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Full Length Research Paper

Molecular xenomonitoring of trypanosomes in tsetse flies

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Monitoring trypanosomes infections in wild-caught tsetse flies in a given area, is important in prediction of epidemic outbreaks and spread of disease, and could help focus control programs for areas requiring immediate attention in order to limit disease transmission and spread. The main objective of this study is to evaluate the recently developed RIME LAMP and PanTryp LAMP for screening large numbers of tsetse flies for trypanosomes and to assess their sensitivities and specificities for trypanosomes in endemic areas. Wild-caught tsetse flies were dissected and the mid-guts examined by microscopy. The mid-guts were pooled in fives (including one infected gut where applicable), homogenised and DNA extracted by Quiagen kits. TBR- and ITS-PCRs were carried out and examined under ethidium bromide-stained agarose gels while RIMELAMP and PanTryp LAMP were carried out and stained with SYBR green and also observed under ethidium bromide stained agarose gels. A total of 14912 tsetse flies identified as *Glossina fuscipes fuscipes, Glossina. pallidipes, Glossina morsitans, Glossina. swynnertoni*, *Glossina fuscipes quazensis* were trapped from the six different countries. Of these, 8789 were dissected. Both males and female tsetse flies had equal infection rates (12.2%) although overall infection rates varied with country. The highest number of infected tsetse flies was obtained by PanTryp LAMP followed by RIME LAMP, ITS-PCR, TBR-PCR and microscopy respectively. PanTryp LAMP was the most sensitive method followed by ITS-PCR, RIME LAMP and TBR-PCR respectively. However, ITS-PCR was the most specific followed by TBR-PCR, RIME LAMP and PanTryp LAMP respectively. Carrying out LAMP tests in the field provides the simplest and quickest means to estimate trypanosome infection rates in the vector tsetse flies.

Key words: Xenomonitoring, trypanosome, tsetse fly, LAMP.

INTRODUCTION

Human African trypanosomiasis (HAT) is an important public health problem that affects rural populations of sub-Saharan Africa. The epidemiology of sleeping sickness disease is mediated by the interactions of trypanosomes with the vectors (tsetse flies) which transmit the disease to humans and animal hosts within a particular environment. The disease is usually confined in spatially limited areas referred to as " foci" of the disease found in remote rural areas in Sub Saharan Africa (Simarro et al., 2010). The risk of getting infected with the disease is, therefore, through the bite of a human being by an infected tsetyse fly. Consequently, deploying integrated control methods in areas infested with infected tsetse flies would drastically reduce the prevalence of the disease. With limited resources experienced by endemic countries, methods that would indicate such areas would be very useful to interrupt disease transmission (Franco et al., 2014). After continued control efforts in many of the endemic countries, the number of sleeping sickness cases reported in 2009 dropped below 10,000 for the first time in 50 years. This trend has been maintained in 2010 with 7,139 new cases reported (WHO Fact sheet No 259, January, 2012). In 2010, only the Democratic Republic of the Congo (DRC) reported over 500 new cases per year while Angola, Central African Republic, Chad, Sudan and Uganda reported between 100 and 500 new cases per year. Other countries such as Cameroon, Congo, Cote d'Ivoire, Equatorial Guinea, Gabon Guinea, Malawi, Nigeria, Tanzania, Zambia and Zimbabwe reported fewer than 100 new cases per year. However, current estimates indicated an annual incidence of between 50,000 and 70,000 cases (WHO, Fact sheet No 259, January 2012).

The disease in the DRC and Congo are due to *Trypanosoma brucei gambiense* that causes the chronic form of HAT while Tanzania, Malawi and Uganda are endemic for the acute form called *Trypanosoma brucei rhodesiense,* which may cause disease within weeks (Thomson et al., 2009). Uganda is the only country with both the chronic and the acute form of HAT whose foci are distinct but are feared to overlap hence complicating the diagnosis and treatment regime (Picozzi et al., 2005). HAT is invariably fatal if left untreated and major efforts to control the disease rely on strategic control which involves diagnosis and treatment of infected cases coupled with control of both the vector and reservoir (Simo et al., 2012).

Diagnostic tools appropriate for undertaking interventions to control trypanosome infections are key element to their success. Many diagnostic tests for trypanosome infection in the vector have unsatisfactory performance characteristics and are not well suited for use in the parasite control programs that are being increasingly implemented. It was argued that PCR techniques would simplify analysis of tsetse collected in the field (Moser et al., 1989; Majiwa and Otieno, 1990; Radwanska et al., 2002; Njiru et al, 2005; Adams et al., 2006), but was later noted that such studies tend to exaggerate both mature and immature fly infections rates when compared with microscopy (Farikou et al., 2010 and Simo et al., 2012). The presence of trypanosome DNA in a tsetse fly does not necessarily indicate a mature infection or even an established mid-gut infection as the trypanosome DNA could be from some blood meal taken just prior to analysis (Macleod et al., 2007). Furthermore, Farikou et al. (2010) state that a mid-gut infection does not indicate a mature infection that will be transmitted. However, for control purposes any tsetse fly infection is important for deployment of control strategy instead of waiting until infection is established in humans or animals. Although the application of modern laboratory research techniques to improve diagnostics for trypanosome infection has resulted in some technical advances, uptake has not been uniform. Frequently, pilot or proof of concept studies of promising diagnostic technologies have not been followed by much needed product development, and in many settings diagnosis continues to rely on insensitive and unsatisfactory parasitological (Woo, 1969; Molyneaux, 1975; Nantulya, 1990) or serodiagnostic techniques (Boakye et al., 1999; Njiokou et al., 2004). In contrast, Loop mediated isothermal amplification (LAMP)-based (Notomi et al., 2000; Kuboki et al., 2003; Njiru et al., 2008a) detection of trypanosomes in the tsetse vectors will result in critical advances in the control of both HAT and Animal African Trypanosomiasis (AAT).

Monitoring parasite prevalence in wild-caught vector populations in a given area (known as "xenomonitoring") has often relied on dissection of insect vectors and observation of parasites under a microscope, a process that is time consuming and depends on the skill of the microscopist (Auty et al., 2012). PCR techniques have now gained more use for example: *Plasmodium sp* (Snounou et al., 1993), *Leishmania spp* (Aransay et al., 2000; Dyab et al., 2015), *Oncocerca vulvulus* (Katholi et al., 1995; Rodriguez-Perez et al 1999). Xenomonitoring is important in prediction of epidemic outbreaks of disease and could help focus control programs to areas requiring immediate attention in order to halt disease transmission. This study aimed to evaluate the recently developed RIME LAMP and Pantryp LAMP for screening large numbers of tsetse flies for trypanosomes and to assess

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their sensitivities and specificities for trypanosomes in endemic areas.

METHODOLOGY

Tsetse collection and examination

During this study, tsetse flies were collected during field surveys between 2011 and 2012 in different sites for each country (Uganda – Kaberamaido and Dokolo, Tororo and Arua; Tanzania – Serengeti and Urambo; Malawi – Kasungu, Liwonde and Nkhotakota; Republic of Congo, Ngabe and Mboka Lefini, the Democratic Republic of Congo- Itubi and Bandundu). The tsetse flies were tested for *Trypanozoon* infection by dissecting out the mid guts of parasites by light microscopy and then pooled in groups of five (1 infected plus 4 non-infected or five non-infected) and kept in 1× PBS in liquid nitrogen tanks (Malele et al., 2013). The samples were then transported from the field sites to the research laboratories in respective countries. Upon arrival the specimens were stored at -80°C till processed. In the field lab, the tsetse flies were classified as positive if parasites were observed in the mid gut, but were classified as non-infected if no trypanosomes were observed in the mid gut by microscopy.

Isolation of parasite DNA

The mid guts were homogenized by a pestle in eppendorf tubes. Parasite DNA was extracted from the pooled midguts using the Qiagen kit (Crawley, UK) as per the manufacturer's instructions. The DNA was eluted in 50µL of Tris-EDTA (TE, Sigma Aldrich, Dorset, UK) and stored at -20°C until further analysis.

Polymerase chain reaction for the detection of parasite DNA

The nucleic acid extracts were analysed with TBR PCR and ITS-PCR, Both PCR assays were performed in a DNA thermal Cycler (Perkin–Elmer Cetus, Norwalk, CT, USA). TBR-PCR assay used two oligonucleotide primers which allowed the amplification of the TBR repeat (284bp) as described by Masiga et al. (1992) that is specific to *Trypanosoma brucei* subspecies. The sequences of these primers are: TBR1F: 5'--CGAATGAATATTAAACAATGCGCAG -3' (25-mer); TBR1R: 5'-AGAACCATTTATTAGCTTTGTTGC -3' (24-mer). Each amplification reaction was made in a final volume of 25µL containing 10 mM Tris–HCl pH 9.2, 1.5 mM $MgCl_2$, 75 mM KCl, 1.25 mM of each dNTP, 12.5pmol of each oligonucleotide primer for TBR-PCR, 1 U of Phusion High-Fidelity DNA polymerase (Thermo scientific, Waltham, USA) and 2µl of DNA template. The temperature program for the TBR-PCR was 1 min at 98°C, followed by 35 cycles of 30 sec at 98°C, 30 sec at 62°C, 2 min at 72°C and a final cycle of 7 min at 72°C. After PCR, 10µL of each sample was run on a 2% agarose gel and stained with ethidium bromide. The remaining volume was stored at -20°C. The ITS-PCR used two oligonucleotide primers with the following sequences: ITSBR 5'-TTG CTG CGT TCT TCA ACG AA-3' (20 mer) and ITSCF 5'-CCG GAA GTT CAC CGA TAT TG-3' (20 mer) for amplification of Internal transcribed spacer (ITS 1) as described by Njiru et al. (2005). Each amplification reaction was made in a final volume of 25µL containing 10 mM Tris–HCl pH 9.2, 1.5 mM $MgCl_2$, 75 mM KCl, 1.25 mM of each dNTP, 100 pmol of each oligonucleotide primer for ITS- PCR 1 U of Phusion High-Fidelity DNA polymerase and 2µl DNA template. The temperature program for the ITS-PCR was 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C and a final cycle of 5 min at 72°C. After PCR, 10 µl of each sample was run on a 2% agarose gel and stained with ethidium bromide. The remaining volume was stored at -20°C.

Detection of trypanosome DNA using RIME LAMP and PanTryp LAMP

The nucleic acid extracts were analyzed with RIME LAMP (Njiru et al., 2008b) and PanTryp LAMP. The 25 µl LAMP reactions were standardized for optimal reagent concentration. Briefly, the reactions were carried out at 2 µM for FIP and BIP primers, 0.8 µM for loop primer (LF and LB), 0.2 µM for F3 and B3 outer primers, master mix and 8U of Bst DNA polymerase large fragment (New England Biolabs). The reactions were carried out for 1 h at 62°C using the DNA thermal Cycler (Perkin–Elmer Cetus, Norwalk, CT, USA), and terminated by increasing the temperature to 80°C for 5 min. The amplification products are detected by direct visual inspection of the LAMP product after addition of 1 µl of 1/10 dilution of SYBR Green I (Invitrogen), and ethidium bromide stained agarose gels. Nucleic acids extracted from an in-vitro culture of procyclic *T. b. rhodesiense* were used as a positive control. Ultrapure water and gut extract of non-infected laboratory tsetse were used as negative controls. A test was considered positive if a green colour was visible and if the agarose gel showed the characteristic ladder pattern (Njiru et al., 2010).

RESULTS AND DISCUSSION

The tsetse flies

In this study, a total of 14,912 tsetse flies were trapped. However, only 8,789 live mature flies were dissected. Tenerals and dead flies were kept frozen and not analyzed any further. The high percentage of dead flies could be due to the high temperatures experienced during fly trapping and exhaustion as flies continually try to escape from the traps. In total, therefore, 8 789 tsetse flies were dissected of which 2,444 (27.8 %) were males and 6,345 (72.2%) were females. Overall, both male and females tsetse flies were equally infected with trypanosomes with a 12.2% infection rate for each sex. Of the total tsetse catches dissected, 1,359 were *G. f. fuscipes* of which 21 (1.5%) were infected; 2,726 were *G. pallidipes*, of which 68 (2.5%) were infected; 992 were *G. morsitans* of which 693 (69.9%) were infected; 622 were *G. swynnertoni* of which 10 (1.6%) were infected; 3090 were *G. f. quazensis* of which 280 (9.1%) were infected with trypanosomes. However, tsetse infection rates with trypanosomes varied from country to country (Table 1). Serengeti II (Tanzania) had the lowest infection rate (1.43%) while Liwonde (Malawi) showed the highest infection rate (94.5%). These data are in agreement with findings reported by Simo et al. (2012) and Auty et al. (2012). Table 2 shows that overall, Pan Tryp LAMP detected more positives (83.1%) followed by RIME-LAMP (67.1%), ITS-PCR (54.3%), TBR-PCR (51.9%) and then microscopy on pools of 5 wild tsetse flies.

Sensitivity and specificity of TBR- and ITS- PCRs and RIME and PanTryp LAMP tests for the detection of trypanosome DNA

A summary of the sensitivity and specificities of the index tests on the pooled tsetse gut specimens is presented in

Table 1. Detection of trypanosomes in tsetse fly midguts by dissection and light microscopy in various countries.

Country	Tsetse sp	Total catches	Dissected			Infected		Inf rate (%)
			M	F	Total	M	F	
Uganda								
Ka'maido+ Dokolo	*Glossina fuscipes*	2391	474	885	1359	5	16	1.55
Tororo	*G. pallidipes*	1000	285	363	648	8	12	3.09
Tanzania								
Serengeti I	*G. pallidipes*	1141	91	616	707	0	11	1.56
Serengeti II	*G. swynertoni*	1417	2	620	622	2	8	1.43
Urambo I	*G. pallidipes*	1329	251	440	691	9	12	3.04
Urambo II	*G. pallidipes*	1088	172	508	680	2	14	2.78
Malawi								
Kasungu	*G. morsitans*	521	121	322	443	51	146	44.46
Liwonde	*G. morsitans*	539	54	253	307	51	239	94.46
Nkotakota	*G. morsitans*	257	109	133	242	90	116	85.12
DRC								
Itubi	*G. f. quanzensis*	2319	561	1409	1970	50	129	9.09
Congo B								
Ngabe, Mboka-Lefini	*G. f. quanzensis*	2910	324	796	1120	31	70	9.02
Total		14912	2444	6345	8789	299	773	-

Male (M), Female (F), Infected Males (Inf M), Infected Females (Inf F), Infection rate (Inf Rate).

Table 2. Performance of TBR-PCR, ITS-PCR, RIME LAMP and Pan Tryp LAMP assays on Pools of 5 wild tsetse midguts collected in 2012.

Institute/country	Disease Form (HAT)	No. of pools tsetse	No. of pools positive (%) by each test				
			Microscopy	TBR-PCR	ITS-PCR	RIME-LAMP	Pan Tryp-LAMP
Makerere-NEUganda	*T.b.rhod*	184	16 (8.7)	160 (86.9)	163 (88.6)	179 (97.3)	182 (98.9)
Uganda-Kenya border	*T.b.rhod*	129	20 (15.5)	98 (75.9)	109 (84.5)	113 (87.6)	124 (96.1)
TTRI-Tanzania-Serengti	*T.b.rhod*	102	12 (11.8)	35 (34.5)	14 (13.7)	38 (37.2)	56 (54.9)
TTRI-Tanzania-Urambo	*T.b.rhod*	98	19 (19.4)	33 (33.7)	16 (16.3)	40 (40.8)	70 (71.4)
Malawi	*T.b.rhod*	214	197 (92.0)	137 (64.0)	129 (60.3)	199 (92.9)	212 (99.0)
NPHAT-DRC	*T.b.gam*	130	60 (46.2)	9 (6.9)	45 (64.6)	17 (13.1)	95 (73.1)
LSNP-Congo	*T.b.gam*	78	24 (30.8)	14 (17.9)	32 (40.0)	41 (52.6)	38 (48.7)
Total (%)	**-**	935	348 (37.2)	486 (51.9)	508 (54.3)	627 (67.1)	777 (83.1)

Tsetse midguts were pooled as follows: 1 infected plus 4 non-infected or five non-infected

Table 3. As shown in Table 3, PanTryp LAMP was the most sensitive at 99.9%, followed by ITS-PCR (88.7%), RIME LAMP (87.1%) and TBR-PCR (77.0%). However, ITS-PCR was the most specific at 67.4%, followed by TBR-PCR (65.5%), RIME LAMP (51.4%) and PanTryp LAMP at 33.6% in all countries participating in this study. TBR PCR appeared to be a problem in Malawi, Democratic Republic of Congo (DRC) and the Republic of Congo where it was less sensitive than microscopy. This could be attributed to problems with technical aspects of the methodology as these laboratories were newly set up and staff had limited experienced.

Table 3: Sensitivities (Sen), specificities (Spe), positive predictive (PP), and negative predictive (NP) values of the 4 molecular tests against microscopy in detecting trypanosomes in tsetse flies

Country	TBR-PCR				ITS-PCR				RIME- LAMP				Pan Tryp-LAMP			
	Sen	Spe	PP	NP	Sen	Spe	PP	NP	Sen	Spe	PP	NP	Sen	Spe	PP	NP
Uganda	100.0	14.3	100.0	100.0	100.0	12.5	9.8	100.0	100.0	2.9	8.9	100.0	100.0	1.2	8.7	100.0
U-K Border	100.0	28.4	24.4	100.0	100.0	18.4	18.4	100.0	100.0	14.7	17.7	100.0	100.0	4.6	16.1	100.0
Tanzania-S	100.0	74.4	34.3	100.0	100.0	97.8	85.7	100.0	100.0	71.1	31.6	100.0	100.0	51.1	21.4	100.0
Tanzania-U	100.0	82.3	57.6	100.0	84.2	100.0	100.0	89.6	100.0	73.4	47.5	100.0	100.0	35.4	27.1	100.0
Malawi	65.9	58.8	94.9	13.0	61.9	58.8	94.6	11.7	94.9	29.4	93.9	33.3	99.5	5.8	92.5	50.0
DRC	15.0	100.0	100.0	57.8	75.0	100.0	100.0	82.3	15.0	100.0	100.0	57.8	100.0	53.3	63.2	100.0
Congo	58.3	100.0	100.0	84.8	100.0	84.1	75.0	100.0	100.0	68.5	68.5	100.0	100.0	84.1	75.0	100.0
Average	77.0	65.5	73.0	79.3	88.7	67.4	69.1	83.4	87.1	51.4	51.2	84.4	99.9	33.6	43.4	92.8

Whenever the prevalence of trypanosomes in tsetse flies is very low (especially in *T. brucei* species), the positive predictive value is never close to 1 even if both the sensitivity and specificity are high. Thus in screening tsetse in the field, it is inevitable that many with positive test results in LAMP will be false positives. Both positive and negative predictive values of a test will depend on the infection rates in tsetse flies.

Mitashi et al. (2012) compared LAMP to PCR in patient diagnosis and reported that the positive predictive value of a test is low in low incidence settings. This study therefore suggest that the variation observed in each country using the same test protocols for each test may be related to variation in trypanosome infection rates in tsetse (Table1, field results). The predictive value indicated the usefulness of each of the test for xenomonitoring of trypanosomes in tsetse flies. Where the infection rates of trypanosomes in tsetse flies were high, both the positive and negative predictive values were comparatively higher. The kappa value of a diagnostic test agreement (not shown in the table) was correspondingly higher than in those with low infection rates.

Unlike other insect vectors such as mosquitoes, black flies and sand flies, both male and female tsetse feed on blood and therefore both are trypanosome vectors. In this study both male and female tsetse were equally infected with trypanosomes at 12.2% infection rates. This is in contrast to earlier reports where male flies (*Glossina morsitans morsitans, G. pallidipes, G. fuscipes fuscipes*) showed higher rates of infection with *T. brucei* than females (Dale et al., 1995; Mauldin et al., 1991; Moloo et al., 1992). In the management of African trypanosomiasis a suite of diagnostics for host-level trypanosomiasis have been developed (Chapuis et al., 2005; Enyaru et al., 2010; Wastling and Welburn, 2011), each with its imperfections (Mitashi et al., 2012). This is coupled to the need to undertake cost-effective trypanomiasis mapping. There is therefore a need to develop novel techniques to overcome the shortcomings of the diagnostics in use.

The major aim of the present study was to evaluate the performance of relatively new rapid tests (RIME LAMP and PanTryp LAMP assays) for the detection of trypanosome DNA in wild-caught tsetse flies. These tests could be used for surveillance to indicate areas with high tsetse infection rates where control measures could be focused. The study data clearly demonstrate that the LAMP DNA detection method is simpler and more sensitive than microscopy and TBR- and ITS- PCRs. This data is also supported by the preliminary laboratory evaluation of the LAMP method using mid guts of laboratory-reared tsetse (Malele et al., 2013). Therefore, LAMP has a superior sensitivity in detecting trypanosome DNA from tsetse midguts in comparison to TBR- and ITS-PCRs respectively. This could be attributed to high copy numbers of RIME, estimated at 500 copies per haploid genome (Bhattacharya et al., 2002) while the ITS region is estimated to have 100 to 200 copies (Desquesnes and Davila, 2002) and TBR about 1000 copies (Masiga et al., 1992). Indeed, the LAMP detection method is more rapid (60 minutes compared to 150 minutes for PCR methods). Like PCR, it is sequence specific, producing sharp and clear bands in trypanosome-positive samples. More importantly, the LAMP detection method does not require expensive laboratory equipment and has the potential to function efficiently in the hands of a moderately trained technician. These factors considerably

overcome some of the many constraints that hamper surveillance and control efforts in trypanosomiasis endemic countries.

Conclusion

Xenomonitoring of trypanosomes in tsetse using RIME and Pan Tryp LAMP techniques would fast highlight areas of potential disease outbreak and help focus control programs. Therefore, combining field data from the LAMP tests and later confirming with PCR tests in the laboratory would be the simplest and practical means to estimate and map trypanosome infection rates in the tsetse flies.

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Conflicts of interests

The authors declare that they have no conflicts of interest.

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Full Length Research Paper

Parasitic occurrence in the giant freshwater prawn *Macrobrachium rosenbergii* from coastal West Bengal, India

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In recent years, culture of *Macrobrachium rosenbergii* is expanding in India and West Bengal state in particular, due to its relatively fast growth rate, high market price and export demand. However, the detail study on parasitic diseases has received less attention on cultivable *M. rosenbergii* compared to the penaied prawns. The present parasitic survey on *M. rosenbergii* from coastal West Bengal state in India encountered 14 species of parasites. Among the protozoan parasites, *Zoothamnium* sp., *Amphileptus* sp., *Dileptus* sp., *Chilodonella* sp., *Balladyna* sp., *Epistylis* sp., *Vorticella* sp. and *Gregarina* sp. were recovered; of which 4 were ecto-commensals and other 4 were endo-commensals. Metazoan parasites represented by *Myxobolus* sp., *Rhabdochona* sp., *Indocucullanus* sp., *Procamallanus* sp., *Cucullanus* sp. and *Acanthogyrus* sp; of which 1 were ecto-commensals and other 5 were endo-commensals. In West Bengal state, inland culture and capture based fishery activities, mainly rural based, are operated by poor farmers. Developing the right kind of intervention and managemnt practice can prevent adverse impact of diseases and assist poor farmers for sustainable production.

Key words: *Macrobrachium rosenbergii*, epibionts, parasites, West Bengal.

INTRODUCTION

The giant freshwater prawn *Macrobrachium rosenbergii* has a wide distribution throughout the Indo-Pacific region and most favoured for farming in tropical and subtropical areas of the world (New, 2005). In India, its culture was developed few decades ago as an alternative to the *Penaeus monodon* and to compensate for the substantial losses due to the epidemics of white spot syndrome in penaeid shrimp farming, hypothesizing that *M. rosenbergii* is resistance to white spot disease (Sahul et al., 2000). This freshwater palaemonid prawn is popularly known as 'scampi' in Indian trade, farmed chiefly in small to medium-sized earthen ponds in the states of West Bengal, Andhra Pradesh, Tamil Nadu and Kerala states in India (Nair and Salin, 2012).

In its culture and natural settings, crustaceans serve as hosts for a wide range of protozoan and metazoan

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parasites, some of them cause considerable pathogenicity by affecting the growth and reproductive performance of the hosts (Jayasree et al., 2001). In Asia, the viral, bacterial and parasitic infections of *M. rosenbergii* were found to be important causes of significant pond production losses (Chu et al., 2011). However, the study on parasites and diseases has received considerable attention on cultivable penaeid prawns and *Penaeus monodon* in particular, but non-penaied prawns have received less attention in this regard. Viral diseases of the *M. rosenbergii* were extensively reviewed by Bonami and Widada (2011). Studies on protozoans and metazoans parasites from *M. rosenbergii* were carried out from various parts of the world (Beck, 1980; Rohde, 1984; Schuldt and Rodrigues-Capitulo, 1985; Areerat, 1988; Nash, 1989; Johnson, 1995; Johnson and Bueno, 2000; Rodriguez et al., 2001; Montoya, 2003) but majorities were focused either on individuals or groups of parasites. In India, several authors reported parasitic occurrences in *M. rosenbergii* (Santhakumari and Gopalan, 1980; Sankoli et al., 1982; Saha et al., 1988; Shanvas et al., 1989, Jayasree et al., 2001; Mariappan et al., 2003).

In recent years *M. rosenbergii* is expanding in India due to its relatively fast growth rate, high market price and export value. The total scampi production from India in 2010 to 2011 was about 8778 metric tons and the West Bengal state was the leading producer with a production of 2906 metric tons (MPEDA, 2011). In the year 2011 to 2012, India exported 2723 metric tons *M. rosenbergii* with an increase of 31.61% in quantity than the previous years (MPEDA, 2011). In view of the facts, information on parasites is essential to prevent any disease outbreak in culture setting and to draw sustainable management plan to avoid any parasitic outbreak. In continuation to the fact, an investigation of the ecto and endo parasites was carried out to benchmark the parasitic occurrences of farmed *M. rosenbergii* from two major prawn producing districts of West Bengal state in India.

MATERIALS AND METHODS

The present study was done during the period of May to July, 2014. Live *M. rosenbergii* (n = 75) (length: 3.95-8.25 inch; weight: 35.6-102.05 g) was randomly sampled from different freshwater as well as brackish water impoundments of South and North. 24 Parganas districts of West Bengal were brought to the laboratory in oxygenated poythene bags for detail investigations. The water quality parameters of the water bodies like temperature, salinity, dissolved oxygen and pH ranged from 28.5 to 32.5°C, 0 to 5 ppt, 3.5 to 7.5 ppm and 7.8 to 8.5 respectively. The stocking density of the prawn in this traditional farming system was generally 5 to 10 pcs/m^2. All prawns were dissected, and scrapings from cuticles, appendages, gills, digestive tracts, intestine and hepatopancreas were taken on clean slides with saline solution (0.75% NaCl) and examined (Mondal et al., 2014). The smeared slides were air dried, fixed in acetone free methanol and stained with Giemsa (HiMedia, Mumbai) (Chakraborti and Bandyapadhyay, 2010). Standard methods were employed for fixation and staining of parasites. The prepared slides were examined under Carlzeiss stereo microscope using 10×, 40× and 100× objectives. The identification of parasites was done based on Couch (1983), Kabata (1985), Lightner (1996) and Mehlhorn (2008). After that, the slides were deposited at the Laboratory of Aquaculture Management and Technology, Vidyasagar University, India.

RESULTS AND DISCUSSION

During the present investigation, a total 14 species of parasites were recorded from different organs of *M. rosenbergii* (Table 1, Figures 1 and 2). Protozoans belonging to two groups were mainly encountered, namely the epibiontic ciliates and the gut dwelling gregarines (Chakraborti and Bandyapadhyay, 2011). Among the 7 ciliates, peritrichous ciliates belonging to the genera *Zoothamnium, Epistylis* and *Vorticella* were found to predominant in pleopods, uropods, gills and intestine. 1 species of gregarines were found to restrict in intestine. Among the observed protozoan parasites, 4 were ecto-commensals and other 4 were endo-commensals. Among the 6 metazoan parasites, 4 species belonging to phylum nematode was predominant only in digestive tracts. 1 species of cnidarian ecto-commensal and acanthocephalan endo-commensal, respectively were also encountered during the present parasitic survey.

A wide variety of epicommensal have been reported from the gills and external surfaces in postlarval and adult fresh water prawn (Johnson and Bueno, 2000). Ciliate infections, in particular *Zoothamnium*, *Epistylis* and *Vorticella* were recorded earlier from penaeid as well as non-penaeid prawns and from several geographical regimes (Brock, 1983; Colorni, 1985; Overstreet, 1987). In consistent with the present findings, several authors reported peritrichous ciliates from India dealing with their occurrence, pathogenicity and epizootiology (Rajendran et al., 1982; Felix et al., 1994; Nandi and Das, 1995; Rajendran, 1997; Jayasree et al., 2001; Chakraborti and Bandyapadhyay, 2011). The heavy infections of ectosymbionts peritrichous ciliates ultimately causing death to the host was discussed by Overstreet (1973).

The seasonal incidence of *Zoothamnium* infections and their relation with salinity and rainfall was also discussed by Jayasree et al. (2001). Gut dwelling gregarine parasites are all pathogenic to the decapod crustaceans and may lead to reduced absorption of food or occasional intestinal blockage and possibly mortality of their host (Sprague and Couchi, 1971; Lightner, 1993). The infection rate with gregarinids is high in summer than winter as discussed by Timofeev (2001). In India, Jayasree et al. (2001) reported two species of gregarines from *Metapenaeus dobsoni* but those were completely absent in *M. rosenbergii*. Nematode infection recorded rarely from *M. rosenbergii* (Nash, 1989). It is contacted through foraging on infected copepods that serve as intermediate hosts for these helminths. There is thus every potential for some human helminth infections to be

Table 1. The detail lists of parasites recovered from *Macrobrachium rosenbergii* and their site of infection.

S/N	Groups	Parasites	Organ specificity
Protozoan			
1	Ciliates	*Zoothamnium* sp.	Gill, appendage
2		*Amphileptus* sp.	Gill
3		*Dileptus* sp.	Intestine
4		*Chilodonella* sp.	Gill, appendage
5		*Balladyna* sp.	Gill, appendage
6		*Epistylis* sp.	Intestine
7		*Vorticella* sp.	Intestine
8	Gregarines	*Gregarina* sp.	Intestine
Metazoan			
9	Myxosporadians	*Myxobolus* sp.	Gill
10	Nematodes	*Rhabdochona* sp.	Intestine
11		*Indocucullanus* sp.	Intestine
12		*Procamallanus* sp.	Intestine
13		*Cucullanus* sp.	Intestine
14	Acanthocephalan	*Acanthogyrus* sp.	Intestine

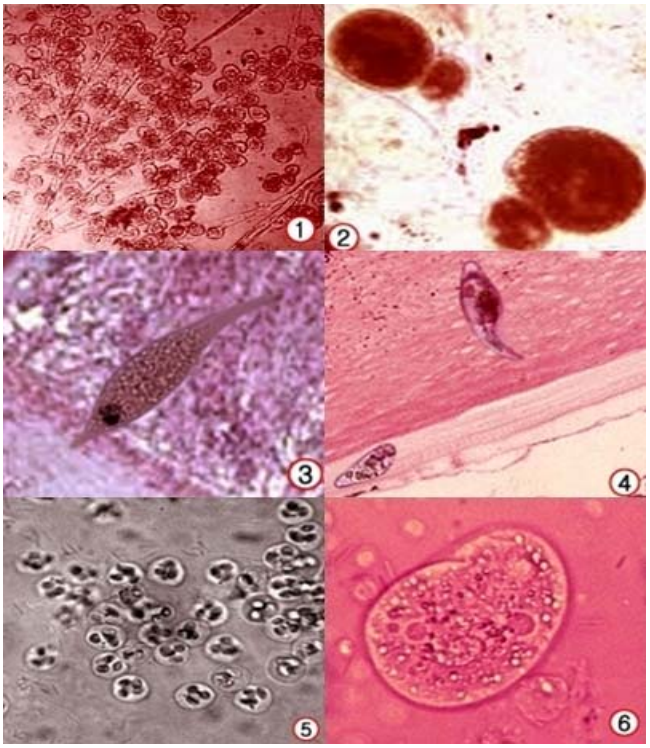

Figure 1. (1) Colonies of Zoothamnium sp. attached to the gills, 150×; (2) *Gregarina* sp. attached in the intestine, 150×; (3) *Amphileptus* sp. attached in the gill, 150×; (4) *Dileptus* sp. attached in the intestine, 150×; (5) *Myxobolus* sp. attached in gill, 150×; (6) *Chilodonella* sp. attached to the appendages, 150×.

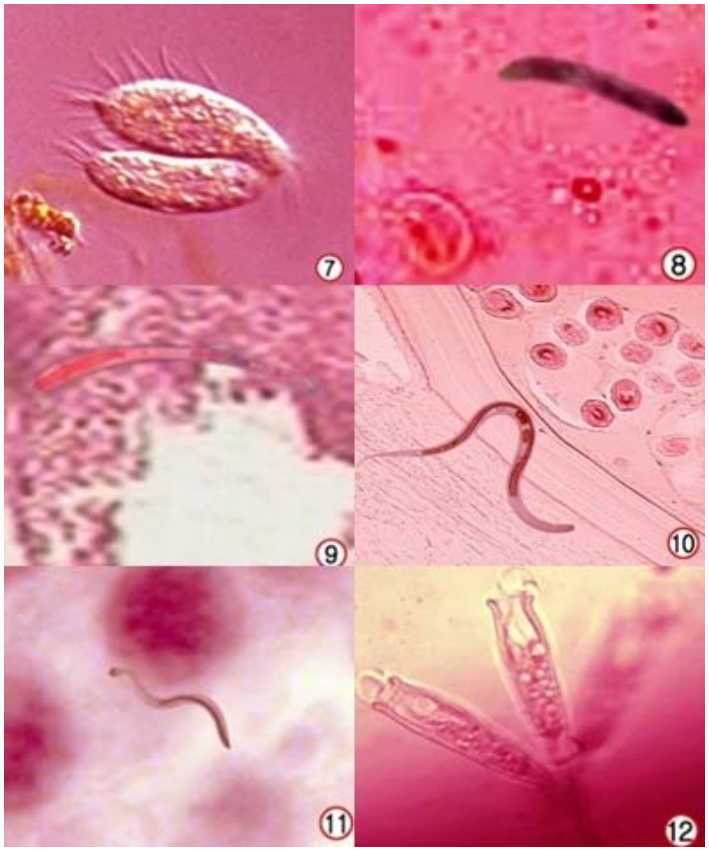

Figure 2. (7) Colonies of *Balladyna* sp. attached to the appendages, 100×; (8) *Rhabdochona* sp. attached in the intestine, 100×; (9) *Indocucullanus* sp. attached in the intestine, 150×; (10) *Procamallanus* sp.attached in the intestine, 150×; (11) *Cucullanus* sp. attached in intestine, 150×; (12) *Epistylis* sp. attached to the intestine, 150×.

carried through freshwater prawns as described by Sen-Hai and Kenneth (1994).

Conclusion

The present study demonstrated that the giant freshwater prawn *M. rosenbergii* serve as hosts for a wide range of epibionts and parasites. Diseases are the most serious limiting factors in fishery sector and prime cause for chronic mortalities and poor growth which affects yield and marketability of aquatic animals. The floodplain wetlands in West Bengal are mostly eutrophicated and clogged with aquatic vegetation resulting sub-optimal water quality, which ultimately affected the general health condition of the aquatic animals. However, the finding of the present study will serve as a baseline for designing parasite specific extensive study in future. In West Bengal, inland culture and capture based fishery activities were mainly rural based and operated by poor farmers. Developing better management practices through pre–stocking pond management, stocking of specific pathagen free seeds, water quality management , better feed managemnt etc can prevent advaerse impact of diseases and assist poor farmers for sustainable production.

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Conflicts of interest

The authors declared that they have no conflicts of interest.

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Full Length Research Paper

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

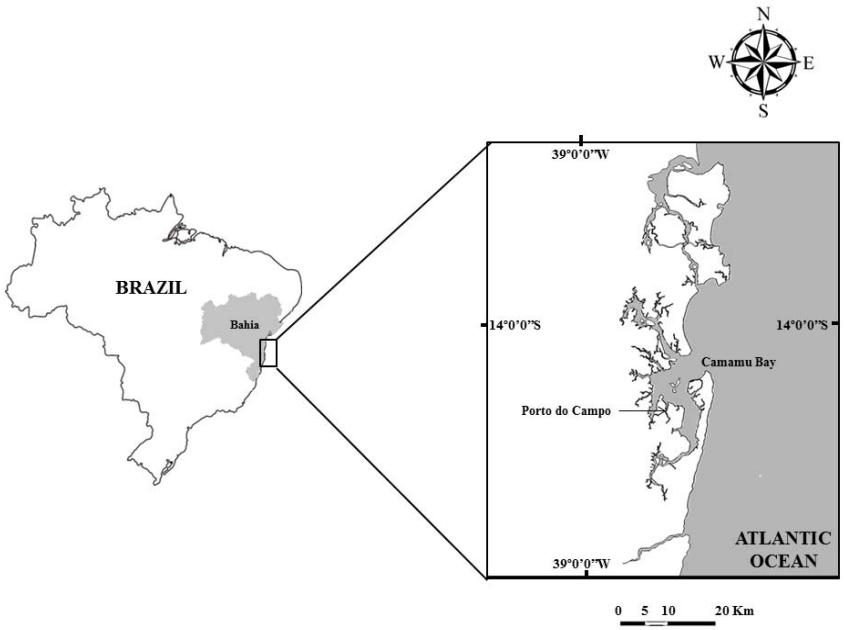

Figure 1. Map of the study area, with the collection point (Porto do Campo, Bay of Camamu, Bahia, Brazil) for the oyster *Crassostrea rhizophorae* in farmed and natural stocks.

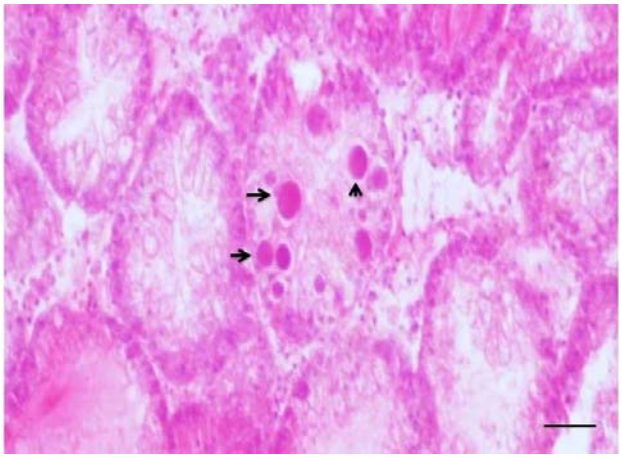

Figure 2. *Rickettsiae*-like organisms (RLOs) (arrows) in *Crassostrea rhizophorae* in the epithelium of the digestive gland;bar = 10 µm.

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-

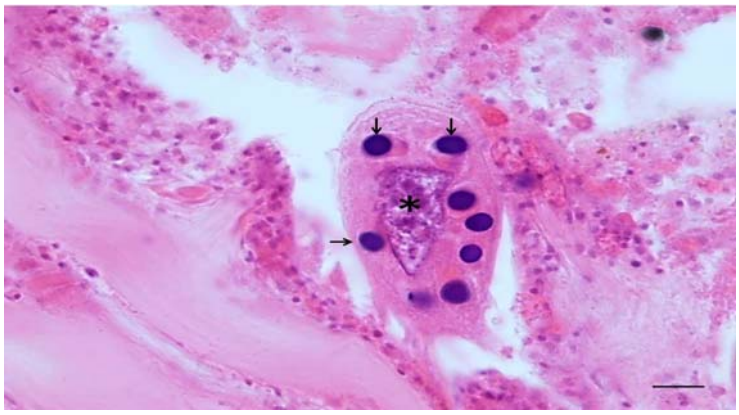

Figure 3. *Sphenophrya* sp. in the gill epithelium with xenoma formation; arrows = parasite; * = nucleus of the host cell;bar = 10 µm.

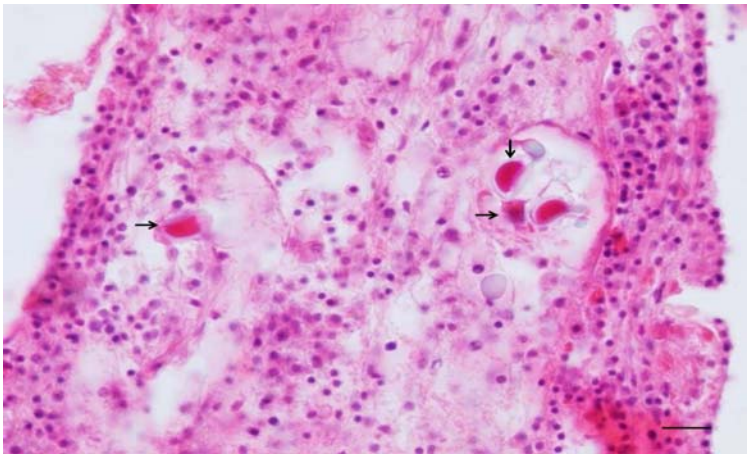

Figure 4. Intrahemocytic oocysts of *Nematopsis* sp. in the gill region (arrows);bar = 10 µm.

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,

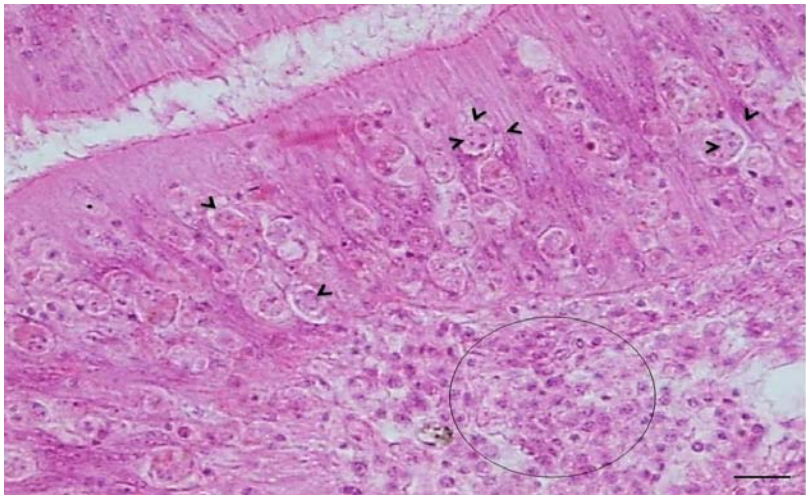

Figure 5. Trophozoites of *Perkinsus* sp.(arrowheads) in the epithelium of the intestine and hemocyte infiltration (indicated by the circle); bar = 10 µm.

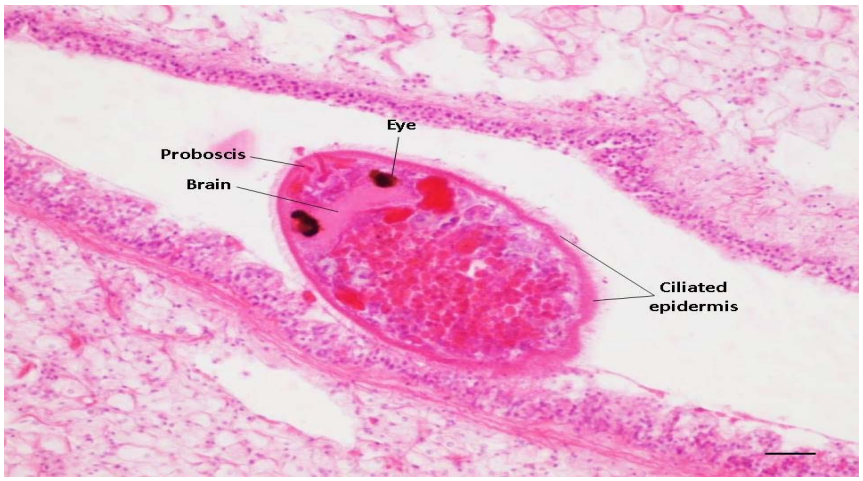

Figure 6. *Urastoma* sp. between the gill filaments;bar = 10 µm.

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,

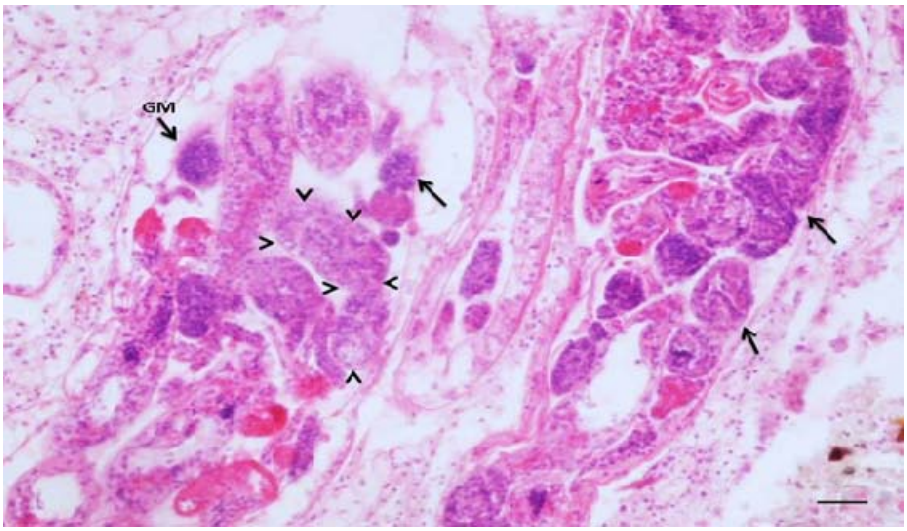

Figure 7. Sporocysts of *Bucephalus* sp. containing germ masses (arrows) and cercariae (arrowheads);bar = 25 µm.

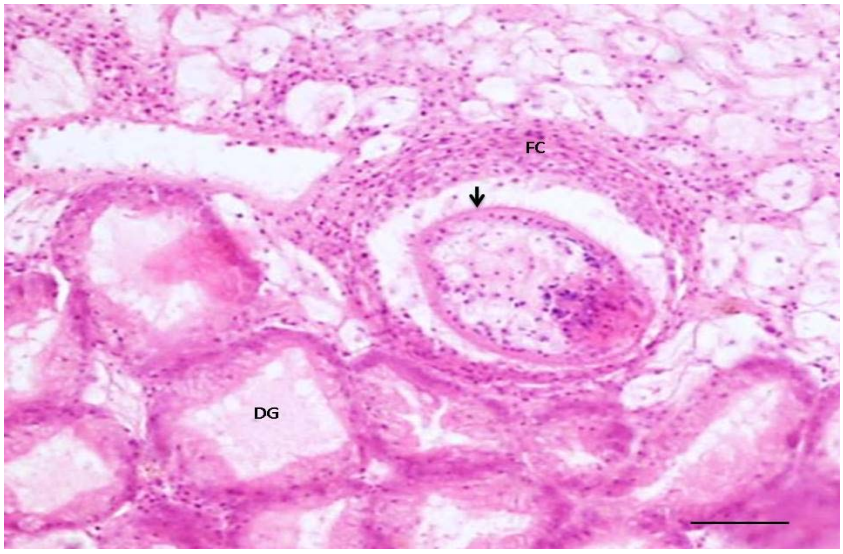

Figure 8. *Tylocephalum* (arrow) in the peripheral region of the digestive gland (DG), with the formation of a fibrous capsule (FC);bar = 25 µm.

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*

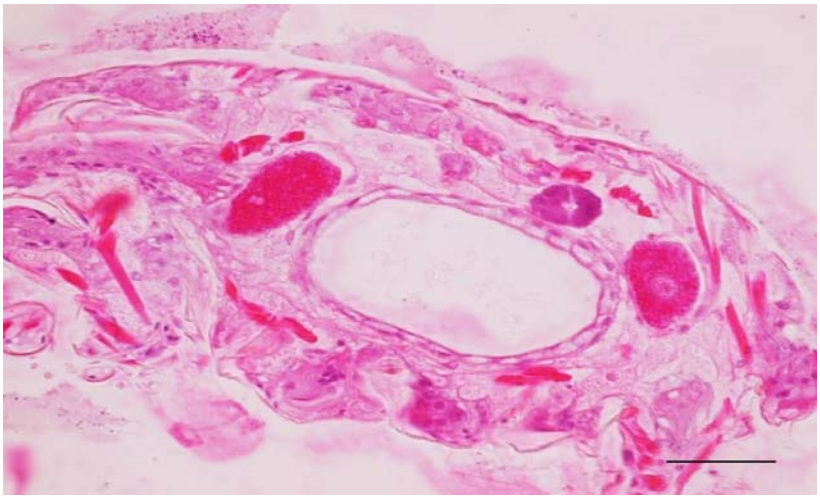

Figure 9. Unidentified copepod in the region near the gills; bar = 25 µm.

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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