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Investigation on parasites of *Octopus cyanea* in Tanzania

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Abstract. *Msongole PL, Kimbita EN. 2020. Investigation on parasites of* Octopus cyanea *in Tanzania. Ocean Life 4: 24-36.* This study aimed to investigate parasites in *Octopus cyanea* (Gray, 1849) or Day octopus along the Indian Ocean of the Tanga region in Tanzania. Information from fishermen was required to determine their awareness of octopuses' parasitic infections. Twenty-five percent (25.3%) agreed on the presence of parasites in octopus skin and muscles, but these were not observed during laboratory investigation. Gamogony and sporogony of *Aggregata* sp. (Apicomplexa: Aggregatidae) were observed during histopathological examination of the digestive tracts of octopuses collected along the Tanga coastal area. The *O. cyanea* was infected with the coccidian parasite, *Aggregata* spp., with a prevalence of 41.1% (23 of 56 hosts examined). Oocysts were sub-spherical in the mucosa wall of the intestine and caecum, measuring 263-279 µm. Sporocysts were smooth-surfaced, dark-staining, spherical, typically 10-15 µm wide, and contained 9-22 banana-shaped sporozoites with a size of 2-6 µm long and 0.2-0.3 µm wide. The coccidian infection in octopuses is accompanied replacement of the infected cells with sporocysts. Parasite infection of *Aggregata* spp. was not significantly correlated with the weight of the octopus, study site, or sex of the host. Molecular analysis was used to confirm the parasite species, in which 68% of samples were positive. Molecular characterization revealed that the coccidian was the *Aggregata octopiana* (Schneider, 1875) Frenzel, 1885. Alignments revealed that the coccidian from *O. cyanea* resembled 89% with *A. octopiana* isolated from common Octopus in Spain. Despite the presence of *A. octopiana* in *O. cyanea*, the parasite does not cause effects on consumers unless the octopus is infected with another epizootiology agent.

Keywords: Coccidian infection, molecular characterization, octopus, parasite, Tanga

INTRODUCTION

Octopus fishing along the coast of Tanzania plays an important role as a source of protein and income that may improve fishermen's livelihood (Jiddawi and Ohman 2002). Octopus has been processed and sold in the local market and can be exported (Guard 2009). Local coastal communities of Tanzania mainly deal with artisanal fishing for White-spotted octopus (*Octopus chromatus* Heilprin, 1888), common octopus (*O. vulgaris* Cuvier, 1797), and Day octopus (*O. cyanea* Gray, 1849) as their source of employment and income generation (Guard and Mgaya 2002; Guard 2009; Mshana and Sekadende 2014) and protein source (Bultel et al. 1950; Estevez et al. 1996; Gestal et al. 2007).

Octopus belongs to the Family of Octopodidae (Emery et al. 2016), with the genus *Octopus* consisting of more than 100 species (Ignatius and Srinivasan 2006). Many *O. cyanea* is mostly found on the East African coast and Madagascar to south-eastern Asia and Hawaii (Guard and Mgaya 2002; Benbow et al. 2014). *O. cyanea* is a predatory cephalopod foraging and feeding on other invertebrates, such as crustaceans (Mshana and Sekadende 2014). The size of male and female *O. cyanea* differs at sexual maturity; females may reach 0.6 kg to 5 kg at spawning, while males may reach 300 g at sexual maturity (Van Heukelem 1973). Cannibalism can happen to small male octopuses during mating (Hanlon and Forsythe 2008).

Octopus fishing has been increasing in recent years due to demand and price increases in local and international markets (Sauer et al. 2011). However, global market growth can generally lead to heavy and unsustainable fishing of *O. cyanea* in Tanzania and East Africa (Guard and Mgaya 2002; Eriksson et al. 2012).

Despite the importance of octopuses in East Africa, there is not enough information concerning their threats. Most studies on octopus diseases and parasites have been conducted in European countries. Coccidiosis is among the most important diseases reported in octopuses, leading to malabsorption syndrome (Pascual et al. 2010). The disease is caused by the protozoa, an intracellular parasite that affects both wild and cultured octopus through food-web relationships (Gestal et al. 1999; Vidal and Haimovici 1999). The enteric infection does not directly cause the octopus's death, but it exposes the octopus to the risk of being affected by other disease-causing agents (Pascual et al. 2007).

Apart from protozoa, also octopus has been parasitized by isopods, digenean, nematodes, cestodes, and copepods (Pascual et al. 2007). Dicyemida, Spirurid nematodes, and *Cystidicola* sp. has been reported to cause infection in the stomach of octopus (Pascual et al. 1996; González et al. 2003). Ectoparasites like copepods (*Octopicola* spp. and *Pennella* spp.) can cause a serious pathological conditions in the gills. The parasite and cephalopod relationship are common in all oceans; cephalopod species have associated parasites (Pascual et al. 2007).

Despite the increased octopus fishing in East Africa in recent years, research in this field is still low, with limited published papers. Octopus diseases and parasites are not important (Vidal and Haimovici 1999). However, a parasitic infection (like coccidiosis) has been reported in

octopuses in the Mediterranean, Atlantic, and Pacific Oceans (Gestal et al. 2007). The *Aggregata* coccidian has been reported to infect the digestive tract of more than 13 species of octopus worldwide (Estevez et al. 1996).

This study aimed to investigate the possibility of *O. cyanea* hosting parasites, investigating the endoparasites and the ectoparasites along the coastal area of Tanga, Tanzania.

MATERIAL AND METHODS

Location and duration

Octopuses were purchased from fishermen in four sites in the Tanga Region, Tanzania districts from December 2017 to January 2018. The study sites were Kwale, Sahare, Kigombe, and Pangani Villages, located in Tanga Region (05004', 39006' E). The Tanga Region has four coastal districts, which were selected as the study area of this research (Figure 1).

Study design

A cross-sectional study design was used; with purposive sampling, four landing sites with n were calculated as sample size:

$n = Z\alpha^2 \times p\ (1-q)^2 / d^2$

Where, Zα (from the table) at Type I error of p= 0.05, q= 1.96, d= effect size.

Sampling strategy and sample collection

Samples were collected in four districts actively involved in octopus artisanal fishing. A total of 56 *O. cyanea* (about 12 from each site), with an average weight of 400.51 g, were collected from four districts (one coastal village per district). Artisanal gear used by local fishermen to catch octopuses. A small piece of kidney, liver, and caecum from each octopus was taken and fixed in 10% buffered formalin for histological purposes. Octopuses were frozen for preservation, transported in a sterile cool box to the laboratory, and thawed before necropsy at the Sokoine University of Agriculture, Morogoro, for parasite examination and identification.

Questionnaire administration

A total of 156 fishermen were selected randomly from four villages, Kwale, Sahare, Kigombe, and Pangani, for the questionnaire. Thirty-nine (39) fishermen over 18 from each selected site answered prepared questions concerning octopus parasites, fishing activities, habits of octopus consumption, type of octopus captured, and processing activities.

Although octopus fishing activities in these areas are seasonal, it was possible to visit fishermen and get some information concerning octopus fishing and parasites. The sites were chosen to gain information on fishermen's perceptions of the presence of parasites on octopuses, which octopus species they preferred, ways of octopus processing for consumption, and how they overcame any side effects caused by consuming octopus.

The structured questionnaire was in Swahili. For the fishermen who could not read and answer the questions on their own, each question was read to them to get some information about the fishing activities of octopuses and octopus parasites or diseases. The structured questions were open- and closed-ended, and the respondents were free to discuss during the interview.

Collected data were grouped according to the group age of the respondents and sex. Few respondents were female (age group 30-53) and mostly octopus dealers. The survey also asked about infectious agents, such as bacteria and fungi. Respondents were required to give their opinions on the effects of any parasites of octopuses on a human being. Some questions were asked on the measures they took to prevent parasites, bacterial or fungal effects.

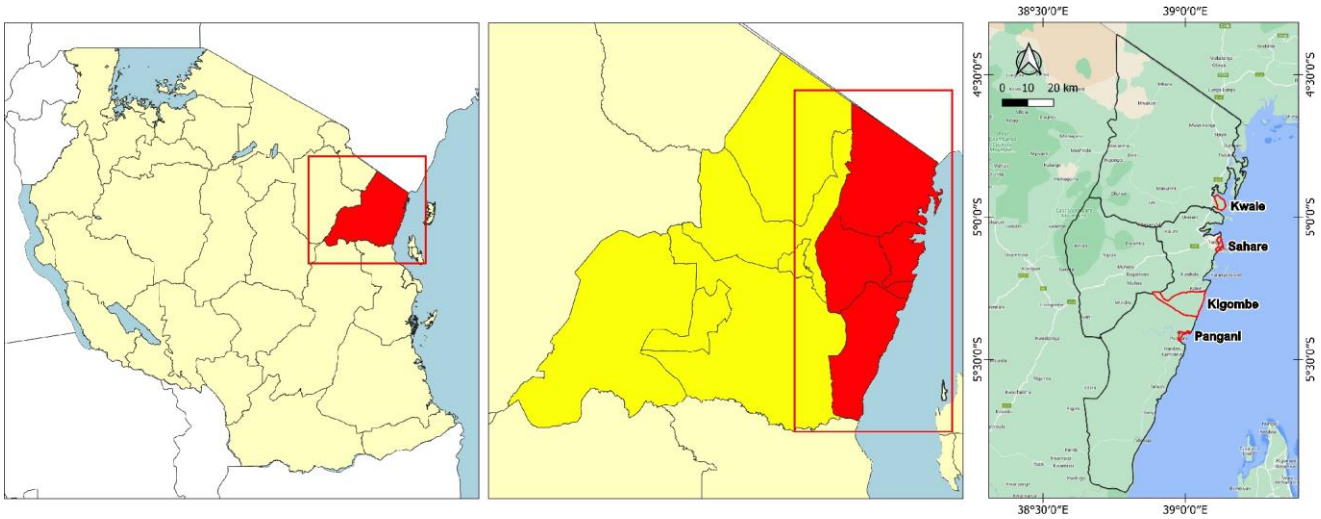

Figure 1. Maps showing the study sites in Tanga Region, Tanzania

Parasite examination

Gross examination

After mantle dissection, a macroscopic examination of parasites on the body surface and intestine for each octopus was done in the field. The entire octopus mantle and body surface were observed for the ectoparasites and endoparasites. The mantle was cut with scissors to observe endoparasites which the naked eye could see in the internal organs.

Laboratory work- examination for ectoparasites

Scrapings from the skin and gills of the specimen were smeared on clean glass slides, covered with cover slides, and examined under light microscopes for ectoparasites. Each sample was examined independently for parasites according to the protocol outlined previously by Obiekezie and Ekanem (1995). Skin scrapings and wet mounts from skin and gills were examined for abundance and distribution of the larval stage of worms and other ectoparasites like leaches. Gills were taken into a petri dish with tap water and placed under a dissecting microscope for larval worm examination.

Examination of intestinal parasites (endo-parasites)

In the laboratory, specimens were weighed using a weighing balance, and their weight was recorded in grams (g). Then, octopuses were dissected to get intestinal contents, caecum, liver, kidney, and fecal samples. The intestinal contents of the weighed octopus were taken into a beaker, mixed with concentrated salt solution, filtered, poured into a test tube then covered with a cover slip for the floatation process to recover the parasite's oocysts. After 10 minutes, a cover slip was removed for microscopic examination. The intestinal contents were also examined for adult worms.

Tissue parasites

The kidney, caecum, and liver were isolated in each dissected octopus. Small pieces of liver, kidney, and caecum were smeared on glass microscope slides, which were immediately fixed in methanol, and some pieces were stored in 70% ethanol. The smears were then stained in Giemsa solution (Bruno et al. 2006) for coccidian oocyst and sporocysts examination. Squashing was the means of detecting parasites from the octopus tissue. Squash preparations of fixed tissues from the intestine and caecum were examined by light microscopy. The parasites' sizes were measured using a calibrated ocular micrometer and expressed in micrometers (μm).

Tissue processing and histopathology

Heavily parasitized tissue of some samples' gills, kidneys, liver, and caecum were fixed in 10% buffered formalin and then trimmed in processing cassettes. The trimmed kidney, gills, liver, and caecum tissues in processing cassettes were fixed in 10% NBF for 48 hours, then processed routinely and embedded in paraffin wax using the standard paraffin procedure. The standard paraffin process (tissue processing) starts with fixation that preserves the tissue. That was followed by dehydration through graded ethanol (70%, 90%, and absolute), clearing in chloroform, and finally, infiltration of the tissue with molten paraffin wax. It was followed by embedding, and then sectioning was done using a rotary microtome (Baird and Tatlock) to produce 4 μm thin sections placed on paraffin section mounting bath (Electrothermal) microscope slides. Sections (4 μm) were stained with H & E following standard procedures (Culling et al. 1985). These sections were dried overnight in a paraffin oven (Electrolux) at 50°C.

Molecular identification

Isolation and purification of parasite

Infected digestive tracts were dissected and homogenized in 10 ml of distilled water with 1% Tween20 using a motor and paste. Tissue homogenates were filtered twice with nylon meshes of 100 μm and 41 μm, respectively, to remove tissue fragments, and then concentrated NaCl was added to make the sporocysts and oocyst float. The mixture was centrifuged at 1000 x g for 5 min in a centrifuge KUBOTA S100. The sporocyst was purified by density gradient centrifugation method according to Gestal et al. (1999) and preserved in 70% ethanol for DNA extraction.

DNA extraction

Genomic DNA was extracted from *Aggregata* parasite sporocysts isolated from the digestive tract of *O. cyanea* using the ZYMO Research DNA extraction kit following the manufacturer's procedures. Sporocysts were suspended in 500 μL of extraction buffer (NaCl 100mM, EDTA 25mM pH 8, SDS 0.5%) and opened by sonication on ice (5 cycles, 40W, 50 s) to release sporozoites. After Proteinase K (Sigma) digestion (1mg ml-1) at 37ºC overnight, the DNA was purified following the alcohol extraction method, as described by Sambrook et al. (1989). DNA was precipitated with ethanol and sodium acetate overnight at -20°C. Finally, the precipitated pellet was resuspended in 50 μL of Tris-EDTA (TE) buffer.

DNA amplification

The small subunit 18S rRNA gene with the size of 970 bp of extracted coccidian was amplified by PCR using conserved primers designed for *Aggregata* spp. (Kopečná et al. 2006), and derived from GenBank sequences: (*Aggregata* 1-F: 5 - ATGATGAAACTGCGAAGAGC-3; *Aggregata* 2-R: 5 - CGACGGTATCTGATCGTCTT-3); PCR reactions were performed in a total volume of 25 μL containing 1 μL of10mM dNTP mix, 0.25 μL Taq DNA polymerase (Roche), 2.5 μL Taq10x buffer, 1 μL 2.5 mM MgCl2, 1 μL of each primer and 1 μL of template DNA at 100 ng μL^{-1}. The temperature profile for primers 1-2 included an initial denaturation at 94°C for 10 min, 35 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min. For primers 3-4, we used an annealing temperature of 55°C. PCR products were separated on 1.5% agarose gels, stained with Gel Red including a 100- bp ladder size standard (Invitrogen), and visualized using a UV transilluminator.

DNA cloning and sequencing

PCR products were cloned using TOPO® Cloning Kit (Invitrogen) according to the protocol supplied by the manufacturers and transformed into TOP 10 F competent bacteria *Escherichia coli* (Mig., 1895) (Invitrogen). Screening of clones carrying 18S rRNA-coding region fragments was performed by PCR, adding the positive colony directly to the PCR mixture reaction using the corresponding *Aggregata* primers. Positive clones were purified by Microcon-centrifugal filter YM-50 (Millipore).

The purified PCR products were bi-directionally sequenced using BigDyeâ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) following manufacturers' procedures, using the 15 positive sequences from PCR. First, DNA sequencing was performed on an ABI 3500XL DNA Analyzer (Applied Biosystems), which uses a 24-capillary at Mbeya Zonal Referral Hospital. Next, the sequenced fragment was assembled and arranged using Genius Software into consensus sequences, then entered in a GenBank for alignment with other *Aggregata* spp.

Phylogenetic analysis

The aligned sequence products were used to construct a phylogenetic tree using sequences of other Apicomplexa taxa to determine the species of the *Aggregata* isolated from *O. cyanea*. The phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis version 7.0 (MEGA-7 software) (Kumar et al. 2016).

Data analysis

Questionnaire data

Survey data were collected, entered, and stored in the spreadsheet using Microsoft Excel version 12 and analyzed using a Statistical Package for Social Sciences (SPSS) version 16.0. Qualitative descriptive analysis was used to describe the awareness of respondents on octopus parasites. In addition, a chi-square test was done to test the goodness for the observed frequencies, such as the number of respondents on parasite existence, yielding statistical differences with the age group of fishermen at p-value of 0.05.

Parasite infection

To describe parasite infection in *O. cyanea*, the prevalence of parasite infection was calculated according to Mitchell et al. (2000).

$$\text{Prevalence} = \frac{\text{number of individuals of hosts infected with parasites}}{\text{Number of hosts examined}} \times 100$$

Oneway ANOVA (performed using the PAST program) was used for normally distributed data to compare the mean differences in parasite infection between sites. Two sample t-tests were performed to determine if there was a statistically significant difference in parasite infection between the host's sex (male and female). Linear regression analysis was carried out to determine if there is a significant relationship in the rate of infections between individuals (*r* applied to determine the degree of association between parasite intensity and host-related factors such as octopus weight). Quantitative analysis was performed for statistically significant ectoparasites and the site of infection. A p-value of ≤0.05 was considered statistically significant. Parasite populations between sites of sample collection were analyzed using prevalence (P, %) of infection as a parameter according to Mitchell et al. (2000). DNA sequence data analysis was done using the Predictive Analysis Software PASW Statistics 19.0 statistical program and presented as a percentage.

RESULTS AND DISCUSSION

Questionnaire administration (field survey)

Profile of the interviewed fishermen and octopus dealers

A total of 156 fishermen from four study sites were interviewed; most were men (94.87%). The respondent's mean age was 40.07 years, with most of them aged 31-53. According to their responses, it was observed that only 32% were able to read the questionnaire (25% primary education, 7% secondary education), while 68% were not educated and unable to read the questionnaire (Table 1). Most fishermen along the Tanga coastline were found to have poor knowledge of parasites in general. 80% of the fishermen responded that they fished only one species of octopus, which is *O. cyanea*, even though they didn't know the name of the octopus they fish. Most fishermen identified octopus by the local name "pweza."

Perception of fishermen on parasite existence in octopus

The results from fishermen and octopus dealers' responses were grouped according to the respondent's age and sex. Age and sex were used as one criterion. According to the data obtained from respondents, the issue of parasites in octopuses is not well known (Table 2).

During the survey, we observed that experienced fishermen understood some abnormal conditions in octopuses, including their diseases. Therefore, elders experienced in fishing were expected to give some good information about parasite infections and diseases in octopuses.

In Figure 2, respondents were asked if the octopus they caught harbored or if there was any observable parasite. Again, most respondents (97/156) didn't know octopuses could harbor parasites, while a small minority knew parasites on an octopus.

Table 1. General features of the respondents who participated in the survey

Characteristic	Sampling site N (% of respondents)				Total
	Sahare	Kigombe	Pangani	Kwale	
Age group					
Young (18-30)	7(17.1)	11(26.8)	16(35.6)	10(34.5)	44(28.2)
Middle (31-53)	23(56.1)	20(48.8)	24(53.3)	16(55.2)	83(53.2)
Old (>54)	11(26.8)	10(24.4)	5(11.1)	3(10.3)	29(18.6)
Sex					
Male	39(95.1)	41(100)	41(91.1)	27(93.1)	148(94.9)
Female	2(4.9)	-	4(8.9)	2(6.9)	8(5.12)
	41	41	45	29	156

Table 2. Fishermen's opinions on parasites in octopus (n=156) expressed as a percentage of respondents

Characteristic	Parasite status (% of respondents)			Total
	Present	Absent	Don't know	
Age group				
Young (18-30)	2(4.54)	23(52.45)	19 (43)	44
Middle (31-53)	5(6.02)	42(50.6)	36(43.4)	83
Old (>54)	8(2.8)	10(34.5)	11(37.9)	29
Sex				
Male	13(86.7)	69(92)	66(100)	148
Female	2(13.3)	6 (8)	0(0)	8
	15	75	66	156

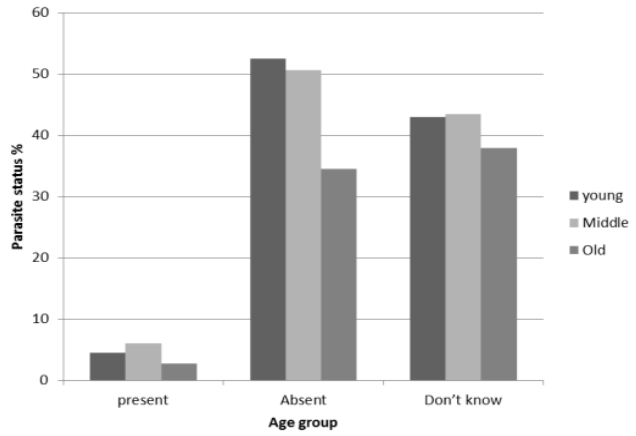

Figure 2. Respondent's opinions on the existence of parasites as the question answered by different age groups

Response on type of pathogen (parasite) occurring in octopus

Most fishermen in each age group had no idea about the kind of parasite infecting octopus. Fishermen were asked to describe the appearance of any parasites they saw in octopus during fishing and processing (Table 3). The respondents answered if the parasites have any effects on consumers.

Fishermen's perceptions of octopus disease in general

Fishermen and octopus dealers were asked to mention and describe any disease in octopuses. The responses to the question were recorded based on the age group of the respondents. The respondent age group was statistical significance at 0.05 levels (X2 = 2.72, p = 0.033) to octopus disease status.

Laboratory analysis of parasite infestation

Among fifty-six (56) *O. cyanea* collected, only 41% (23) were infested with parasitic protozoa, the coccidian *Aggregata* spp., all of which were found in the digestive gland (liver), kidney, and intestine. Table 4 shows the octopus samples examined and the number infested at each selected study site. Cysts were found on the octopus body and digestive tract (the cecum). As much as 96% of the infected octopuses weighed above 500g, while 89.3% below this weight were found uninfected by parasites. The bigger the octopus's size, the higher the probability of having a parasite, especially a gastrointestinal protozoan.

Table 3. Fishermen's responses on the kind of cause of disease harbored by octopus along the coastline of Tanga, Tanzania

Age group	Cause of disease (n=156)				Total
	Worms	Bacteria	Fungus	Don't know	
Young (18-30)	6	0	0	41	47
Middle(31-53)	5	1	3	71	80
Elders (>54)	3	1	1	24	29
Total	14	2	4	136	156

Table 4. Shows the number of octopuses examined for parasites, prevalence, and number of octopuses infected with parasites

Site	No. examined	No. infected	Prevalence %
Kwale	14	5	35.7
Kigombe	15	8	53.3
Sahare	13	3	23.1
Pangani	14	7	43.8
Total	56	23	41

External lesions were observed in some octopuses, while some had been injured with their arms chopped. The lesions could result from ectoparasite invasion having attached, fed, and detached, leaving some wounds on their host's body. Fishermen have blamed the predator eel fish for being the main reason for causing lesions and cutting the arms of octopuses.

Histopathological evaluation of octopus intestine, liver, kidney, and gills showed substitution of tissues with developed oocysts with sporocysts and gametes in some tissues. The gametes were mostly observed in the intestinal mucosa, especially the caecum, while no gametes were found in the gills, liver, and kidney tissues in the intestinal mucosa, substituted by many oocysts. Histological sections of the kidney and liver showed little protozoa infections compared to the digestive tracts, especially the caecum. However, the mucosa of many infected octopuses was found to have lesions caused by invasion of the protozoa oocysts, which replaced the host mucosal cells.

Gross examination

White cysts containing *Aggregata octopiana* (Schneider, 1875) Frenzel, 1885 were commonly found by gross observation in the wall of digestive tracts of some octopuses sampled from Kwale, Pangani, Kigombe, and Sahare (Figures 3 and 4). Most cysts were seen in the intestines, especially in the cecum. In Figures 3 and 4, cysts were seen as white spots in the wall of the intestine, and sometimes they were observed in the digestive gland of heavily infected octopus. Apart from the observable white cysts, no other parasite was observed on the mantle or intestine surface. No ectoparasites were found during the examination of parasites. A fresh smear of eyes, skin, and gills showed no worms in octopuses collected from all selected study sites.

Fresh smear examination

In fresh smear preparation, some of the sporocysts had already raptured, as shown in Figure 5, with the sporozoites being easily seen with a light microscope with different magnifications. The sporocysts were ovoidal and smooth, containing a large number of sporozoites.

Fresh squash examination

The results for Giemsa stain squash preparation of the intestine and caecum showed spherical sporocysts, ranging from 10-15 µm containing sporozoites (Figures 6 and 7). The Light Microscope observation of fresh tissue squash showed that the intestine and kidney were infected with parasites, while the liver was less infected.

Prevalence of parasite infection

The prevalence of parasites was calculated after a light microscope examination for the parasite using a smear, squash preparation, and histopathology examination. The results were grouped depending on the number of hosts examined concerning the site where samples were collected (Table 5).

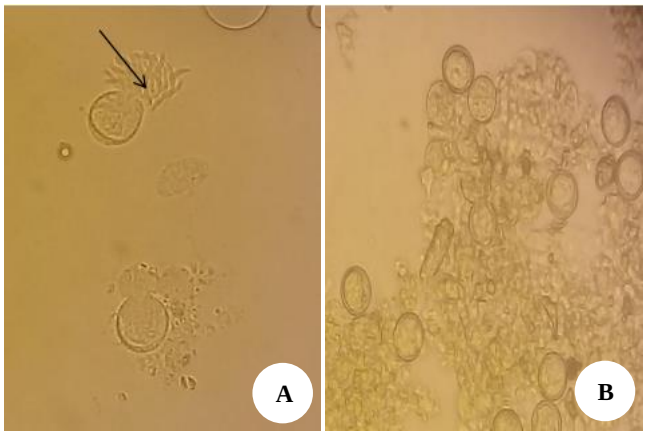

Figure 5. Light microscopic examination of the fresh smear of intestinal contents. A. showing the sporocyst raptured to release the banana-shaped sporozoites. B. Several sporocysts with sporozoites inside

Figure 3. A macroscopic view of dissected *Octopus cyanea* showing heavy infection with Coccidian parasite in the intestine, white cysts (arrow)

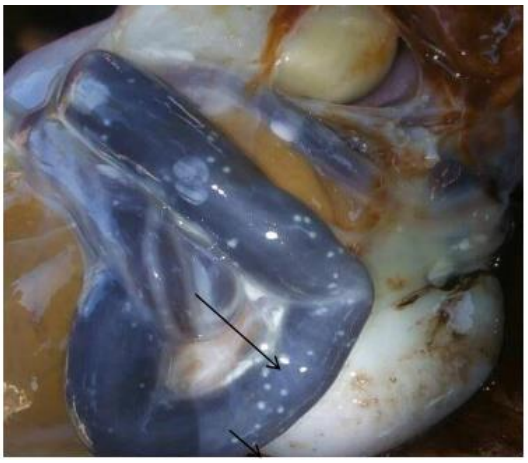

Figure 4. Photograph showing a macroscopic view of an octopus intestine with a heavily infected condition. Note the number of white cysts (arrows) infecting the intestine and caecum of the octopus

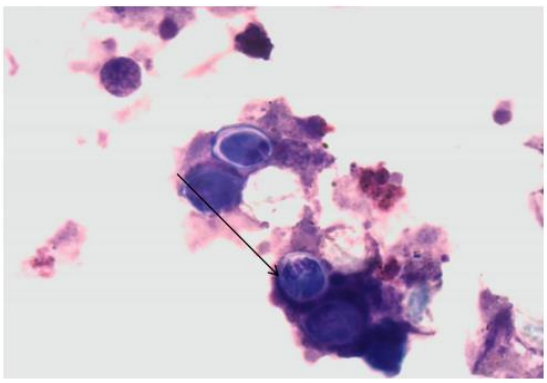

Figure 6. Fresh squash preparation of the octopus caecum infected with coccidian parasites. The figure shows the sporocysts with sporozoites (arrow)

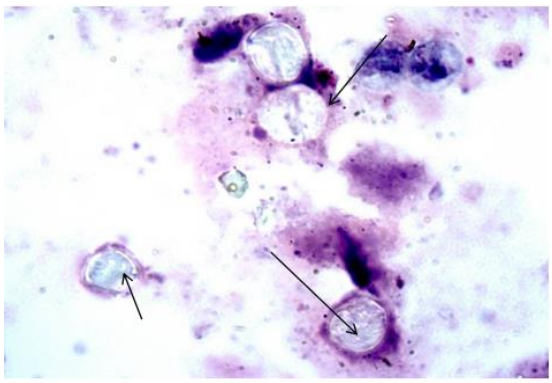

Figure 7. Fresh squash preparation of the intestine shows sporocysts with sporozoites inside (arrows)

Histopathology examination

Histopathological study showed that each organ of the infected octopus had several huge oocysts containing sporozoites and gametes. The gamogony stage was observed in the intestine and the caecum, where the microgametes and macrogametes were seen, as shown in Figure 8 (observe arrows). The parasites were mostly found in the submucosa and muscular layers (Figure 9). The detachment of epithelial cells was observed, especially during the sporogonic stage of *Aggregata* sp. (Figure 11). Some nuclear displacements were observed in gills and mucosal cells due to the presence of many sporocysts and developing macrogametes (Figure 10). However, there was less tissue destruction in the gills and kidneys compared to the intestine and caecum.

Sporocysts counting

During sporocysts isolation, large numbers were observed under the light microscope. With the aid of a light microscope, several sporocysts were recorded using the Neubers chamber, as shown in Table 5. Also, during sporocysts counting, some had already released the sporozoites (Figure 5).

Analysis results

Prevalence of infection by individuals

The comparison was conducted to any association between infections' density and individual octopuses' weight. The data were normally distributed, and a linear regression analysis was performed.

The correlation between the weight of an individual octopus and the number of sporocysts was not statistically significant at the 0.05 level (r= 0.18927, p= 0.16238). The plot of several sporocysts and the weight of hosts showed a weak positive correlation. A significant difference was not found. The number of sporocysts depends on the infective dose of parasites that the host ingested from the intermediate host and not its weight (Figure 12).

Regression equation:

$y = 7.157 – 2288.5x$,

$r = 0.18927$

$r^2 = 0.035825$ (the host's weight can explain, i.e., only 3.5825% of the variation in infection density).

$t = 1.4165$, $p = 0.16238$ (not significant).

Infestation between sites

A one-way ANOVA was performed on the normally distributed data to compare the means of sporocysts between study sites. The means of infections were not significantly different among sites at the 0.05 level (F= 1.63, p= 0.1936). A Tukey's Honest Significant Difference test indicated that parasite infestation in samples from Kigombe was higher than those from other sites. Since there was no statistical significance between parasite infestations in octopuses among sites, post hoc testing was not required. The parasite infection prevalence was positively and significantly correlated among sites (r= 0.98, p= 0.008, df= 3) (Figure 13).

Table 5. Details on octopuses examined, state of infection, body weight, and location of octopus collection

Host no.	BW (g)	Locality	HS	I	No. sporocyst/g	Site of infection
O 3	387	KW	F	+	2 x10^3	Caecum
O 5	586	KW	F	+	1.2 x 10^3	Digestive tract
O 1	962	S	M	+	3 x 10^3	Digestive tract
O 4	1532	KW	F	+	8 x 10^2	Digestive tract
O 5	1540	K	F	-		
O 6	442	K	M	-		
O8	261	P	M	-		
O 7	253.5	KW	M	-		
O 9	567	P	F	+	5 x 10^2	Caecum
O 10	262	P	M	-		
O2	317	P	F	-		
O1	217.6	K	F	-		
O5	255.4	P	F	-		
O1	250	P	M	-		
O7	261	P	M	-		
O2	263	S	M	-		
O9	567	KW	M	-		
O3	341.8	P	M	+	6 x 10^3	Caecum
O4	512	K	M	-		
O6	442	KW	F	-		
O11	511.4	P	M	+	8 x 10^2	Digestive tract
O6	347	S	F	-		
O3	712	K	F	+	1.6 x 10^3	Caecum
O7	422	K	F	+	6 x 10^2	Digestive tract
O 1	387	KW	M	-		
O10	262	KW	M	-		
O4	267	S	F	-		
O3	253	S	M	-		
O2	337	K	F	+	8 x 10^3	Digestive tract
O6	250.5	P	F	+	3 x 10^3	Digestive tract
O10	312.8	S	M	-		
O9	251.4	K	F	-		
O7	560	S	M	-		
O12	712.6	K	M	+	5 x 10^3	Kidney, digestive tract
O11	461	S	F	-		
O13	502.3	P	M	+	1 x 10^4	Digestive tract
O14	281	KW	F	-		
O15	637	P	F	+	4 x 10^3	Digestive tract
O16	511.9	P	F	-		
O4	252	P	F	-		
O12	251.8	P	M	-		
O10	524	K	M	+	6 x 10^3	Digestive tract
O9	378.2	S	F	-		
O12	428	KW	M	+	1.2 x 10^3	Digestive tract, kidney
O14	390.3	K	F	+	8 x 10^2	Digestive tract
O11	442	KW	F	-		
O12	571	S	F	+	1 x 10^3	Digestive tract
O15	419	K	F	+	8 x 10^2	Digestive tract
O5	267.6	S	M	-		
O13	704.7	KW	M	-		
O11	672	K	F	+	2 x 10^3	Digestive tract
O13	254.8	S	M	-		
O5	1540	KW	M	+	3.4 x 10^3	Caecum
O14	734.2	P	M	+	2.8 x 10^3	Digestive tract
O13	302	K	M	-		
O2	586	KW	M	-		

Note: M: male host; F: female host; O: host numbers; KW: Kwale; P: Pangani; S: Sahare; K: Kigombe; BW: Octopuses body weight; HS: host sex; I: Infection

Infection between male and female hosts

The results showed that the infection was higher in male octopuses than in female octopuses (Figure 14). However, the comparison of means between the two sexes of hosts was performed using two-sample t-tests, and there was no statistically significant difference in parasite infection between the two sexes.

Molecular analysis

A total of 25 samples of coccidian parasites isolated from the intestine of *O. cyanea* were run in a TAKARA PCR Thermo cycler machine using a pair of primers to confirm the presence of *Aggregata* species in the isolated intestinal parasites. Seventeen samples were positive for the 1F-2R primers, and eight were negative. In Figure 15 are representative samples that show positive results in the first pair of primers visible in 1.5% agarose gel.

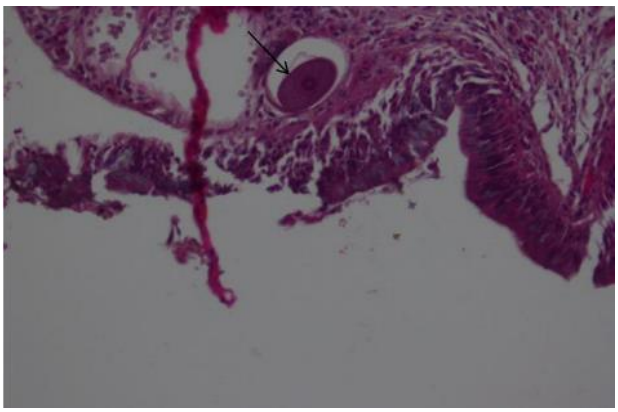

Figure 8. Macrogamete with spongy cytoplasm in the microvilli of the intestine of *Octopus cyanea*

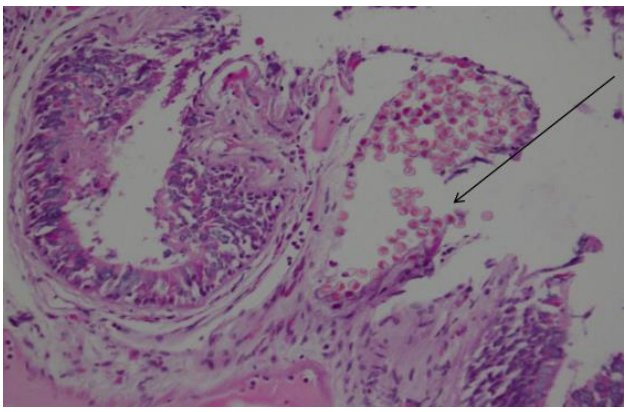

Figure 11. Detachment of the epithelial cell seen (arrow)

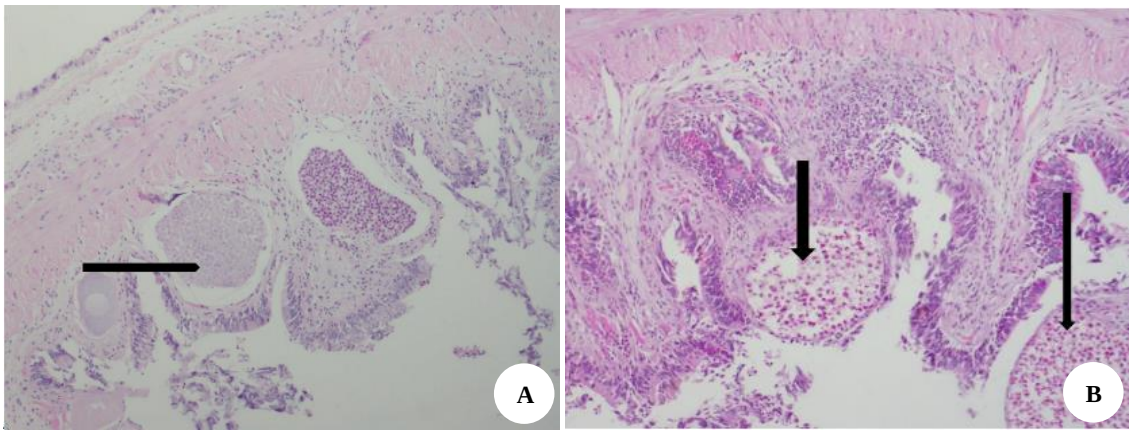

Figure 9. (A and B) H & E section showing the mucosal cells of the intestine replaced with large Oocysts containing many sporocysts (X10). An arrow shows Oocyst within the intestinal villi

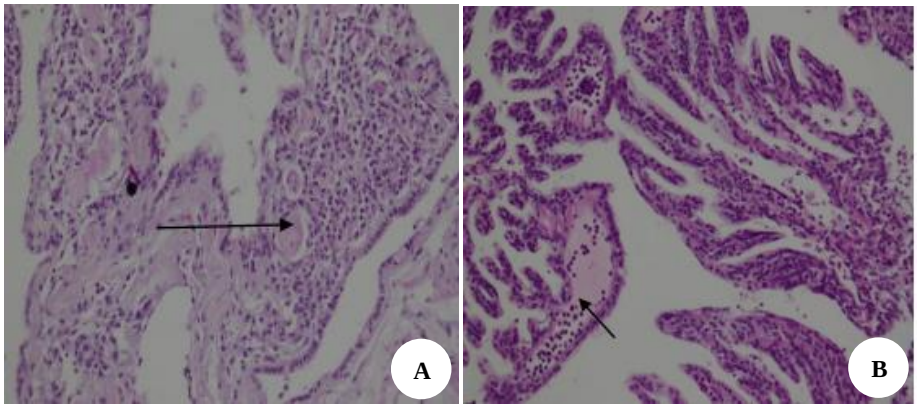

Figure 10. (A and B) H&E stain section LM examination of the octopus gill-infected oocysts of the coccidian parasite

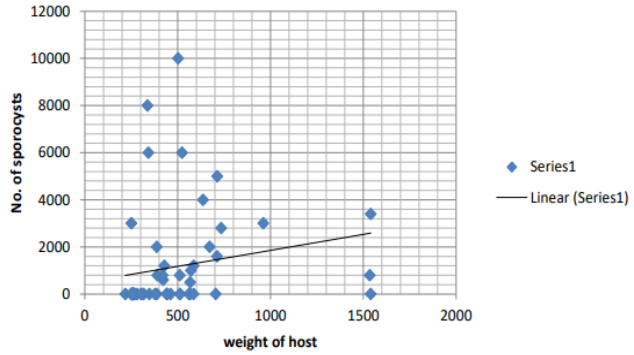

Figure 12. Relationship between the number of sporocysts in an individual host and the weight of the infected hosts

	Sum of sqrs	df	Mean square	F	p-value
Between groups:	2.21581E07	3	7.38602E06	1.63	0.1936
Within groups:	2.35562E08	52	4.53003E06		
Total:	2.5772E08	55			

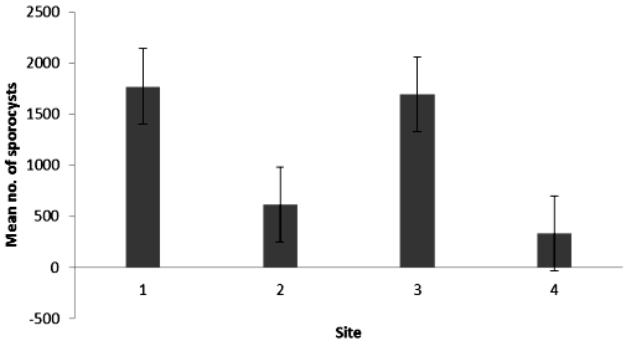

Figure 13. Prevalence of parasite infestation in octopuses among the sites where octopuses were collected (1- Kigombe, 2-Kwale, 3-Pangani, 4- Sahare)

Female		Male	
N:	27	N:	29
Mean:	974.07	Mean:	1317.2
95%:	(284.9 1663.2)	95%:	(360.44 2274)
Var.:	3.0351E06	Var.:	6.3272E06

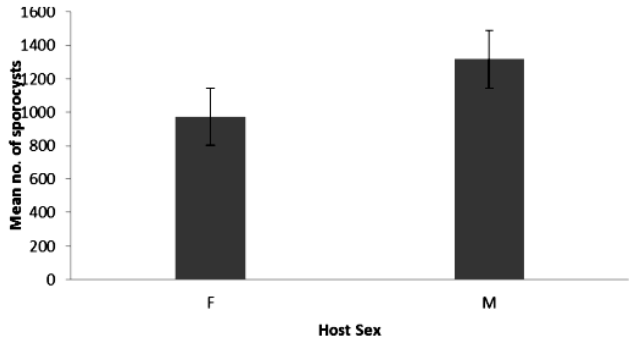

Figure 14. A variation on coccidian infection in male and female *Octopus cyanea*

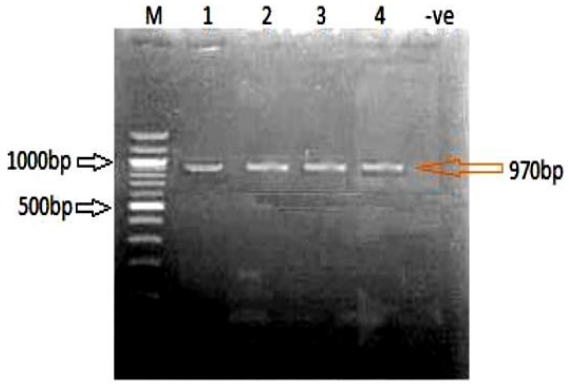

Figure 15. Photograph showing PCR amplification of *Aggregata* species, using universal primers, *Aggregata* primers 1F and 2R were used to amplify an 18S rRNA gene. The positive product 970bp is visible in 1.5% agarose gel. M is a 100bp Marker, and the gel was stained with Gel Red

.

Aggregata sp. DNA was found in pooled intestinal samples collected from 23 infected *O. cyanea* on the Indian Ocean coast of Africa. In proportion, 68% of DNA samples performed (amplified) in a PCR were positive. The positive PCR products were cloned for sequencing to confirm the *Aggregata* spp.

Seventeen of the 25 samples reacted positively with the designed *Aggregata* primer pair (Table 6). The remaining eight samples had no identifiable PCR amplicons with the designed primers. Of the 17 positive samples, two of them were weak, and they were not selected.

Sequencing identification of coccidian parasite

Histology and microscopic examination aided in the phylogenetic identification of *Aggregata* spp. Cloning and sequencing were done for the 15 strongly positive DNA samples to confirm the parasite species. The sequenced samples were edited and assembled in Genius Software to form a consensus sequence aligned in a GenBank. The alignment showed that all the sequences were identical to *A. octopiana* isolate H1 18S ribosomal RNA gene for 89% with Accession number KC188342 followed by *A. eberthi* gene isolate RV2 18S ribosomal RNA for 84% with Accession number KC188343.

Table 7 shows the summary of alignment of the consensus sequences during BLAST analysis and the percentage of identity with the *A. octopiana* sequences from a GenBank.

Discussion

Octopus cyanea represents an important economic artisanal fishery product around the Indian Ocean coast of East Africa, providing national income and a source of proteins to East African countries (Guard and Mgaya 2002). The *O. cyanea* artisanal catch comprises about 99% of octopus caught off the coast of Tanzania (Guard 2009).

The fishermen's community depends on octopus fisheries as their source of protein and employment. It was observed that a variety of people dealt with artisanal octopus fishing as their source of income and food, as reported by Jiddawi and Ohman (2002).

Even though octopus fishing has been a major activity in coastal areas, the community lacks knowledge of octopus parasites and diseases (Pascual and Guerra 2001). Furthermore, knowledge of the octopus-parasite relationship in the wild is poor since coccidian has not been reported to cause any harm to consumers (Bentacor et al. 2013). Even does not cause direct mortality in octopuses (Pascual et al. 1996; Pascual and Guerra 2001; Gestal et al. 2007; Castellanos-Martínez and Gestal 2013). For this reason, the fishermen's communities and even the consumers can't recognize the parasitic infections in octopuses easily.

According to fishermen, its observed octopuses are infected with some unknown agents that cause lesions on their bodies and arms. Biotic and abiotic factors can cause a lesion in the environment where octopuses live. Pascual et al. (2006) reported that injuries could be caused by parasites, bacterial or viral infections, or sometimes by any substance that can cause injury to the animal in its natural environment. Fishermen proposed that injuries in octopuses were mainly caused by predators like eel fish and other carnivores that hunt on an octopus. . Still, more studies are needed to determine the association between eel fish and octopus.

Table 6. Results for molecular analysis showing the positive and negative samples for the PCR reaction for 1F and 2R primers

S/N	Specimen name	Specimen no.	DNA Results
1	OP	3	+
2	OK	12	-
3	OKW	3	-
4	OP	9	-
5	OS	3	+
6	OS	1	+
7	OP	14	-
8	OK	10	-
9	OKW	5	+
10	OK	3	+
11	OK	15	+
12	OK	7	+
13	OKW	5	+
14	OS	12	+
15	OK	11	+
16	OP	13	+
17	OK	14	-
18	OS	8	+
19	OK	12	+
20	OP	6	+
21	OS	7	+
22	OKW	4	+
23	OP	15	+
24	OP	11	-
25	OK	2	-

Note: OKW: octopus from Kwale, OP: Octopus from Pangani, OS: octopus from Sahare, OK: octopus from Kigombe. (+) - DNA present, (-) - DNA absent

Table 7. GenBank reference sequences of *Aggregata* spp. used in the construction of phylogenetic trees

Field sequence	Name	Isolate	Accession no.	% Identity	Country	Host	Year
S1	*A. octopiana*	RV1	KC188342.1	86	Spain	*O. vulgaris*	2013
	A. eberthi	RV2	KC188343.1	83	Spain	*S.officinalis*	2013
	A. octopiana		DQ096837.1	82	Czech Republic	*O. vulgaris*	2006
	A. eberthi		DQ096838.1	81	Czech Republic	*S.officinalis*	2006
S2	*A. octopiana*	RV1	KC188342.1	86	Spain	*O. vulgaris*	2013
	A. eberthi	RV2	KC188343.1	84	Spain	*S.officinalis*	2013
	A. octopiana		DQ096837.1	82		*O. vulgaris*	2006
	C. ubiquitum	JFM10	AB697056.1	77	Japan	*A. speciosus*	2013
S3	*A. octopiana*	RV1	KC188342.1	86	Spain	*O. vulgaris*	2013
	A. eberthi	RV2	KC188343.1	84	Spain	*S.officinalis*	2013
	A. octopiana		DQ096837.1	82	Czech Republic	*O. vulgaris*	2006
	A. octopiana	H1	LC186909.1	89	Spain	*O. vulgaris*	2017
S4	*A. octopiana*	RV1	KC188342.1	85	Spain	*O. vulgaris*	2013
	A. eberthi	RV2	KC188343.1	83	Spain	*S.officinalis*	2013
	A. octopiana		DQ096837.1	82	Czech Republic	*O. vulgaris*	2006
	A. eberthi		DQ096838.1	81	Czech Republic	*S.officinalis*	2006
S5	*A. octopiana*	RV1	KC188342.1	87	Spain	*O. vulgaris*	2013
	A. eberthi	RV2	KC188343.1	84	Spain	*S.officinalis*	2013
	A. octopiana		DQ096837.1	83	Czech Republic	*O. vulgaris*	2006
	A. eberthi		DQ096838.1	82	Czech Republic	*O. vulgaris*	2006
S6	*A. octopiana*	RV1	KC188342.1	86	Spain	*O. vulgaris*	2013
	A. eberthi	RV2	KC188343.1	83	Spain	*S.officinalis*	2013
	A. octopiana		DQ096837.1	82	Czech Republic	*O. vulgaris*	2006
	A. eberthi		DQ096838.1	81	Czech Republic	*S.officinalis*	2006

Note: O: octopus; S: Sepia; A: Apodemus; A: aggregata; C: cryptosporidium; S: Consensus sequences

The fishermen's opinions of parasite existence in octopuses were observed by categorizing them by age groups. That was intended to get different opinions depending on the respondent's age and experience in octopus fishing. Age groups helped in gaining information concerning parasite infection in octopuses. Fishermen in the middle age group (31-53) were more knowledgeable about octopus parasite existence than other age groups. Most of the fishermen interviewed perceived that octopus had neither been infected by any parasite nor had any disease, while some knew nothing about parasites supporting that the knowledge of parasites and disease in octopuses is limited (Pascual and Guerra 2001).

It was possible to get some information about the effects of octopus consumption in the fishing community. In the survey, some fishermen confirmed the occurrence of allergies in some people after eating octopus resulting in rashes, stomachaches, and vomiting. According to previous reports on octopus diseases and parasites, these allergies could be associated with some worms reported in octopuses, such as nematodes, *Anisakis* sp. (Angelucci et al. 2011; Tsabouri et al. 2012). However, according to the experiences of the fishermen's community, they overcame the allergic reaction by cooking octopus meat or stopping the allergen victim from eating octopus.

The reported results for the histopathology study and fresh squash examinations of the liver, gills, kidney, and intestine, especially the cecum of *O. cyanea*, were likely like those reported by Licciardo et al. (2005) and Betancor et al. (2013) in the intestine of common octopus (*O. vulgaris*). This study found no association between parasite load and octopus weight, although host size has been reported to contribute to parasite infection (Catalano et al. 2014). The weight of the octopus influences parasite pathology, as described by Betancor et al. (2013). Octopuses with small body weights and sizes were observed to be no or slightly infected, as described by Storero and Narvarte (2013). The larger in size and weight of the octopus, the greater the possibility of harboring parasites or being heavily infected with protozoan parasites, as discussed by Storero and Narvarte (2013).

In this study, no ectoparasites were found, even though some were reported in octopuses, such as copepods *Pennella* spp. and *Octopicola* spp. located in the gills of common octopuses (Pascual et al. 1996). Nematode larvae or Monogenean trematodes like *Diphyllobothrium* sp. were also expected to be attached in gills, as Pascual et al. (1996) reported in common octopus. But the finding was different as the results revealed no ectoparasite observed in *O. cyanea* captured along the coast of Tanga. Although ectoparasites were not found, some octopuses had lesions on their skin, possibly due to parasites or any other mechanical damage that occurred during octopus catch.

Recent papers indicate that enteric coccidian infection in octopuses is the most important consequence since they have been causing an economic loss in reared cephalopods (Gestal et al. 1999). In this study, almost all the infected octopuses examined were found to have an enteric coccidian infection.

Compared with other *Aggregata* spp., the *Aggregata* sp. found in *O. cyanea* had sporocysts with more than eight sporozoites, as shown in Figure 5. Poynton et al. (1992) reported *Aggregata dobelli* in *Octopus dofleini* (Wülker, 1910) from the Northeast Pacific Ocean, with sporocysts having more than eight sporozoites as those observed in this study. The sporocysts of *Aggregata* sp. in octopuses from the coastal area of the Tanga region were found to have a cover resembling that of *A. octopiana* for 86% compared to other coccidian species but having 9-22 numbers of sporozoites.

The caecum is the usual site of infection with coccidian, as described by Estevez et al. (1996) and Gestal et al. (2002). Some pathological effects of the coccidian parasite noticed in histopathological study of the intestine, caecum, and gills were the same as those described by Gestal et al. (2000). In heavily infected octopus, the mucosal and epithelial cells were replaced by coccidian parasites (Pascual et al. 2007). Betancor et al. (2013) reported pathological reactions of *A. octopiana* in the caecum, intestine, and gills of wild and reared octopus, similar to the infected *O. cyanea* in this study.

The replacement and destruction of mucosal and epithelial cells with sporocysts can cause malabsorption syndrome and loss of intestinal and caecum epithelium (Baldascino et al. 2017). Pathology caused by these coccidian parasites is reported to weaken the host and increase the susceptibility to other pathogens (Pascual et al. 2007). The host and parasite (*Aggregata* sp.) have been reported to influence the pathological state (Gestal et al. 2002). This study noted that parasite pathology was not high in wild *O. cyanea* compared to that reported by Betancor et al. (2013) in reared and wild common octopuses.

Apart from *Aggregata* sp., Dicyemid mesozoans were also reported to be host specific and found in the Northern Indian Ocean with no evidence in the Eastern Indian Ocean by Castellanos-Martínez et al. (2011). None of the octopuses examined was found to be infected with these mesozoans. Most of the described mesozoan parasites have been reported to be host-specific (Castellanos-Martínez et al. 2011), so it could be possible to find one in *O. cyanea*, but only *Aggregata* sp. was observed in octopus. Therefore, it cannot be concluded that mesozoan parasites are completely absent in this specie of octopus; more work must be done to prove the reality of dicyemids' condition along the Indian Ocean.

The existence of visible cysts in the wall of the digestive tract, oocysts having a large number of sporocysts, the shape of oocysts and sporocysts used as evidence and key taxonomic features of identifying gastrointestinal protozoan as *Aggregata* sp. since they are only coccidian parasites infecting cephalopods including octopuses (Pascual et al. 2007; Baldascino et al. 2017). Pathology of the parasite in the host intestine was useful for parasite identification in this case since it is the well-known common feature of any coccidian parasite infection in their host gastrointestinal tissue (Pascual et al. 2007).

This report is the first of parasite existence in *O. cyanea* along the Indian Ocean of Tanzania coast. Many

studies have reported *A. octopiana* infections in European countries in common octopus, *O. vulgaris* (Gestal et al. 2005). Isolating *A. octopiana* in *O. cyanea* is a new finding. The task of this study was to determine parasites affecting *O. cyanea*, but only one parasite, *A. octopiana*, was identified and confirmed by molecular techniques.

The prevalence of coccidian parasites found in examined octopuses differed depending on the geographical location (site) where they were collected, as shown in Table 2. Geographical location has a great influence on the distribution of parasites, as reported by Castellanos-Martínez et al. (2011), Storero and Narvarte (2013), and Catalano et al. (2014). In this study, the location where the octopus is collected can be one factor leading to parasite abundance variation. González et al. (2003) documented that ecosystem structure has something to do with parasite richness, so this could be the reason for the variation of parasite prevalence among the octopuses collected from four study sites.

Molecular identification showed that the isolates coccidian parasite of *O. cyanea* was the *A. octopiana*. Morphological and molecular identification of the parasites were compared with other published findings. The results from this study confirmed the presence of coccidian parasites in *O. cyanea*. Combining morphological and molecular identification revealed that the enteric coccidian parasite was the *A. octopiana*, also found in the common octopus, *O. vulgaris* (Gestal et al. 2002). More studies should be done on the parasite of *O. cyanea* to prove the coccidian parasite inhabiting this cephalopod species.

From the confirmed 23 coccidian-infected octopuses, 17 microscopically positive animals were confirmed by PCR. The PCR was the most specific technique for diagnosing the parasite than using the microscope, which showed 23 animals were positive. The PCR results showed that four octopuses from Pangani, five from Sahare, three from Kwale, and five from Kigombe were positive, less than the positive number obtained from the microscopic examination. In most diseased cases, PCR has been used and reported to be the most sensitive technique of disease confirmation than the less sensitive microscopic method (Johnston et al. 2006).

In addition, the absence of morphological and structural information on helminths, crustaceans, and coccidian parasites affecting *O. cyanea* made it difficult to identify them. Therefore, there could be some confusion in identifying the parasite in *O. cyanea* due to the absence of detailed information on the parasite harboring them. Moreover, further investigation is needed in our coastal area.

In conclusion, like other octopus species, octopuses along the Indian Ocean coast of Tanzania have been infected by parasites. About 41% of the collected octopuses were infected with the coccidian parasite. The parasites are dangerous when it comes to octopus rearing as they can cause mortality to the infected host once exposed to other biotic or abiotic stressors. The parasite does not cause any disease to consumers, but it is advised the octopus must be well cooked to prevent any disease-causing agents. Combining this study with previous reports on octopus parasites, this must consider a serious problem to bring more awareness to East African countries on the existence of parasites in octopuses along the Indian Ocean. The expectation is that more research can be done on investigating more parasite species found in all *octopus* spp. in Tanzania, where the species is being exploited. Although the *A. octopiana* was found as the only parasite in the present study, more stains of coccidian or *Aggregata* spp. from *O. cyanea*, even in other cephalopod species, are expected to be discovered in the Indian Ocean as many species have been described in other countries. That is the first time octopuses have been investigated for parasites along the Tanzanian coast. Literature for references was difficult to assess, but this challenge will be reduced if more research is conducted on octopus to simplify future work.

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