal areas of Korea (Lee et al. 2001).

In addition to the unidentified ovarian parasites reported from Santa Catarina Island, Brazil (Da Silva et al. 2012, Ivachuk 2013), Becker & Pauley (1968) detected an unusual parasite in the oocytes of

C. gigas from Humboldt Bay, California, USA. In British Columbia, Canada, a similar unidentified ovarian parasite was encountered in a *C. gigas* specimen (G. R. Meyer & S. M. Bower unpubl.; further details provided in the [Discussion] section).

During recent histopathological surveys (2009 and 2010) of commercially important bivalves from southern Brazil, a protistan parasite was detected in the oocytes of ripe female oysters. The morphology of this parasite differed from that of *Steinhausia* spp. and *M. chungmuensis*, and the prevalence of this unidentified ovarian parasite in some oyster populations in Brazil was high. Concern about the potential impact of this parasite on bivalve production in Brazil prompted investigations into the identity of the parasite including reassessment of all bivalves included in the recent surveys for the presence of this parasite.

MATERIALS AND METHODS

Sampling and location

To evaluate the presence or absence of the oocyte parasites in cultured and wild bivalve molluscs from Santa Catarina State (SC), southern Brazil, a total of 480 bivalves collected in winter (August/2009) and summer (January/2010) were examined histologically (Table 1).

Specimens belonging to 4 species of cultured bivalves were obtained. Mangrove oysters *Crassostrea gasar*, brown mussels *Perna perna* and wing pearl oysters *Pteria hirundo* were sampled from culture systems within the experimental area of the Federal University of Santa Catarina (UFSC), Ponta do Sambaqui beach (27° 29' 18.8" S, 48° 32' 12.9" W), Florianópolis, SC. The lion's paw scallops *Nodipecten nodosus* were from lantern-net cultures at the experimental area of Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina (Company of Agricultural Research and Rural Extension of Santa Catarina), located on Bombinhas beach (27° 12' 5.4" S, 48° 30' 47.7" W), Bombinhas, SC.

Two species of wild molluscs were randomly selected from intertidal areas in Florianópolis (SC). The mangrove oysters *C. rhizophorae* were from a rocky shore site (approximately 100 m²) at Ponta do Sambaqui beach (27° 29' 26.5" S, 48° 32' 17.9" W), and the carib pointed venus clams *Anomalocardia brasiliiana* were from another site (approximately 200 m²) within the Reserva Marinha Extrativista do Pirajubaé-REMAPI (Marine Extractive Reserve of Pirajubaé; 27° 29' 18.8" S, 48° 32' 12.9" W).

Table 1. Details pertaining to the female bivalve molluscs from Santa Catarina State, Brazil, that were examined by histology: wing pearl oyster *Pteria hirundo*, mussel *Perna perna*, oysters *Crassostrea gasar* and *C. rhizophorae*, scallop *Nodipecten nodosus* and clam *Anomalocardia brasiliana*. Length is mean (\pm SD) shell length for all individuals in a sample. Note that the species *N. nodosus* is a simultaneous hermaphrodite. The stages of gonadal development are gametogenesis (GA), pre-spawning (PS), spawning (SP) and resting (RE). Unk.: Unknown

Species	Sampling season	Age (yr)	Length (mm)	No. of females (total)	Female gonad stage				Parasitized females (n)			
					GA	PS	SP	RE	GA	PS	SP	RE
From culture												
<i>P. hirundo</i>	Summer 2010	3	60.18 \pm 1.60	12 (30)	0	91.7	8.3	0	–	0	0	–
<i>P. perna</i>	Winter 2009	1	71.11 \pm 4.03	18 (40)	38.9	11.1	11.1	38.9	0	0	0	0
	Summer 2010	1	70.53 \pm 3.63	11 (40)	0	0	90.9	9.1	–	–	0	0
<i>C. gasar</i>	Winter 2009	1	52.01 \pm 8.35	3 (40)	0	0	0	100	–	–	–	0
	Summer 2010	1	52.67 \pm 4.52	19 (40)	0	0	100	0	–	–	19	–
<i>N. nodosus</i>	Winter 2009	1.5	67.08 \pm 5.70	40 (40)	2.5	35.0	47.5	15.0	0	0	0	0
	Summer 2010	1.5	66.42 \pm 3.85	40 (40)	45.0	52.5	0.0	2.5	0	0	0	0
From wild												
<i>C. rhizophorae</i>	Winter 2009	Unk.	60.55 \pm 7.90	24 (40)	4.2	8.3	62.5	25.0	0	1	15	0
	Summer 2010	Unk.	61.34 \pm 4.54	23 (40)	4.3	87.0	8.7	0	0	2	2	–
<i>A. brasiliana</i>	Winter 2009	Unk.	21.63 \pm 4.29	17 (40)	0	0	0	100	–	–	–	0
	Summer 2010	Unk.	22.43 \pm 2.31	16 (40)	0	0	87.5	12.5	–	–	0	0

During sampling, visual assessments for mortality at each site were made. Bivalves were transported on ice to the Nucleus for Aquatic Pathology Studies laboratory of UFSC, for dissection within 2 h of collection. The maximum shell length (hinge to bill) was recorded, and each bivalve was dissected using sterile techniques. During the dissection, the following macroscopic features were recorded: colouration of the gills, digestive gland, mantle and gonad; presence of nodules in the digestive gland, mantle and gonad; and appearance of the valves.

Histology

Cross sections and/or pieces of tissue about 1 cm thick that represented all major organs were fixed for 48 h in Davidson's fixative (Shaw & Battle 1957), then dehydrated and embedded in paraffin. Histological sections (5 μ m thick) were stained with haematoxylin-eosin (H&E) and examined using light microscopy. The entire gonadal area of each bivalve within each histological section was examined under 100, 400 and 1000 \times magnification. The sex, stage of gonadal development (gametogenesis, pre-spawning [ripe], spawning and resting [spent]), the presence of haemocyte infiltration and occurrence of oocyte parasites were recorded for each specimen. Prevalence was calculated according to Bush et al. (1997), and the intensity of infection was calculated by stereological analysis using a Weibel graticule with 21 short lines and 2 account-points in each extremity (42

account-points in total; Weibel et al. 1966). Briefly, under 400 \times magnification, 5 random and non-overlapping fields were selected in the gonad area and the parasites at each account-point were counted. The size of the unknown ovarian parasite (UOP) was determined from heavily infected oysters. The greatest diameter of 25 parasites containing the nucleus and parasome in the cross-section (see Fig. 3) as well as the diameter of the nucleus and parasome were measured. In addition, parasites showing evidence of division were photographed and a proposed sequence of division assembled.

DNA extraction

A small piece of gonad tissue was dissected from 4 species (*P. perna*, *C. gasar*, *N. nodosus* and *A. brasiliana* that were examined by histology) using sterile techniques and preserved in 95% undenatured ethanol at 4°C. DNA was extracted from the samples using a DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's instructions. DNA concentration and purity were measured using a NanoVue® spectrophotometer.

PCR

Some morphological features of the UOP were generally similar to those of *Paramoeba* (syn. *Neoparamoeba*, according to Feehan et al. 2013) spp.,

Perkinsus spp. and *Marteilioides* spp. Molecular probes were therefore used to detect parasites in these genera as well as endosymbionts or parasomes of *Paramoeba* spp. (genus *Perkinsela*). Table 2 lists the oligonucleotide primers and probe used to specifically amplify parasite DNA by conventional and real-time PCR. The negative control was nuclease-free water. Assay details pertaining to each parasite are as follows.

Paramoeba spp.

The real-time PCR was performed on DNA samples from the gonad of 6 heavily infected *C. gasar* according to Fringuelli et al. (2012), using the primer set for the 18S rRNA gene of *Paramoeba* spp. (UN for, UN rev and UN probe as described in Table 2). The reaction mixture included 10 µl of Sybr Green Kappa (Applied Biosystems), 0.6 µM of forward and 0.9 µM of reverse primers, 0.1 µM of probe and 2 µl of DNA template at a concentration of 100 ng µl⁻¹ in a total volume of 20 µl. The real-time PCR was carried out using a 7900HT Real-Time PCR System (Applied Biosystems) under the following conditions: (1) pre-denaturation at 95°C for 15 min; and (2) 45 amplification cycles of denaturation at 95°C for 15 s and annealing/extension at 56°C for 30 s each. Fluorescence in the VIC channel was obtained during the annealing/extension stage. The positive control for the *Paramoeba* spp. assays was a DNA solution

extracted from the gill of Atlantic salmon infected with *P. perurans*.

The conventional PCR was performed on DNA samples from the gonad of the same 6 heavily infected *C. gasar* to test for the presence of *P. perurans*. The PCR was performed according to Young et al. (2008) with modifications to the amplification conditions. The primer set used was N.perurF and N.perurR (Table 2). The PCR reaction with a total volume of 25 µl was made up of 20 µl of PCR Super-Mix, 1 µl of each primer, 1 µl of ddH₂O and 2 µl of DNA template at a concentration of 100 ng µl⁻¹. The amplification conditions were as follows: (1) initial denaturation at 94°C for 3 min; (2) 35 amplification cycles of 94°C for 45 s, 50°C for 45 s and 72°C for 90 s; and (3) final elongation at 72°C for 10 min. The same positive control used in the real-time PCR assay for *Paramoeba* spp. was employed. The PCR products were run on a 1% agarose gel (100 V for 40 min) and the presence or absence of amplified DNA of the expected product size for *P. perurans* (Table 2) was assessed by comparison to product obtained for the positive control.

Endosymbiont of *Paramoeba* spp.

Endosymbiont-specific PCR was performed according to Dyková et al. (2008) with modifications to the amplification conditions as indicated below. The primer set used was PERK-F and PERK-R (Table 2).

Table 2. Primer sequences for conventional PCR to evaluate the presence or absence of *Marteilioides chungmuensis*, *Perkinsus* spp., *Paramoeba* (syn. *Neoparamoeba*) *perurans* and *Perkinsela amoebae*-like organism, and for real-time PCR to evaluate the presence or absence of *Paramoeba* spp. F: forward; R: reverse

Target parasite	Primer name	Sequence (5'–3')	Dir.	Product size (bp)	Reference
Real-time PCR					
<i>Paramoeba</i> spp.	UN for	CGA TCA GAT ACC GTC GTA GTC	F	150	Fringuelli et al. (2012)
	UN rev	CAG CCT TGC GAC CAT ACT C	R		
	UN probe	VIC-CTC CTT CAG TAC CTT ACG AG			
Conventional PCR					
<i>Paramoeba perurans</i>	N.perurF	ATC TTG ACY GGT TCT TTC GRG A	F	636	Young et al. (2008)
	N.perurR	ATA GGT CTG CTT ATC ACT YAT TCT	R		
<i>Perkinsela amoebae</i> -like organism	PERK-F	GGA TAA CTT CGC GAA ACG CGA	F	1365	Dyková et al. (2008)
	PERK-R	GCT TTG ATG AAG CAC GTC TAC	R		
<i>Perkinsus</i> spp.	PerkITS-85	CCG CTT TGT TTG GAT CCC	F	703	Audemard et al. (2004)
	PerkITS-750	ACA TCA GGC CTT CTA ATG ATG	R		
<i>Marteilioides chungmuensis</i>	OPF-2	CCG CGT TTA CAC CTG TGA CC	F	672	Itoh et al. (2003)
	OPR-2	GAC CTT CCG ATT ATC CGC CC	R		
	OPF-3	GGC TGA ATA CCT CTG CC	F	447	
	OPR-3	CCT CTT GAC GAC GAC AC	R		

The PCR reaction (total volume of 25 μl) was made up of 20 μl of PCR SuperMix, 1 μl of each primer, 1 μl of ddH₂O and 2 μl of DNA template at a concentration of 100 ng μl^{-1} . The amplification conditions were as follows: (1) initial denaturation at 95°C for 3 min; (2) 35 amplification cycles of 94°C for 45 s, 60°C for 45 s and 72°C for 2 min; and (3) final elongation at 72°C for 10 min. The positive control was genomic DNA extracted from Atlantic salmon gills infected with *P. perurans* and previously shown by PCR to be positive for *Perkinsella amoebae*-like organisms. The PCR products were run on a 1% agarose gel (100 V for 40 min), and the presence or absence of amplified DNA of the expected product size for *P. amoebae* (Table 2) was assessed by comparison to the product obtained for the positive control.

Perkinsus spp.

DNA samples from the gonads (n = 6) and a mix of gills and digestive gland (n = 1) from heavily infected *C. gasar* were assayed by PCR according to Audemard et al. (2004) and OIE (2013), using the primers PerkITS-85 and PerkITS-750 (Table 2). The PCR reaction included 45 μl of PCR SuperMix, 1 μl of each primer, 1 μl of ddH₂O and 2 μl of DNA template at a concentration of 100 ng μl^{-1} , in a total volume of 50 μl . The amplification was carried under the following conditions: (1) initial denaturation at 95°C for 4 min; (2) 40 amplification cycles of 95°C for 1 min, 55°C for 1 min and 65°C for 3 min; and (3) final elongation at 65°C for 5 min. The PCR products were run on a 1.5% agarose gel (100 V for 40 min), and the presence or absence of amplified DNA of the expected product size (703 bp) was assessed by comparison to products obtained for positive controls of *Perkinsus marinus* and *P. olseni* (extracted DNA suspension, provided by G. Lowe, Pacific Biological Station, Canada).

Marteilioides chungmuensis

A nested PCR was performed according to Itoh et al. (2003), on DNA from gonad samples of *P. perna* (n = 2), *C. gasar* infected with the UOP (n = 2), *N. nodosus* (n = 2) and *A. brasiliensis* (n = 2) using a first-round primer set OPF-2/OPR-2 and a second-round PCR primer set OPF-3/OPR-3 (Table 2). Both PCR reactions included 45 μl of PCR SuperMix (Applied Biosystems), 1 μl of each primer, 1 μl of ddH₂O and 2 μl of DNA template at a concentration

of 100 ng μl^{-1} , using a Mastercycler gradient (Eppendorf) in a total volume of 50 μl . The first-round PCR amplification was carried out as follows: (1) initial denaturation at 94°C for 5 min; (2) 35 amplification cycles of 94°C for 1 min, 59°C for 30 s, and 72°C for 1 min; and (3) final elongation at 72°C for 10 min. The second-round PCR used the same thermocycling conditions, with the exception of an amplification annealing temperature of 55°C rather than 59°C. The electrophoresis was performed using a Bioanalyzer DNA Chips Agilent 2100 (Agilent Technologies), according to the manufacturer's instructions. The presence of amplified DNA of the expected product size in the first PCR (672 bp) and in the second PCR (447 bp) was assessed by comparison to products obtained for positive controls for *M. chungmuensis*-infected oyster DNA (extracted DNA suspension, provided by G. Lowe, Pacific Biological Station, Canada).

RESULTS

Histological analysis of bivalve gonad tissue

The UOP was only observed in females of *Crassostrea gasar* and *C. rhizophorae* (Table 1). The parasite was only detected in the gonads of oysters at spawning and pre-spawning stages of gonadal development. In samples of wild *C. rhizophorae* collected in winter 2009, the UOP was observed in all 15 spawning and in 1 pre-spawning female, but not in the remaining 8 females (overall prevalence = 67%). In summer 2010, infections were observed in 2/2 spawning and in 2/20 pre-spawning females, but not in the remaining females undergoing gametogenesis (overall prevalence = 17%). In samples of cultured *C. gasar* collected in summer 2010, the UOP was detected in all 19 spawning females (100%). The parasite was not detected in a sample of 3 females collected from this site in winter 2009; however, the sex could not be determined in over 90% of the 40 oysters sampled, and all were at the resting stage of gonadal development (Table 1). The mean intensity of oocyte infection for *C. rhizophorae* was similar in the winter and summer samples (2.14 \pm 0.61% and 2.21 \pm 1.65%, respectively, of the oocytes infected). Oocyte infection intensity was greater in *C. gasar* summer samples, where 5.38 \pm 2.96% of the oocytes were infected.

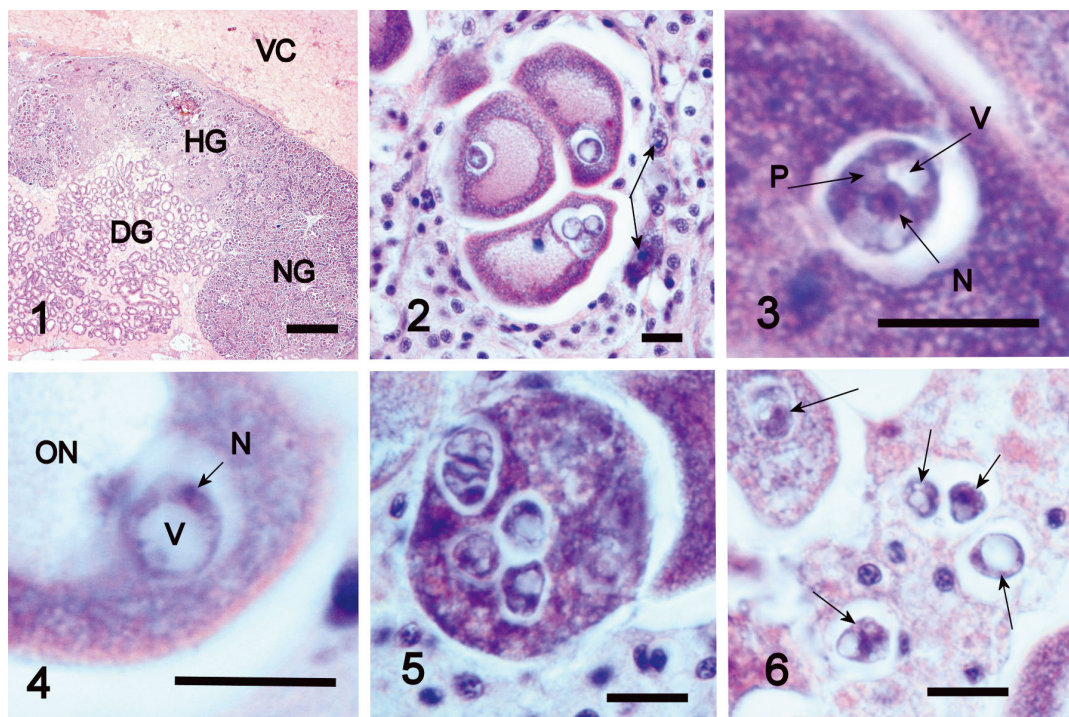
No alterations were observed macroscopically in infected animals, and no oyster mortality was observed during the oyster sampling. However, his-

tologically, all parasitized *C. gasar* and *C. rhizophorae* showed intense haemocytic infiltration in the vicinity of the parasitized oocytes (Figs. 1 & 2). Also, the epithelial layers of the gonadal follicles containing infected oocytes were severely disrupted (Fig. 2). No evidence of destruction or impairment of tissues was observed beyond the foci of infection (Fig. 1). No UOPs were found in the other organs, including the mantle, gills, adductor muscle and digestive gland. However, a few UOPs were observed among haemocytes in the connective tissue between the gonad and the digestive gland.

The parasite was spheroid in shape with an irregular outer membrane. The cytoplasm appeared frothy and contained numerous vacuoles of various sizes and a nucleus with a prominent central nucleolus. A basophilic parasome usually with peripheral chromatin was located near the nucleus (Fig. 3). In *C. gasar*, the mean \pm SD diameter of the UOP ($n = 25$)

was $6.57 \pm 0.73 \mu\text{m}$, the diameter of the nucleus was $1.96 \pm 0.34 \mu\text{m}$, and the diameter of the parasome was $1.88 \pm 0.36 \mu\text{m}$. These measurements were similar in *C. rhizophorae* ($n = 25$): 6.03 ± 0.55 , 1.69 ± 0.25 and $1.68 \pm 0.33 \mu\text{m}$, respectively. In some UOPs, a large central vacuole displaced the nucleus to the periphery of the cell (Fig. 4).

Most infected oocytes contained 1 UOP, usually within the cytoplasm and occasionally in the nucleus. However, in heavily infected oysters, several UOPs (up to 10) were observed in 1 oocyte. The histological staining of the cytoplasm in a few heavily infected oocytes was altered (Fig. 5). The parasite appeared to disrupt the nuclear membranes of its host cell (see Fig. 10). Single haemocytes were occasionally observed in the cytoplasm of infected oocytes (see Fig. 9). Some UOPs also occurred between oocytes among the haemocytes (Fig. 6). In oocytes and between haemocytes, UOPs were usu-



Figs. 1 to 6. Histological sections (haematoxylin and eosin stain) of parasitic amoebae (unknown ovarian parasites, UOPs) in the gonad of a mature female *Crassostrea gasar* from an aquaculture site at Ponta do Sambaqui beach, Florianópolis, Brazil. Fig. 1. Low magnification of a cross-section through an infected oyster with significant histopathology in the infected area of the gonad (HG) in contrast to normal gonadal tissue (NG) and no evidence of pathology in surrounding vesicular connective tissues (VC) and adjacent digestive gland (DG). Fig. 2. Cluster of 3 infected oocytes surrounded by haemocytes with few remnant cells from the gonadal follicle epithelium (arrows). Fig. 3. Classical amoeba profile with a nucleus (N) containing a prominent central nucleolus, a basophilic parasome (P) containing peripheral chromatin, cytoplasmic vacuoles (V) and surrounded by a vacuole-like space in the cytoplasm of an oocyte. Fig. 4. Signet ring morphology of the UOP with a large central vacuole (V) that has displaced the nucleus (N) to the periphery of the parasite in the cytoplasm of the host oocyte adjacent to its nucleus (ON). Fig. 5. Several UOPs in the cytoplasm of an oocyte with unusual staining properties. Fig. 6. UOPs (arrows) among the haemocytes between oocytes, 1 of which is infected (arrow upper left corner). Scale bars = (Fig. 1) 500 μm , (Figs. 2–6) 10 μm

ally surrounded by a vacuole-like space. In most cases, 1 UOP was observed in each vacuole-like space. However, some vacuole-like spaces within the oocytes contained 2 or more UOPs, and up to 10 were counted in 1 vacuole. Dividing stages were occasionally observed within the oocytes (Figs. 7–12). Division occurred by binary fission. Nuclear division (Figs. 7 & 8) was followed by migration of the resulting 2 nuclei to opposite sides of the UOP (Fig. 9). Although division of the parasome was not directly observed, by the time the nuclei were separated, a parasome was again found to be associated with each nucleus (Fig. 10). The cytoplasm cleaved (Fig. 11), and the resulting daughter cells were often observed in close proximity (Fig. 12).

Real-time PCR for *Paramoeba* spp.

Gonad DNA from heavily infected *C. gasar* had a mean cycle threshold value (C_t) of 26.6 ± 0.17 (range, 25.9–27.2). The mean C_t in the no-template control

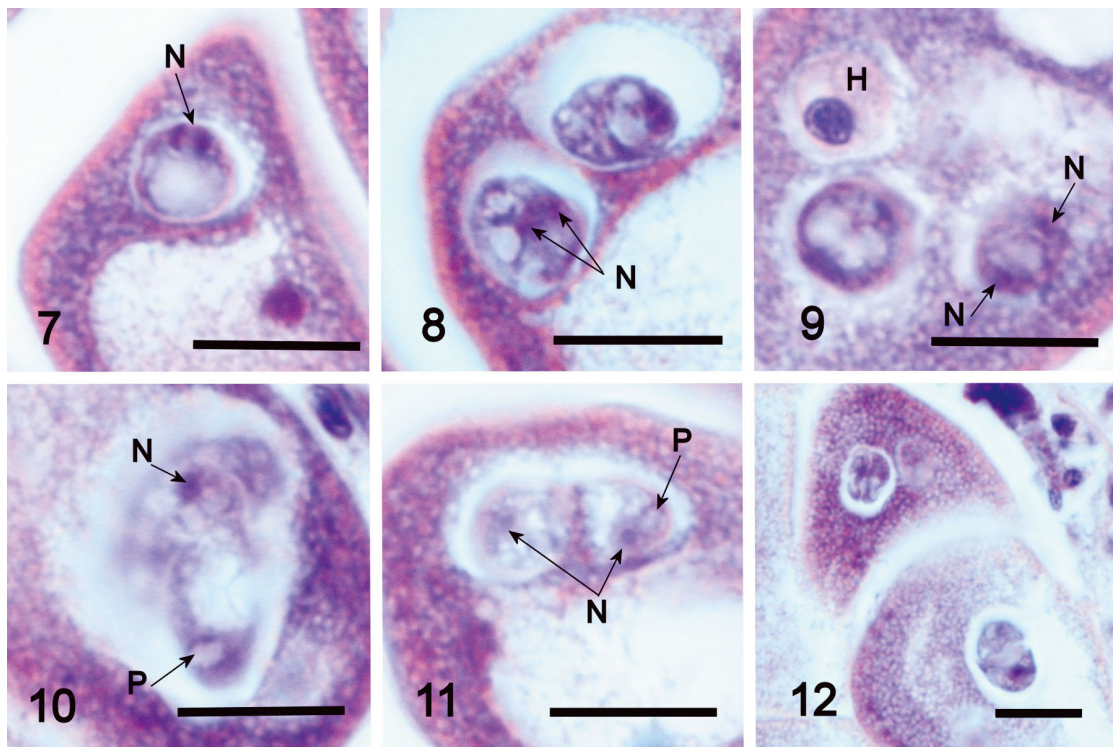
was 34.1 ± 1.25 (range, 29.5–37.1), whereas the positive control for *P. perurans* had a mean C_t value of 22.4 ± 0.4 (range, 21.9–22.9).

PCR for *Marteilioides chungmuensis*, *Perkinsus* spp., *Paramoeba perurans* and the *Paramoeba* endosymbiont

In the nested-PCR assays performed with *P. perna*, *C. gasar*, *N. nodosus* and *A. brasiliensis*, no product was seen, excluding the possibility of *M. chungmuensis* infection.

DNA from gonads, gills and digestive gland of heavily infected *C. gasar* did not amplify in PCR assays for *Perkinsus* spp. Also, there was no product in the PCR assays with *C. gasar* for *P. perurans*.

The endosymbiont of *Paramoeba* spp. PCR product obtained from a heavily infected *C. gasar* was a band of the appropriate size (1365 bp). A product of the same size was produced from the positive control sample.



Figs. 7 to 12. Histological sections (haematoxylin and eosin stain) of dividing amoebae (unknown ovarian parasites, UOPs) in the oocytes of a ripe female *Crassostrea gasar* from an aquaculture site at Ponta do Sambaqui beach, Florianópolis, Brazil. Fig. 7. Nucleus (N) in anaphase stage of mitosis. Fig. 8. One (possibly both) UOP in early telophase stage of parasite division (N). Fig. 9. Nuclei (N) have migrated to opposite sides of the UOP (note that a second UOP and a haemocyte (H) occur in the cytoplasm of this infected oocyte). Fig. 10. Cytoplasm beginning to cleave. At this level of focus, the nucleus (N) at one pole and the parasome (P) at the other are evident; also, the host cell nucleus is degenerating (upper left corner of image). Fig. 11. Cytoplasmic cleavage nearing completion with the nucleus (N) and parasome (P) of 1 UOP visible. Fig. 12. Two pairs of recently divided UOPs occur in close proximity within the cytoplasm of adjacent oocytes. All scale bars = 10 μ m

DISCUSSION

Morphological features and molecular assays confirmed that the parasite in the gonads of ripe female oysters in Brazil is new to science.

Parasites most commonly reported from the ova of bivalves are *Steinhausia* spp. However, the UOP found in the mangrove oysters *Crassostrea gasar* and *C. rhizophorae* described herein had different morphological features evident by light microscopy. Unlike *Steinhausia* spp., the cells of this UOP were larger and less numerous in each vacuole, and no spores were evident. Because *Marteilioides chungmuensis* is of considerable disease concern in areas of the North Pacific Asian coast, we excluded the possibility that the UOP was *M. chungmuensis* when no product was found in the nested-PCR analysis. Also, the UOP did not cause irregular, grossly visible enlargement of the infected gonadal tissues (nodular appearance) characteristic of oysters infected with *M. chungmuensis*, and many histological features typical of *M. chungmuensis* were not evident in the UOP. Although some UOPs had a large vacuole which displaced the nucleus to the periphery of the cell ('signet-ring' appearance), characteristic of *Perkinsus marinus* trophozoites (compare Fig. 9 in Perkins 1996 to Fig. 4 herein), the PCR assay for *Perkinsus* spp. was negative.

The irregular outline of the UOP, the frothy-looking cytoplasm, the prominent centrally located nucleolus in the nucleus and, most importantly, the presence of a parasome suggested that this UOP is related to ubiquitous members of the Order Dactylopodida and may be a species of *Paramoeba* or *Janickina*. Species in these genera are free-living amoebae in the marine environment and facultative parasites of marine vertebrates and invertebrates. The presence of the basophilic parasome in the cytoplasm of the amoeba is an important characteristic for the identification of amoebae in these genera (Page 1983). In these amoebae, the parasome is an endosymbiont related to members of the Kinetoplastida flagellates and variously referred to as *Ichthyobodo necator*-related organism (Caraguel et al. 2007, Tanifuji et al. 2011), *Perkinsiella amoebae* (Hollande 1980, Dyková et al. 2000), *Perkinsiella amoebae*-like endosymbiont (Dyková et al. 2003), and *Perkinsela amoebae*-like organism (Dyková et al. 2008, Feehan et al. 2013). As explained by Dyková et al. (2008), the senior homonym *Perkinsiella* is a genus of insects, thus requiring a replacement name of *Perkinsela*. Amplification observed in the real-time PCR for *Paramoeba* spp. and for the endosymbiont *P. amoebae*-like organism by conventional PCR supported this

view and suggested that the UOP in the mangrove oysters *C. gasar* and *C. rhizophorae* is an amoeba belonging to the Subclass Flabellinia, Order Dactylopodida. The relatively high C_t values obtained with the *Paramoeba*-generic assay is likely the combined result of reduced sequence homology and relatively low abundance of target DNA in the sample. Failure of the *Paramoeba perurans*-specific PCR indicated that this UOP is not *P. perurans*.

Reports of similar looking parasites were described by Da Silva et al. (2012) and Ivachuk (2013). However, these authors observed lower prevalences (2.50 and 2.10%, respectively) in Pacific oysters *C. gigas* from southern Brazil.

Interestingly, Becker & Pauley (1968) reported a gonad parasite, with similar features to the UOP described here, in mature gonads of *C. gigas* from Humboldt Bay, California, which they called Humboldt egg parasite (HEP). Although detected at a relatively high prevalence in female oysters (15 of 21 infected), those authors observed the HEP only in the cytoplasm of the oocytes. They did not identify the taxonomic affiliation of the HEP and considered it as 'Protista Incertae Sedis'. A similar gonad parasite was also observed in a partially spawned female oyster *C. gigas* from Read Island, British Columbia, Canada, in November 2009 (G. R. Meyer & S. M. Bower unpubl. report). Nucleus and parasome were evident in the oocyte parasites observed in histological sections (Fig. 13). Only this single infection has been detected in British Columbia over the past 25 yr, during which time more than 2000 mature female oysters have been examined for parasites and diseases using histology.

The haemocytic infiltration associated with the UOP observed in the present study is a typical immunological response of bivalves. A similar response was reported by Da Silva et al. (2012) and Ivachuk (2013) in the gonads of female *C. gigas* infected with unknown ovarian parasites.

The impact of intracellular infection in oocytes on the reproductive cycle is unknown (Comtet et al. 2004, Bower 2012). However, many authors (e.g. Bower & Figueras 1989, Villalba et al. 1997, Comtet et al. 2004) have suggested that parasites in the oocytes may affect reproduction of infected molluscs. The parasites can distort the nucleus of the gamete and can also cause the destruction of the oocyte (Rybakov & Kholodkovskaya 1987, Bower 2012). Also, the mollusc's fecundity is believed to be inversely related to the intensity of infection. Cunningham & Daszak (1998) associated the extinction of snails *Partula turgida* on Ra'iatea (Society Islands) with a *Steinhausia* sp. infection. Further research is

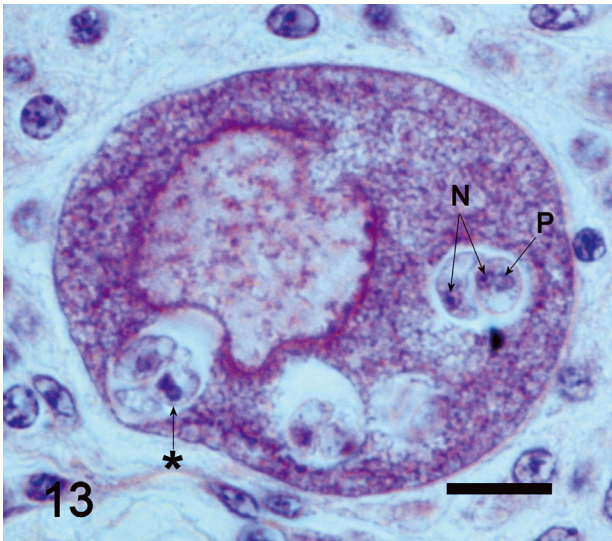


Fig. 13. Histological section (haematoxylin and eosin stain) of a parasitic amoeba in the gonad of a partially spawned female *Crassostrea gigas* from Read Island, BC, Canada. The nucleus (N) and parasome (P) are evident, and 1 specimen appears to be in late stages of division. Another pair of parasites (asterisk) has distorted the nucleus and cell wall of the oyster oocyte. The scale bar is 10 μ m

required to assess the impact of UOPs on oysters in Brazil.

Paramoeba have been described as ectoparasites on the gills of fish (Dyková et al. 2000, Munday et al. 2001) and as endoparasites in several tissues of sea urchins (Jones et al. 1985, Feehan et al. 2013), lobsters (Mullen et al. 2004) and crabs (Johnson 1977). In these hosts, severe infections with paramoebae were often associated with host death. To date, no parasites in the genera *Paramoeba* or *Janickina* have been described from bivalve molluscs. Also, the length of amoeba trophozoites in fish (ranging from 16.3 to 37.5 μ m; Dyková et al. 2000) and in sea urchin (ranging from 9 to 49 μ m; Feehan et al. 2013) are greater than observed for the UOP described here.

To identify the amoeba species observed in the present study, additional research is necessary. For example, the UOP should be isolated *in vitro* and observed actively moving on a surface, because the number of pseudopods separates the genus *Paramoeba* from *Janickina*, which has only 1 active pseudopod. Also, transmission electron microscopy and molecular analysis including sequencing and *in situ* hybridization are recommended to identify additional details relating to morphological and molecular features and to reveal life cycle parameters, which are all necessary for a valid species description. Additional studies are required to examine whether

a relationship exists between the sexual stage of the host and UOP prevalence and to evaluate UOP presence in other organs of the oysters.

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