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Parasites in the oyster *Crassostrea rhizophorae* from farmed and natural stocks in the Bay of Camamu, Bahia, northeastern Brazil

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This study investigated parasites in the oyster *Crassostrea rhizophorae* from a farmed and a natural stock in the Bay of Camamu, Bahia, Brazil. Samples were taken in October and November, 2012 and in January, 2013. 300 oysters were fixed in Davidson's solution and processed by means of conventional histological techniques, and were examined under an optical microscope. The following parasites were associated with *C. rhizophorae*: *Rickettsiae*-like organisms (RLOs), *Sphenophrya* sp. (Ciliophora), *Nematopsis* sp. (Apicomplexa), *Perkinsus* sp. (Perkinsozoa), *Urastoma* sp. (Turbellaria), *Bucephalus* sp. (Digenea), *Tylocephalum* sp. (Cestoda) and an unidentified copepod (Crustacea). *Perkinsus* sp. and *Nematopsis* sp. were prevalent in both environments. *Nematopsis* sp. had greater expression ($p<0.05$) in mangrove oysters than in cultivation, which was related to the more conspicuous presence of crustaceans in the first environment. Disruption of epithelial cells was caused by *Rickettsiae*-like organisms (RLOs) and hemocyte reaction and changes to the epithelium by *Perkinsus* sp. Xenomas were caused by *Sphenophrya* sp. in oysters from the mangrove and parasitic castration was caused by *Bucephalus* sp.

Key words: Histopathology, mangrove oyster, oyster farming, pathogens, *Perkinsus.*

INTRODUCTION

Brazil has more than 8,000 km of coastline and holds 12% of the planet's freshwater reserves. It also has more than two million hectares of mangrove swamps, and there is great potential for farming various types of marine organisms (FAO, 2012). Bivalve molluscs are among the types of organisms that are already farmed, notably the mussel *Perna perna* (Mytilidae) and oysters of the genus *Crassostrea* (Ostreidae). The oyster *Crassostrea rhizophorae* (Guilding, 1828) is found from the southern Caribbean until the Uruguay, which includes the Brazilian coast (Rios, 2009). This oyster inhabits consolidated substrates, including rocks, but mainly the roots of the red mangrove (*Rhizophora mangle*). This oyster is farmed, not intensively, only in a few estuaries of the state of Bahia, mainly involving traditional extractive communities of the coast. Diseases can have a damaging effect on natural and farmed shellfish stocks. According to the review by Boehs et al. (2012) in relation

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relation to the parasites and pathogens associated with the oyster *C. rhizophorae*, a series of inventories were produced over the last decade, at several locations along the Brazilian coastline, especially in the states of Santa Catarina, Ceará, Sergipe and Bahia. The present study investigated parasites that were present in *C. rhizophorae* in farmed stock (long-line) and in a nearby mangrove. The study hypothesis is that the oysters grown in long-line system are slightly different in terms of prevalence and severity of parasitic infection, since in this system the oysters are constantly submerged in comparison with the oysters from the adjacent natural stock, whose immersion is intermittent, being subject to tidal stage. Such comparison is important for attaining sustainability of farming practices.

MATERIALS AND METHODS

The oyster *C. rhizophorae* was collected from a region in Porto do Campo, in the Bay of Camamu, southern Bahia (13°57'S; 39°02'W) (Figure 1), on three different occasions: in October, 2012, November, 2012 and January, 2013. One hundred oysters were obtained on each collection day. Fifty were from a long-line cultivation system and fifty from the roots of the red mangrove *R. mangle*, near to the farmed stock. Temperature and salinity data from the site were measured using a standard mercury thermometer and an Atago S/Mill hand-held optical refractometer, every three days during the period. Rainfall data were obtained from weather station records held by the meteorological station of the Executive Commission for Cocoa Crop Planning (CEPLAC) in Camamu, Bahia, Brazil. The oysters were transported in buckets containing sea water to the State University of Santa Cruz (UESC), where they were processed. The transit time between the collection and the first processing (fixing material) was approximately 4 h.

The first procedure was to measure the dorsoventral axis (height) (Galtsoff, 1964), using digital calipers. The oysters measured from 5 to 7 cm in height. Following biometrics, the oysters (n = 300) were opened and macroscopically examined to ascertain whether there were any presence and clinical signs of parasitic infections, such as weight loss, along with changes to flesh color and texture. A longitudinal section of approximately 5mm was removed using a scalpel and then fixed in Davidson's solution (Shaw and Battle, 1957) for 24 to 30 h. The tissues were then washed in running water and put into 70% ethanol, dehydrated in a series of increasing alcohol concentrations, diaphanized in xylene and embedded in paraffin, at 60°C. Sections of thickness 7µm were cut using a microtome, placed on slides and subsequently stained with Harris's haematoxylin and eosin (H&E). The slides were analyzed using optical microscopy.

A Weibel graticule coupled to the microscope eye piece was used to evaluate the infection intensity by means of the stereology method (Lowe et al., 1994). This evaluates the area occupied by the parasite in the host tissue and makes it possible to classify the infection intensity, as follows: I -mild(area occupied by the parasite < 5%); II -moderate (5-25%); III -high (25-50%); or IV -very high (> 50%). Parasites that were present in low numbers were counted, and the results were expressed as the number of parasites per histological section.

Parasite prevalence was calculated as the number of infected oysters/total number of oysters collected and this was expressed as a percentage (Bush et al., 1997). The size of each parasite was measured using an Olympus CX 35 eye piece with a graduated scale attached to the microscope. The chi-square (χ^2) test was used to compare the prevalence of the most frequent parasites and, to compare the two environments (mangrove swamp and farmed). The significance level used was 95%.

RESULTS

The water temperature ranged from 26 to 31°C (mean = 29.9±2.14°C), salinity was between 25.5 and 31‰ (mean = 28.4±1.5‰) and the total rainfall was 73 mm in October, 78.4mm in November and 224.4mm in January. There was no macroscopic evidence of parasites or clinical signs of diseases. Microscopic analyzes identified bacteria, protozoa and metazoa associated with *C. rhizophorae* (Table 1). *Rickettsiae*-like organisms (RLOs) were observed in all three collections, and the highest prevalence was in farmed oysters in October (8%) (Table 1). They occurred intracellularly, in the epithelium of the digestive gland, in the form of colonies measuring between 4 and 10µm (mean = 7.1±2.7µm; n = 10), generally with 1 to 2 colonies/histological section but with some oysters presenting up to 9 colonies/histological section (Figure 2). In some cases, ruptures were observed in infected cells, but there was no evidence of any hemocytic response.

Sphenophrya sp. (Ciliophora: Sphenophrydae) was observed at low prevalence (2 to 6%), always in the gills, with sizes between 3 and 10µm (mean = 9.8±2.8µm; n = 8). This protozoan was more frequent in the mangrove oysters in relation to cultivation (Table 1). In 85.72% of the cases, it caused a lesion known as xenoma, which consists of hypertrophy in the host cell and its nucleus, caused by this parasite's intracellular presence (Figure 3). Xenomas were found only in mangrove oysters. This lesion was observed both in their early stage and at more developed stages. Up to 19 protozoa were seen in the xenomas within the host cell, and there were 1 to 3 xenomas/histological section. The size of the hypertrophied nuclei ranged from 6 to 15µm and the xenoma, from 27 to 35µm. The lesions were restricted to parasitized cells and there was no apparent hemocytic response from the host.

Oocysts of *Nematopsis* sp. (Apicomplexa: Eugregarinidae), typically containing a single sporozoite, were observed in 69.3±4.44% (n = 150) of the oysters from the mangrove swamp and 51.3±2.8% (n = 150) of the oysters from the farmed stock (Table 1), parasitizing hemocytes. On average, there were 1 to 3 oocysts/phagocyte, but in one specimen up to 8 oocysts/phagocyte were observed. The sporozoites had dimensions of between 3 and 6µm (mean = 5.5±1.7µm; n = 30) and the phagocytes were between 10 and 15µm. This parasite was observed in the mantle, digestive gland, gills (Figure 4) and adductor muscle, and it was most frequent in the digestive gland. The infection intensity was mild (< 5% of the parasitized tissue; n = 10 oysters) to moderate (5 to 25% of the parasitized tissue; n = 20 oysters). The chi-square test showed that there was higher prevalence of *Nematopsis* sp. in the

Table 1. Parasites and their prevalence (%) in the oyster *Crassostrea rhizophorae* in the farmed environment (n = 150) and in the mangrove swamps (n = 150) in Porto do Campo, Bay of Camamu, Bahia, Brazil, in October and November 2012 and January 2013, as shown by means of histology.

Parasites	October		November		January	
	Mangrove (%)	Cultivation (%)	Mangrove (%)	Cultivation (%)	Mangrove (%)	Cultivation (%)
RLOs	6	8	4	-	2	-
Sphenophrya sp.	6	-	6	2	2	-
Nematopsis sp.	94	56	44	38	70	60
Perkinsus sp.	92	100	100	86	94	88
Urastoma sp.	4	4	-	2	-	2
Bucephalus sp.	2	-	-	-	-	-
Tylocephalum sp.	2	-	-	2	-	-
Unidentified Copepod	4	-	2	2	2	2

*Without formingxenoma.

mangrove swamp than in the farmed stock (p = 0.0006), thereby indicating a relationship between the parasite's presence and the environment.

Perkinsus sp. (Perkinsozoa: Perkinsidae) was observed in all the collections and at high prevalence (93.3%). It presented a rounded shape, with vacuoles occupying most of the cell volume, a nucleus in the peripheral region and a prominent nucleolus.

This parasite was recorded in the mantle and in epithelium of the digestive gland, stomach walls (where its presence was most evident) and intestine (Figure 5). Its measurements were between 3 and 10 µm (mean = 6.8 ± 2.2 µm; n = 30). Hemocytic phagocytosis and infiltration were observed wherever there were large numbers of parasites (Figure 5). In these places, schizonts (dividing trophozoites) were also observed, measuring between 2 and 6µm (mean: 4.2 ± 1.8 µm; n = 30), along with brown cells (rhogocytes) around the *Perkinsus* sp. cells, thereby indicating a possible host defense response (not shown). Breakdown of the epithelium cells was observed at sites with large number of parasites, and in some cases, the tissue had become decharacterized and atrophied.

Urastoma sp. (Turbellaria: Urostomidae) was observed in all the months at low prevalence of infection (2 to 4%) and low intensity of infection (1 parasite/histological section). This measured between 43 and 97µm in length (mean = 78.6 ± 19.9 µm; n = 5) and was observed between the oyster's gill filaments in all cases. This turbellarian presented a typical ovoid shape, with thick walls and cilia covering the body, along with prominent ocelli (Figure 6). Sporocysts of *Bucephalus* sp. (Digenea: Bucephalidae) containing cercariae and germ masses (Figure 7) were observed in only one specimen, which was collected from the mangrove swamp in October, 2012.

The presence of furcae in the cercariae made it possible to identify the genus. This trematode was present in the digestive gland, mantle and gonad. There was evidence of parasitic castration, as shown by the destruction of follicles and gametes. The infection level was high (>25 to 50% of the tissue was parasitized). The mean size of the sporocysts was 50.6± 22.6 µm (n = 10).

Tylocephalum sp. (Cestoda: Tetragonocephalidae) was observed in one oyster that was collected in October, 2012 from the mangrove swamp and one from the farmed stock in November, 2012. The parasite, which was seen in a metacestode larval stage, was typically ovoid, and was found in the peripheral region of the digestive gland, surrounded by a fibrous layer of connective tissue from the host and also by hemocytes (Figure 8). It measured between 73 and 80µm (mean = 76.5±3.5 µm; n = 2). In both cases, only one parasite/histological section was view.

One unidentified copepod (Crustacea: Copepoda) was observed in all the collection months at low prevalence of infection (2 to 4%) and low intensity of infection (1 parasite/histological section) in the palial cavity, near the gills (Figure 9).

DISCUSSION

The results obtained from this study were consistent with previous observations made along

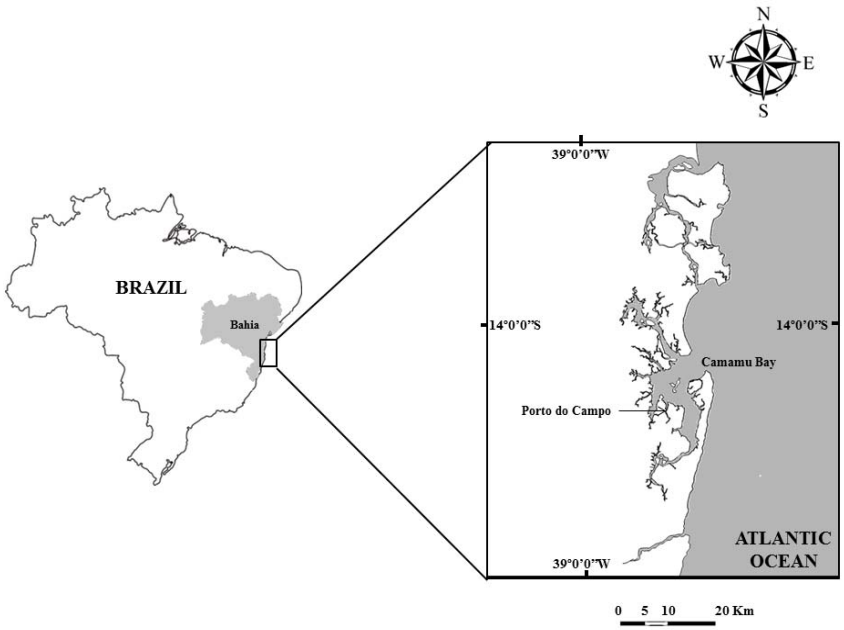

Figure 1. Map of the study area, with the collection point (Porto do Campo, Bay of Camamu, Bahia, Brazil) for the oyster *Crassostrea rhizophorae* in farmed and natural stocks.

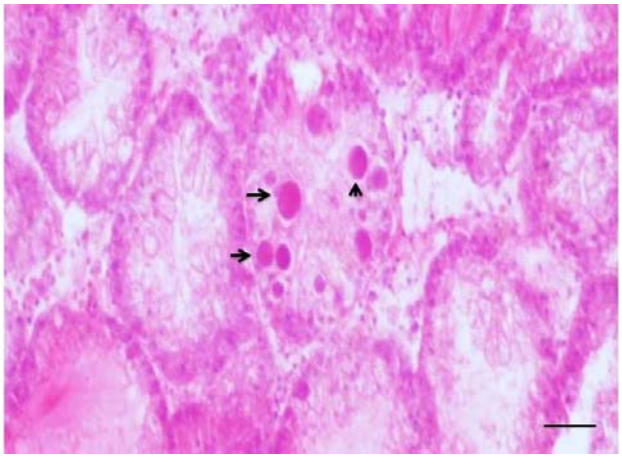

Figure 2. *Rickettsiae*-like organisms (RLOs) (arrows) in *Crassostrea rhizophorae* in the epithelium of the digestive gland;bar = 10 µm.

along the Brazilian coast (Boehs et al., 2012). Regarding the RLOs, although these bacteria were located in the digestive gland at low prevalence, they caused hypertrophy and, in some cases, rupturing of the epithelium at the base of the colonies' growth. This observation was consistent with previous observations on this same species (Zeidan et al., 2012; Sabry et al., 2013; Brandão et al., 2013; Cova et al., 2015). RLOs have also been recorded in *C. gigas* (Sabry et al., 2013), *Mytella guyanensis* (Ceuta and Boehs, 2012) and *Anomalocardia brasiliana* (Boehs et al., 2010), along the Brazilian coast. Cellular lysis, which was observed in some oysters during this study, was also reported in *Pitar rostrata* (Veneridae), in Uruguay (Cremonte et al., 2005). The effect of RLOs seems to be located, not causing severe damage to the host. With regard to *Sphenophrya* sp., this was observed in both environments and during the three collection months. It was located between the gill filaments or intra-

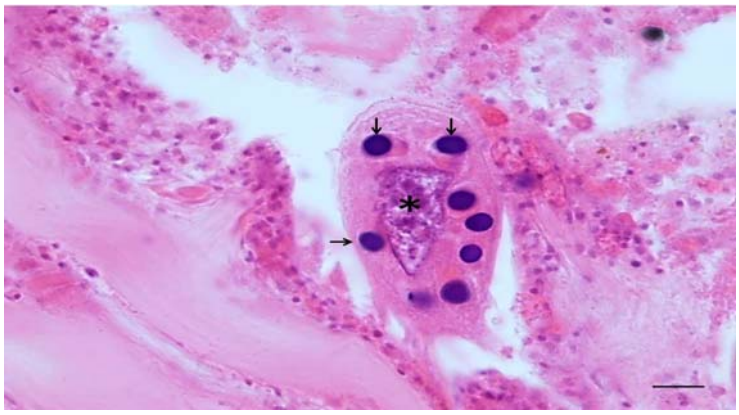

Figure 3. *Sphenophrya* sp. in the gill epithelium with xenoma formation; arrows = parasite; * = nucleus of the host cell;bar = 10 µm.

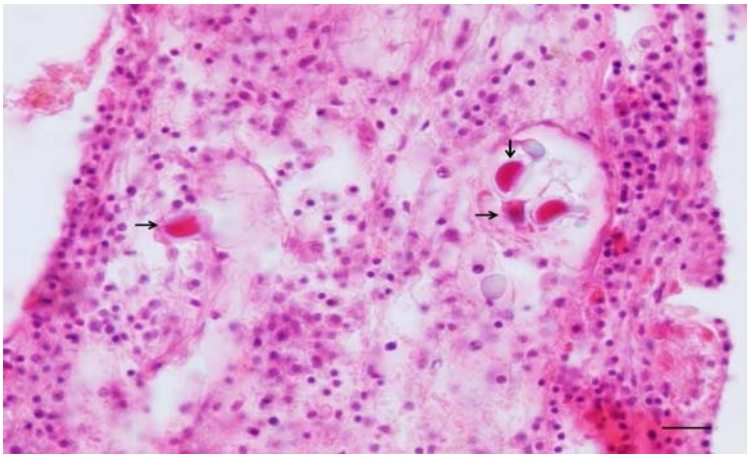

Figure 4. Intrahemocytic oocysts of *Nematopsis* sp. in the gill region (arrows);bar = 10 µm.

cellularly and, in this latter case, it formed xenomas, observed mostly in initial stage. Nascimento et al. (1986) studied *C. rhizophorae* in the *Baía de Todos os Santos* (All Saints' Bay) (Bahia) and found low prevalence (2%) and low intensity of infection by this protozoon. However, they did not report any cases of xenoma. This type of tumor has been reported in previous studies in the northern hemisphere (Bower et al., 1994), while it has only been reported in southern Bahia in Brazil (Boehs et al., 2009; Zeidan et al., 2012). In a study conducted in Florida (USA), Winstead et al. (2004) reported findings of xenomas due to *Sphenophrya* sp. at an advanced level of infection, presenting a length of around 3mm, which were detected in the oyster *C. virginica*. This is unlike what was observed in the present study, in which these lesions were relatively small (around 30 µm) and were undetectable to the naked eye.

Regarding *Nematopsis* sp., this was found at high prevalence level both in farmed stock and in the mangrove swamp. It was located in the gills, mantle and digestive gland of *C. rhizophorae*. The high prevalence of this parasite and its location in the host were also consistent with previous studies on the same species of oyster (Nascimento et al., 1986; Sabry et al., 2013; Cova et al., 2015), and also with studies on other economically important bivalves found along the Brazilian coast (Boehs et al., 2012). This protozoon uses bivalve molluscs as intermediate hosts and completes its life cycle in the intestine of crustaceans (Lauckner, 1983). In this study, the greater expression of this protozoon in oysters from the natural stock was most likely related to major presence of crustaceans in the mangrove swamp. A similar result was observed by Boehs et al. (2010) in the Cachoeira River (Ilhéus, Bahia), who reported that there was greater prevalence of this protozoon in *M. guyanensis* than in *A. brasiliana* and that there was no presence in *Iphigenia brasiliana*, which were respectively, crustaceans are more common. In the present study,

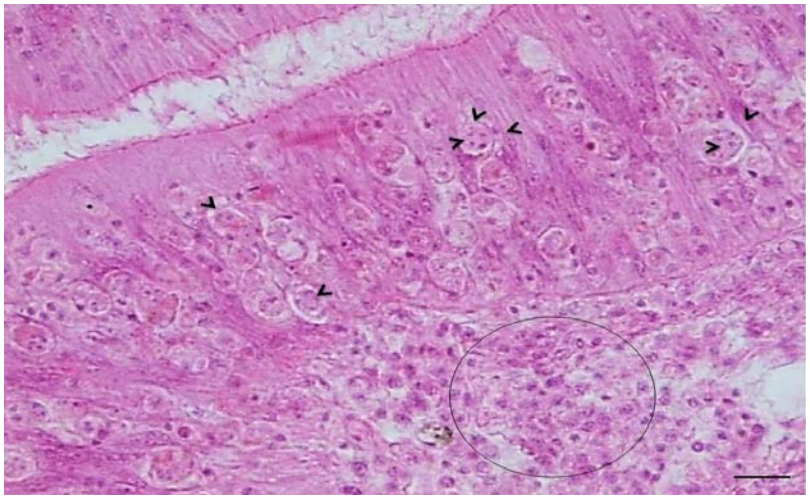

Figure 5. Trophozoites of *Perkinsus* sp.(arrowheads) in the epithelium of the intestine and hemocyte infiltration (indicated by the circle); bar = 10 µm.

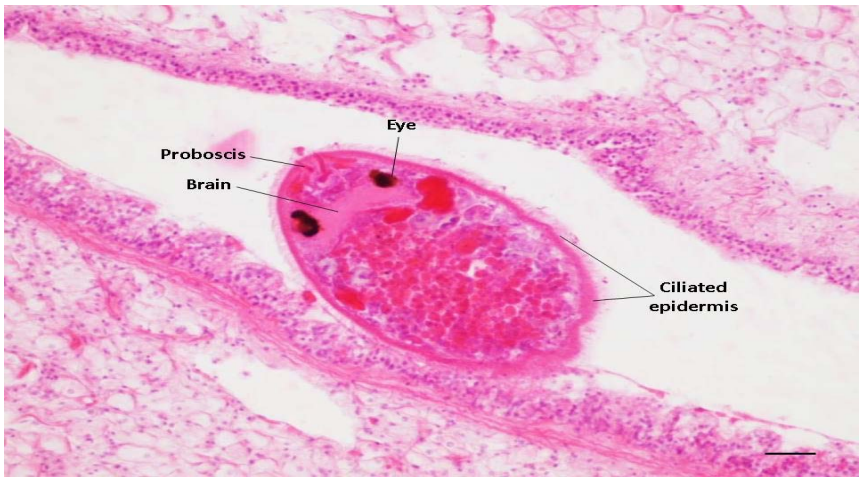

Figure 6. *Urastoma* sp. between the gill filaments;bar = 10 µm.

despite the parasite's high prevalence, no harm, histopathological changes or hemocyte responses were observed in the tissues of *C. rhizophorae*.

With regard to *Perkinsus* sp., the infected organs and tissues were also consistent with previous reports concerning the Brazilian coast (Brandão et al., 2013; Sabry et al., 2013). Histopathological changes caused by the protozoan *Perkinsus* sp. had already been reported by Lee et al. (2001) in *Tapes philippinarum* in Korea, who observed hemocyte infiltration and encapsulation in various tissues and organs, and in some cases atrophy of the digestive epithelium, as was also found in the present study. The presence of brown cells, which was observed in this study, was also identified in the oyster *C. virginica* on the coast of the United States in tissues infected with *P. marinus* (Lauckner, 1983), and those occurrences were correlated with changes to fat metabolism caused by the parasite. Protozoa of the genus *Perkinsus* are responsible for large-scale mortality among molluscs in various parts of the world (Brandão et al., 2013) and have already been reported to be present in gastropods, as well as oysters, scallops, mussels and other bivalves that are farmed for economic gain (Sanil et al., 2010), including in Brazil (Brandão et al., 2013; Queiroga et al., 2013; Sabry et al., 2013).

Metazoa of the phylum Platyhelminthes were observed in this study at low prevalence and low intensity of infection. Regarding *Urastoma* sp., it has not yet been well established what kind of association there is between between turbellarians and bivalve molluscs, that is, whether it is commensal or parasitic (Boehs et al., 2012). However, it is possible that both associations may occur,

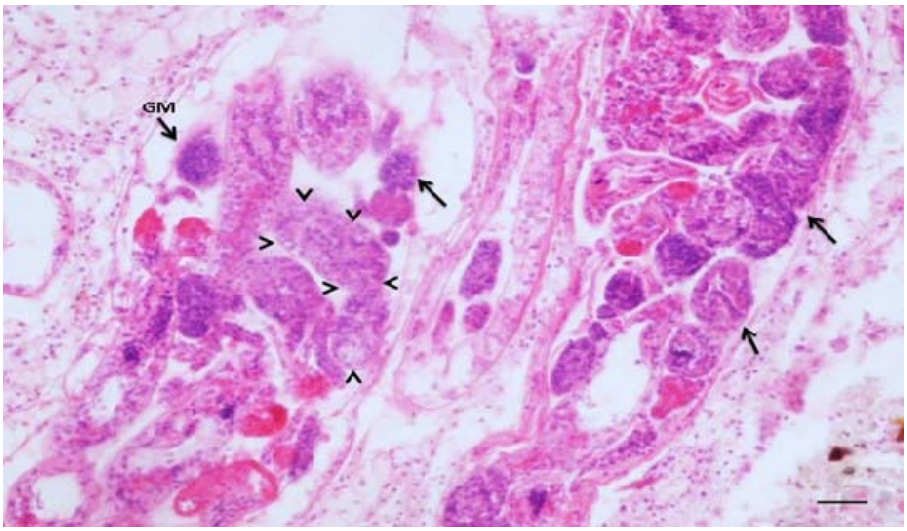

Figure 7. Sporocysts of *Bucephalus* sp. containing germ masses (arrows) and cercariae (arrowheads);bar = 25 µm.

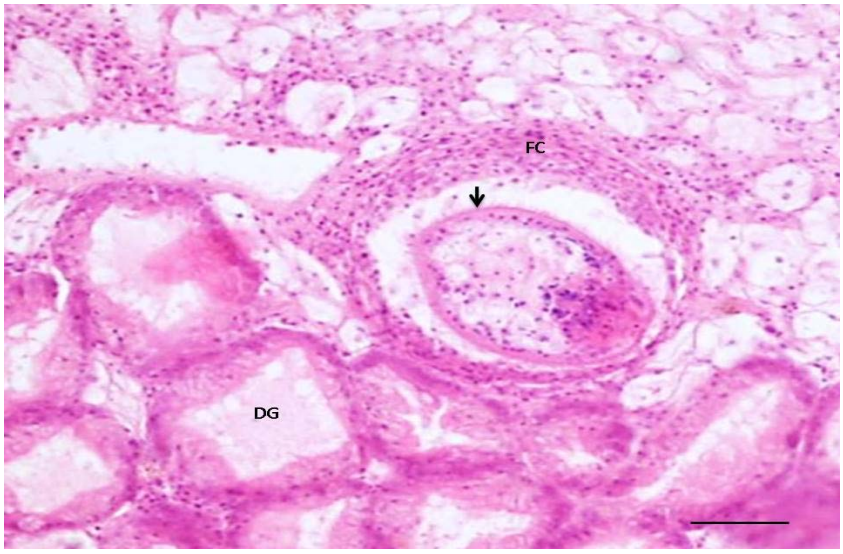

Figure 8. *Tylocephalum* (arrow) in the peripheral region of the digestive gland (DG), with the formation of a fibrous capsule (FC);bar = 25 µm.

depending on the physiological condition of the host and the number of symbionts that are present. This turbellarian was previously observed in *C. rhizophorae* and in *C. gigas* in Santa Catarina, southern Brazil (Sabry et al., 2013) and in *C. rhizophorae* and *M. guyanensis* in Bahia (Zeidan et al., 2012; Cova et al., 2015), both in the mantle cavity and between the gill filaments. In all cases, no apparent harm was caused to the host, or any consequent hemocytic response. This differed from what was observed by Robledo et al. (1994) in *Mytilus galloprovincialis* in an area of Spain (Galicia), where changes were observed in the gill tissue, along with hemocytic infiltration. With regard to *Bucephalus* sp., as was observed in a single oyster from the mangrove swamps, it is known to use bivalves as intermediate hosts (primary or intermediate) and fish as the definitive hosts (Lauckner, 1983). This parasite has already been recorded in *C. rhizophorae* on the coast of Bahia (Brandão et al., 2013) with similar infection pre- valence and intensity, as well as with a lack of defense response from the host. However, it always causes parasitic castration and tissue destruction. Concerning *Tylocephalum*

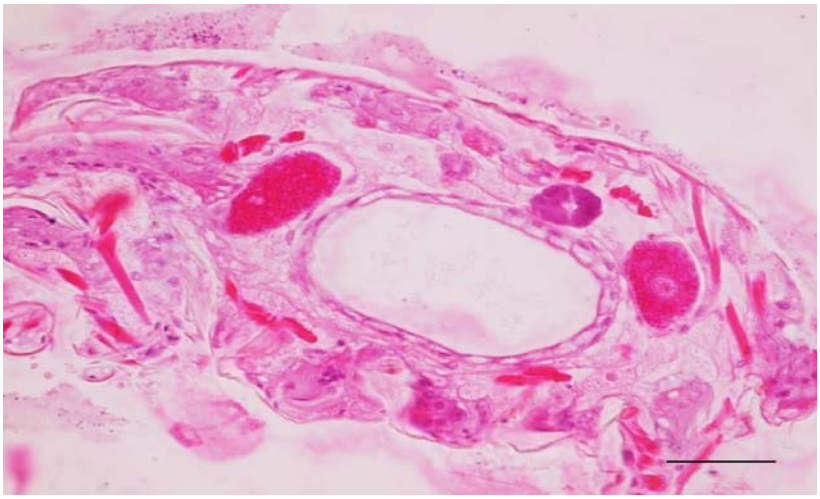

Figure 9. Unidentified copepod in the region near the gills; bar = 25 µm.

sp., records exist regarding this metacestode in *C. rhizophorae* in the state of Ceará (Sabry et al., 2007) and in Bahia (Nascimento et al., 1986; Zeidan et al., 2012; Brandão et al., 2013), documenting that this parasite has low prevalence and intensity of infection. This parasite has also been reported in other bivalves on the Brazilian coast, such as in *A. brasiliana* and *Iphigenia brasiliana* (Boehs et al., 2010) and *M*. *guyanensis* (Ceuta and Boehs, 2012). In all cases, encapsulation by fibers and cells from the host was observed, with no harm to the animals' organs. However, in a study conducted in Baía de Todosos Santos (Bahia), animal tissue rupturing was observed: this was due to penetration of the cestode, thereby causing mechanical harm to the animal (Nascimento et al., 1986). During this study, no histopathological or mechanical harm was detected.

CONCLUSION

Variations in temperature, salinity and rainfall over the course of the period had no apparent influence on the parasites' prevalence or intensity of infection. Regarding the observed harm caused by the parasites, the changes caused by *Sphenophrya* sp. and *Perkinsus* sp. were considered to be of low severity. On the other hand, *Bucephalus* sp. caused destruction that was evident in the tissues; however, this was not a matter for concern given the low prevalence of these parasites. Other associations were not considered relevant from the point of view of the health of *C. rhizophorae*. These included *Nematopsis* sp., which despite its relatively high prevalence, did not seem to significantly affect these oysters. This situation is further eased in farmed stocks, where the occurrence of *Nematopsis* sp. is lower due to the low rate of contact between parasites and crustaceans. Regarding *Perkinsus* sp., despite the high prevalence of this parasite, no macroscopic signs or records of mortality were observed in the oyster population during the study. Nevertheless, for this and other parasites, regular monitoring is recommended, especially in farmed oyster stocks, so that production losses can be reduced.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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