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Original Article

First report of Diplostomidae metacercariae (Trematoda: Digenea) in African catfish *Clarias gariepinus* (Siluriformes: Clariidae) in Brazil

Primeiro relato de metacercárias de Diplostomidae (Trematoda: Digenea) em bagre africano *Clarias gariepinus* (Siluriformes, Clariidae) no Brasil

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Abstract

Clarias gariepinus is a fish from North of South Africa and was later introduced in several countries, including Brazil. The present study aimed to describe the first report of Diplostomidae metacercariae in *C. gariepinus* in Brazil. For this, 30 *C. gariepinus* were captured in a lake in the city of Campos dos Goytacazes, RJ, Brazil. Fishes were euthanized using freezing and necropsied for collection of parasites. The organs were dissected and analyzed for the presence of parasites that were processed for light and scanning electron microscopy. Trematodes were stained with Semichon's Carmine and Gomori's Trichrome, observed and schematized under a light microscope with image analysis software. A total of 190 trematodes were collected from the gills, suprabranchial organs, heart, stomach, intestinal mesentery, liver and body cavity of the fish. The parasites had a foliaceous body divided by a discrete constriction, without genital primordia, and a holdfast organ present at the posterior region, typical of metacercariae of the family Diplostomidae. It was classified as the 'Diplostomulum' morphotype, based on the morphology of the reserve bladder structure. This is the first report of the metacercariae of Diplostomidae parasitizing *C. gariepinus* in the Americas. This fish acts as an intermediate or paratenic host of this digenean in Brazil.

Keywords: Fish, parasite, trematode.

Resumo

Clarias gariepinus é um peixe do Norte da África do Sul e posteriormente introduzido em diversos países, incluindo o Brasil. O presente estudo teve como objetivo descrever o primeiro relato de metacercárias em *C. gariepinus* no Brasil. Para tanto, 30 *C. gariepinus* foram capturados em um lago na cidade de Campos dos Goytacazes, RJ, Brasil. Os peixes foram eutanasiados por congelamento e necropsiados para a coleta de parasitos. Os órgãos foram dissecados e analisados para a presença de parasitos que foram processados para microscopia ótica e electronica de varredura. Os Trematodeos foram corados com carmim de Semichon e tricrômico de Gomori, observados e esquematizados sob microscópio ótico equipado com software de análise de imagens. Um total de 190 trematodeos foram coletados das brânquias, órgãos suprabrânquiais, coração, estômago, mesentério intestinal, fígado e cavidade corporal dos peixes. Os parasitos tinham corpo foliáceo, dividido por uma discrete contrição, sem primórdio genital, e órgão tribocítico presente na região posterior, típico das metacercárias da fámilia Diplostomidae. Foram classificados como do morfotipo 'Diplostomulum', baseado na morfologia da estrutura da bexiga de reserva. Este é o primeiro relato de metacercárias de Diplostomidae parasitando *C. gariepinus* nas Américas. Este peixe atua como hospedeiro intermediário ou paratênico deste Digenea no Brasil.

Palavras-chave: Peixe, parasito, trematodeos.

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Introduction

Clarias gariepinus Burchell, 1822, popularly known as African catfish, is a pulmonary fish from Africa (ERSOY & OZEREN, 2009) which was introduced in Brazil for commercial purposes in the mid-1980s (OZÓRIO et al., 2004), and its breeding in atypical tanks without protection barriers near the river and Lake beds resulted in escape and invasion of the surrounding environment. *Clarias gariepinus* is considered an omnivorous species (TEUGELS, 1986), and there are no studies on infection by metacercariae of digeans in this fish outside Africa.

The metacercariae of the family Diplostomidae are important pathogens that can cause serious impacts on fish health in fish populations from the natural habitat as well as in aquaculture. This larval stage can cause mortality in young fish by capillary hemorrhage and blood vessel obstruction (mainly in the head and brain), loss of vision, growth reduction, and emaciation, deformation of the vertebral column, brain tumor and cellular necrosis, which can lead to death (SZIDAT & NANI, 1951; CHAPPELL et al., 1994; CHAPPELL, 1995; NIEWIADOMSKA, 1996; MACHADO et al., 2005). This study aimed to describe the first report of metacercariae of Diplostomidae in *C. gariepinus* in Brazil.

Materials and Methods

A total of 30 *C. gariepinus* were captured from the Jacaré Lake, district of Tocos, in the city of Campos dos Goytacazes, Rio de Janeiro State, Brazil. Fish were kept in isothermal boxes with ice to reduce the desensitization stress, euthanized by freezing and stored at -20 °C.

The necropsy procedure, parasite collection, fixation and conservation were performed according to Amato et al. (1991) and Eiras et al. (2010). The organs and viscera were removed and separated in Petri dishes with 0.65% saline solution, washed in sieves with 0.025-mm mesh, dissected and analyzed under a stereomicroscope. After removal of all viscera, the coelomic cavity was washed with distilled water and passed in a sieve with the same mesh, and the retained material was observed under a stereomicroscope and analyzed for the presence of parasites.

Light microscopy

Metacercariae were fixed in hot AFA (70° GL ethanol, 93%; formaldehyde, 5%; glacial acetic acid, 2%) for 48 hours and conserved in 70% ethanol. Specimens were stained in Semichon's Carmine and Gomori's trichrome (in this case, worms were hydrated before staining), dehydrated in an increasing ethanol series, differentiated in 2% acetic acid, clarified in clove oil and mounted between lamina and cover slips with Dammar Gum.

Measurements were performed to the nearest micron (range (mean ± S.D.)) and were based on nine specimens from multiple fish. Measurements were conducted with an Axioplan Zeiss light microscope (Carl Zeiss, Germany) equipped with a Canon Power-Shot A640 digital camera (Canon, China) and Zeiss Axion Vision Sample Images Software (Carl Zeiss, Germany) for image analysis. Drawings were performed with the aid of an Axioplan Zeiss light microscope (Carl Zeiss, Germany) equipped with a camera lucida and were digitized using Adobe Photoshop Elements 8.0 software with the aid of an Intuos4 Wacon® pen tablet (Wacon Co. Ltd, Japan).

Scanning electron microscopy

Ten metacercariae specimens were fixed in Karnovsky solution (2.5% glutaraldehyde, 4% freshly prepared paraformaldehyde, 5 mM calcium chloride in 0.1 M cacodylate buffer, pH 7.2), washed in 0.1 M cacodylate buffer, post-fixed in 2% 0.1 M osmium tetroxide, 5 mM calcium chloride and 0.8 potassium ferrocyanide in 0.1 M cacodylate buffer. The samples were dehydrated in an acetone series, critical point dried with CO_2, sputter-coated with gold, and examined in a Zeiss EVO MA 10 scanning electron microscope (SEM) operating at 15 kV.

Results

Two males (7%) and seven females (23%) of *C. gariepinus* were infected with trematode metacercariae. Larvae were collected from the washing of the gills, suprabranchial organs, heart, stomach, intestinal mesentery, liver and body cavity. A total of 190 metacercariae were collected, of which 98 (52%) were in *C. gariepinus* males and 92 (48%) in *C. gariepinus* females. The metacercariae collected from different organs present similar morphology. They were identified as metacercariae of the Diplostomidae of the 'Diplostomulum' morphotype.

The metacercariae of Diplostomoidae had a foliate body (Figure 1a, b), measuring 618 to 913 (761 ± 109.3) long by 197 to 304 (246 ± 38.5) wide. Papillae and denticles were not observed by light microscopy and SEM (Figure 1). The body was composed of a long and discreet forebody, followed by a small hindbody. The forebody measured 560 to 739 (633 ± 77.3) long, where a subterminal oral sucker was observed measuring 46 to 64 (55 ± 6.5) long by 42 to 67 (54 ± 7.0) wide. Muscular pharynx ranging from 23 to 47 (34 ± 7.5) long by 19 to 32 (39 ± 4.4) wide, followed by a short esophagus measuring 26 to 28 (27 ± 0.7) long, which bifurcated into two long intestinal caeca with a blind end near the hindbody. The ventral sucker measured 37 to 51 (44 ± 3.7) long by 35 to 49 (43 ± 4.2) wide, with a distance of 352 to 612 (458 ± 79.5) from the anterior extremity. Holdfast organs present with an elliptical shape and a median fissure observed under light microscopy (Figure 1a, b), located between the ventral sucker and the posterior region of the body, measuring 67 to 115 (87 ± 18.9) long by 56 to 97 (75 ± 14.9) wide. This structure was not observed by SEM (Figure 1c). The hindbody measured 57 to 81 (69 ± 9.7) long. Three main longitudinal canals are present, one medial and two laterals, connected by a commissure in the anterior region of the body, near the level of the pharynx and another posteriorly, anterior to the ventral sucker, with several terminal pockets with rounded excretory bodies distributed throughout the body (Figure 2). Y-shaped excretory vesicle present near the posterior extremity of the body. Genital primordium absent (Figure 1a and Figure 2).

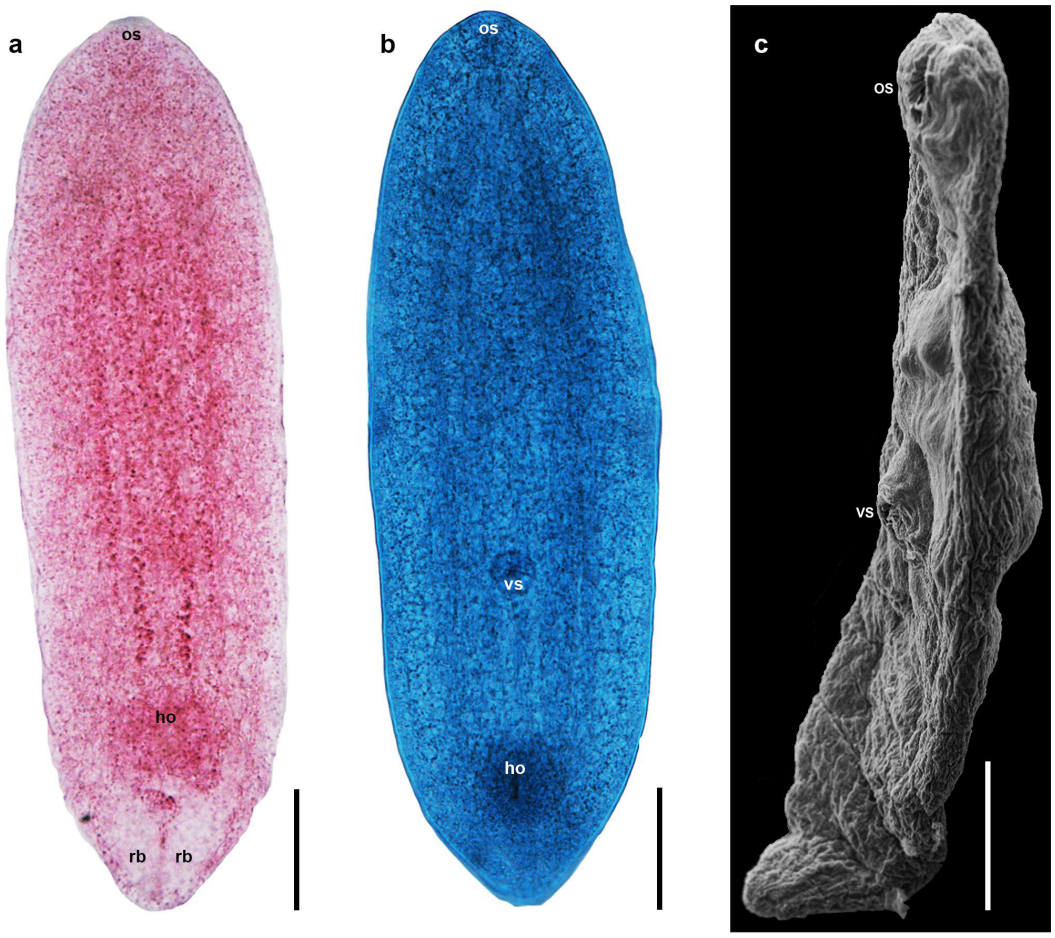

Figure 1. Metacercariae of Diplostomidae in *Clarias gariepinus* from the Jacaré Lake in Campos dos Goytacazes, State of Rio de Janeiro, Brazil. (a) light microscopy of the specimens stained in Semichon's Carmine. Bar 100 µm; (b) light microscopy of specimens stained in Gomori's trichrome. Bar 100 µm; (c) scanning electron microscopy. Oral sucker (os); holdfast organ (ho); reserve bladder (rb); ventral sucker (vs). Bar 200 µm.

Representative specimens were deposited in the Helminthological Collection of the Oswaldo Cruz Institute (CHIOC), Fundação Oswaldo Cruz, Rio de Janeiro, Brazil (CHIOC Nº 38868 a-b).

Discussion

The members of Diplostomidae are distinct from the other groups of the Trematoda due to the presence of a unique holdfast organ (NIEWIADOMSKA, 2002a), which allowed us to characterize the specimens of Trematoda collected from *C. gariepinus* from Brazil as belonging to this family (Figure 1a, b; Figure 2). The metacercariae of Diplostomidae have similar morphologies between the different genera, which makes it impossible to identify them at the generic level. However, several authors classify the metacercariae at a generic level (MWITA & NKWENGULILA, 2004; CHIBWANA & NKWENGULILA, 2010), and specific level (BARSON et al., 2008; ZHOKHOV et al., 2010). Few studies developed molecular analyses for the identification of the metacercariae specimens collected from *C. gariepinus* (MWITA & NKWENGULILA, 2010; CHIBWANA et al., 2013, 2015; MOEMA et al., 2013). In this way, according to Niewiadomska (2002a), these metacercariae are classified into four main morphotypes ('Diplostomulum', 'Naescus', 'Prohemistomulum' and 'Tetracotyle') based on the structure of the reserve bladder, however only one study classified the collected metacercariae from *C. gariepinus* into morphotypes (BARSON et al., 2008). The simplest reserve bladder type is characteristic of the 'Diplostomulum' morphotype, which presents three longitudinal canals (one median and two laterals) connected by a commissure at the anterior region of the body, next to the pharynx level, and another located posteriorly, anterior to the ventral sucker. The ramification system of the longitudinal canals is enlarged at the terminal portions, forming a terminal pocket where the excretory bodies are located, which can be oval or rounded in shape (NIEWIADOMSKA, 2002a). These characteristics were observed in the metacercariae collected from *C. gariepinus* of this study, which allowed inferring that these are of the 'Diplostomulum' morphotype (Figure 2), different to the morphotype resported by Barson et al. (2008), for metacercaria collected in *C. gariepinus* from the Save-Runde River, Zimbebwe.

In freshwater fish, the metacercariae of the family Diplostomidae are found encysted, encapsulated in the tissues or free in the tegument, muscles, eyes and central nervous system and are most

commonly reported in the last two habitats (GIBSON et al., 2002; THATCHER, 2006). Barson et al. (2008) found encysted diplostomid metacercariae in the muscle and not encysted metacercariae in the intestine of *C. gariepinus*. However, in *C. gariepinus* from this study, the metacercariae were observed free in several organs examined, but all of them with similar morphology. There are several reports of the occurrence of diplostomid metacercariae among *Diplostomum mashonense* Beverly-Burton, 1963, *Dolichorchis tregenna* and *Tylodelphys* spp. in the cranial cavity of *C. gariepinus* in Africa (MASHEGO & SAAYMAN, 1989; MWITA & NKWENGULILA, 2004; MUSIBA & NKWENGULILA, 2006; CHIBWANA & NKWENGULILA, 2010; ZHOKHOV et al., 2010; CHIBWANA et al., 2013, 2015; MOEMA et al., 2013). However, the cranial cavity of the fish from the present study was not analyzed during the necropsies.

Chibwana & Nkwengulila (2010) classified the diplostomid metacercariae based on the presence or absence of pseudosuckers, distinguishing three genera: *Diplostomum* and *Dolichorchis* with pseudosuckers and *Tylodelphys* without these structures. However, these authors do not describe the metacercariae in morphotypes according to the reserve bladder structure as described in the key to the Superfamily Diplostomoidea (NIEWIADOMSKA, 2002a). Furthermore, the three genera cited above present pseudosuckers on adult specimens (NIEWIADOMSKA, 2002a). Barson et al. (2008) identified the metacercariae collected from the intestine of *C. gariepinus* as *D. mashonense*, based only on the morphology of these larvae. However, the identification at the generic level is not possible based only on the metacercariae morphology, without knowledge of the complete parasite life cycle (NIEWIADOMSKA, 2002b). The generic and specific diagnosis is based on the morphology of general characters, mainly that of the reproductive system, which are absent in larval stages. Thus, the trematode identification keys are based on adult worms (DUBOIS, 1968; GIBSON et al., 2002). In this way, the identification of the genus of the metacercariae collected from *C. gariepinus* from Brazil was not possible. According to Chibwana & Nkwengulila (2010), only Shigin (1971) apud Chibwana & Nkwengulila (2010) developed a key for the identification of this species class based on the intermediate stage, more precisely for the metacercariae, but this study has been written in Russian and its use is restricted to the Palearctic region.

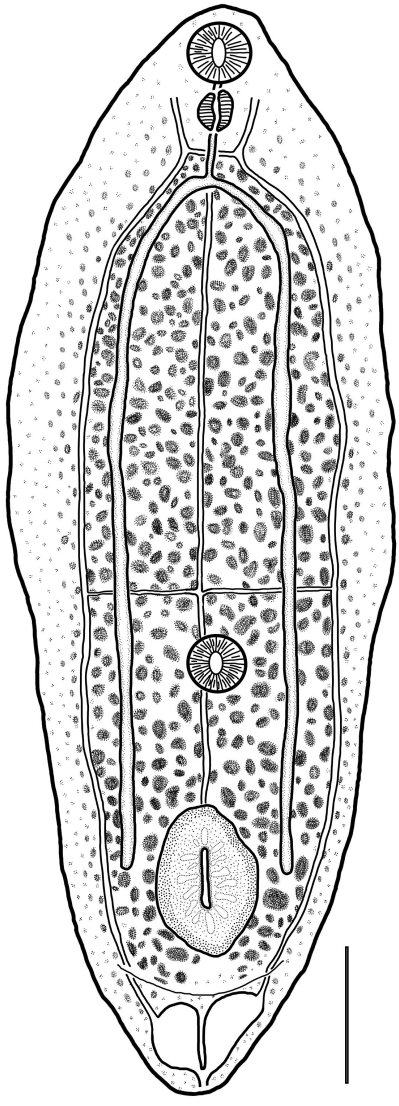

Figure 2. Drawing of metacercariae of Diplostomidae in *Clarias gariepinus* from the Jacaré Lake in Campos dos Goytacazes, State of Rio de Janeiro, Brazil. Bar 100 μm.

Prudhoe & Hussey (1977) described three species of diplostomid metacercariae from *C. gariepinus* in South Africa, two as *Diplostomum* (type I and II) and one as *Neodiplostomum*. According to these authors, the metacercariae of the genera *Diplostomum* presented two pseudosuckers next to the oral sucker, which were not observed in the present study. However, Niewiadomska (2002b) affirms that *Diplostomum* metacercariae can or cannot present pseudosuckers. In *Neodiplostomum* metacercariae, reported by Prudhoe & Hussey (1977), the pseudosuckers were absent, similar to our study.

The species of the genera *Diplostomum*, *Neodiplostomum* and *Alaria* present a 'Diplostomulum' morphotype metacercariae (NIEWIADOMSKA, 2002a), which suggests that *C. gariepinus* from Brazil is an intermediate or paratenic host of at least one of these genera of Trematoda. The metacercariae of the genera *Alaria* and *Neodiplostomum* are found mainly in amphibians (second intermediate hosts) and can also be found in reptiles and mammals (as paratenic hosts). However, *Diplostomum* metacercariae are frequently reported from fish (second intermediate hosts) (NIEWIADOMSKA, 2002b). The definitive hosts for the genera that usually possess the 'Diplostomulum' morphotype metacercariae in its life cycle are piscivorous birds (*Diplostomum* and *Neodiplostomum*) or mammals (*Alaria*) (NIEWIADOMSKA, 2002b), which indicates that the adult stages of the trematode collected from *C. gariepinus* from the present study can be found in birds or mammals (Carnivora, Rodentia, Marsupialia), which can be fed *C. gariepinus* from the studied locality and become infected, thus completing their life cycle.

The comparison of the measurements of the metacercariae from the present study, it was similar to those classified as *D. mashonense* (BARSON et al., 2008; CHIBWANA & NKWENGULILA, 2010), with exception from the specimens described as *D. mashonense* by Moema et al. (2013) that presented a longer total body length and holdfast organ length (Table 1). Chibwana & Nkwengulila (2010)

Table 1. Comparison of measurements (μm) of Diplostomidae metacercariae in *Clarias gariepinus* from the Africa and present.

SPECIMES	Country (Locality)	Measured parameters – Range (Mean)											References
		tbl	mbw	osl	osw	pl	pw	vsl	vsw	d	hol	how	
'Diplostomulum'	Brazil (Lake Jacaré)	618-913 (761)	197-304 (246)	46-64 (55)	42-67 (54)	23-47 (34)	19-32 (39)	37-51 (44)	35-49 (43)	352-612 (458)	67-115 (87)	56-97 (75)	**Present study**
Diplostomum mashonense	Zimbabwe (Save-Runde River)	445-585 (514)	101-255 (185)	47-57 (49)	46-52 (48)	25-32 (28)	10-22 (17)	31-63 (48)	43-60 (52)	-	55-63 (59)	35-47 (42)	Barson et al. (2008)
Diplostomum mashonense	Tanzania (Kilombero River)	790	224	38	42	38	25	39	39	322	100	73	Chibwana & Nkwengulila (2010)
Diplostomum mashonense	Tanzania (Lake Victoria)	699-896 (818)	180-242 (214)	30-46 (36)	29-50 (36)	35-45 (39)	19-31 (26)	29-45 (36)	29-45 (36)	299-360 (330)	100-121 (110)	60-91 (76)	Chibwana & Nkwengulila (2010)
Diplostomum mashonense	Tanzania (Mindu Dam)	831	197	41	46	50	26	44	40	302	97	65	Chibwana & Nkwengulila (2010)
Diplostomum mashonense	Tanzania (Msimbazi River)	1039	240	34	49	30	29	47	49	482	100	84	Chibwana & Nkwengulila (2010)
Diplostomum mashonense	Tanzania (Ruvu River)	799	250	40	40	31	20	26	37	302	92	70	Chibwana & Nkwengulila (2010)
Diplostomum mashonense	South Africa (Supersand Dam)	946-1947 (1409)	240-485 (375)	36-90 (70)	42-96 (72)	24-60 (46)	30-60 (47)	36-90 (71)	30-96 (67)	-	156-198 (180)	24-150 (101)	Moema et al. (2013)
Diplostomum type I	South Africa (Olifants River)	530	460	58	58	-	-	35	43	180	87	87	Prudhoe & Hussey (1977)
Diplostomum type II	South Africa (Olifants River)	810-850	200-220	40-50	40-50	25	20	50	45	290-320	125	70	Prudhoe & Hussey (1977)
Dolochorchis tregenna	Ethiopia (Lake Tana)	1010-1330 (1170)	260-320 (300)	66-90 (80)	60-72 (70)	54-72 (60)	24-36 (33)	60-78 (63)	60-72 (60)	-	114-180 (160)	108-180 (130)	Zhokhov et al. (2010)
Neodiplostomum type	South Africa (Olifants River)	80-1220	-	45-50	30-50	37	30	67-75	87-100	-	120-160	100-120	Prudhoe & Hussey (1977)
Tylodelphys sp. X	Tanzania (Lake Victoria)	940-1180	160-240	48-58	60-71	-	-	25-35	23-37	-	83-117	44-69	Mwita & Nkwengulila (2004)
Tylodelphys sp. Y	Tanzania (Lake Victoria)	624-860	165-250	37-50	31-55	-	-	23-28	23-28	-	69-81	42-74	Mwita & Nkwengulila (2004)
Tylodelphys sp. 1	Tanzania (Kilombero River)	1043	214	41	41	41	32	38	37	555	117	63	Chibwana & Nkwengulila (2010)
Tylodelphys sp. 1	Tanzania (Lake Victoria)	889-1070 (1022)	180-240 (217)	30-41 (37)	31-41 (37)	30-50 (40)	20-36 (29)	30-40 (36)	21-43 (36)	470-520 (496)	71-120 (104)	50-76 (65)	Chibwana & Nkwengulila (2010)
Tylodelphys sp. 1	Tanzania (Ruvu River)	1231	194	61	59	51	43	44	41	792	163	62	Chibwana & Nkwengulila (2010)
Tylodelphys sp. 2	Tanzania (Kilombero River)	560	111	30	29	22	19	21	21	282	61	31	Chibwana & Nkwengulila (2010)

Abbreviations: total body length (**tbl**); maximum body width (**mbw**); oral sucker length (**osl**); oral sucker width (**osw**); pharynx lentgh (**pl**); pharynx width (**pw**); ventral sucker lentgh (**vsl**); ventral sucker width (**vsw**); distance of ventral sucker from the anterior extremity (**d**); holdfast organ length (**hol**); holdfast organ width (**how**).

Table 1. Continued...

SPECIMES	Country (Locality)	Measured parameters – Range (Mean)											References
		tbl	**mbw**	**osl**	**osw**	**pl**	**pw**	**vsl**	**vsw**	**d**	**hol**	**how**	
Tylodelphys sp. 2	Tanzania (Lake Victoria)	351-650 (495)	70-150 (100)	20-38 (28)	19-37 (28)	16-32 (23)	15-22 (19)	16-27 (21)	16-73 (21)	135-333 (248)	41-84 (61)	27-49 (37)	Chibwana & Nkwengulila (2010)
Tylodelphys sp. 2	Tanzania (Ruvu River)	667	128	31	31	21	19	21	22	366	60	22	Chibwana & Nkwengulila (2010)
Tylodelphys grandis n. sp	Ethiopia (Lake Tana)	1150-1870 (1600)	360-470 (390)	60-84 (74)	48-66 (55)	42-66 (56)	18-30 (24)	18-30 (24)	54-60 (55)	-	114-198 (163)	24-96 (74)	Zhokhov et al. (2010)
'Tetracotyle'	Zimbabwe (Save-Runde River)	300-438 (361)	244-320 (286)	18-50 (36)	21-49 (36)	30-48 (39)	23-36 (29)	26-50 (36)	28-59 (44)	-	40-55 (48)	60-105 (77)	Barson et al. (2008)

Abbreviations: total body length (**tbl**); maximum body width (**mbw**); oral sucker length (**osl**); oral sucker width (**osw**); pharynx lentgh (**pl**); pharynx width (**pw**); ventral sucker lentgh (**vsl**); ventral sucker width (**vsw**); distance of ventral sucker from the anterior extremity (**d**); holdfast organ length (**hol**); holdfast organ width (**how**).

Table 2. Metacercariae of Diplostomidae reported in *Clarias gariepinus* from Africa, compared to those of this host from Jacaré Lake in Campos dos Goytacazes, State of Rio de Janeiro, Brazil.

DIPLOSTOMIDAE	ENCOLOGICAL INDEX			AUTHORS
	PREVALENCE %	MEAN INTENSITY	MEAN ABUNDANCE	
Diplostomun mashonense	93.0	2391	-	Mashego & Saayman (1989)
	88.0	538.5	473.2	Mwita & Nkwengulila (2004)
	97.0	619.5	598.9	Musiba & Nkwengulila (2006)
	11.0	-	-	Barson et al. (2008)
Tylodelphys grandis	35.0	-	-	Zhokhov et al. (2010)
Tylodelphys sp.	31.0	98.4	30	Mwita & Nkwengulila (2004)
Tylodelphys sp. 1	75.0	76.7	57.2	Musiba & Nkwengulila (2006)
Tylodelphys sp. 2	94.0	393.5	370.2	Musiba & Nkwengulila (2006)
Metacercariae	**30.0**	**21.1**	**6.3**	**Present study**

reported the occurrence two types of *Tylodelphys* sp. metacercariae, being all the type 2 metacercariae collected from different location from Tanzania, presented smaller measurements and those from the type 1 (Kilombero River and Ruvu River) presented a higher total body length, when compared with this study (Table 1). From all the studies analyzes form morphological comparison, only two species metacercariae presented the intestinal caeca ending near the holdfast organ level (MWITA & NKWENGULILA, 2004), similar to our metacercariae. All other studies, the caeca exceeds the holdfast organ (BARSON et al., 2008; CHIBWANA & NKWENGULILA, 2010; ZHOKHOV et al., 2010; MOEMA et al., 2013).

In the present study, a low prevalence of infection by Diplostomidae metacercariae was observed compared with studies carried out in *C. gariepinus* from the Africa. However, Barson et al. (2008) reported a lower prevalence than that observed in Brazil. Mwita & Nkwengulila (2004) and Zhokhov et al. (2010) found a prevalence similar to our study however, these authors, despite similar prevalence, observed a higher mean intensity and mean abundance compared to our study. Several studies performed in Africa report high values of mean intensity and abundance in comparison to the values found in *C. gariepinus* from Brazil (Table 2). This first study on Diplostomidae metacercariae in *C. gariepinus* from the Brazil suggested that this fish can act as a paratenic host to this native digenean species from Brazil.

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