UNIVERSIDADE PAULISTA

FISH PROTOZOAN AS A BIOLOGICAL INDICATOR OF CLIMATE CHANGE

Dissertação apresentada ao Programa de Pós-Graduação em Patologia Ambiental e Experimental da Universidade Paulista – UNIP para obtenção do título de Mestre em Patologia Ambiental e Experimental.

RICARDO JOSÉ TEIXEIRA

SÃO PAULO

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Orientadora: Profª. Drª. Maria Anete Lallo

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DEDICATÓRIA

Dedico este trabalho à:

Primeiramente a Deus, por ser essencial em minha vida, autor do meυ destino, meυ guia, socorro presente nas horas de angústia, ao meυ pai Alberto, minha mãe Lourdes e a meus irmãos.

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SUMÁRIO

Applied and Environmental Microbiology

Fish protozoan as a biological indicator of climate change

Protozoan as a biological indicator (running title)

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Abstract

Parasites of fish are useful biomarkers and appear to be more sensitive to environmental stressors than are the fish themselves. For this reason, we evaluated the prevalence of parasites in freshwater fish collected before and after a long drought. We analyzed 150 specimens of fish collected from three different water reservoirs - Ibirapuera Lake (lake of urban park), Biritiba Dam (water reservoir for human consumption) and Recreational Fishing Lake (Lake of fish farming for fishing). Prior to necropsy, fish was anaesthetized by immersion solution of tricaine methane sulfonate until paralysis of the operculum. The parasites were surveyed by the fresh smears of gills and feces, or by histopathological examination with Hematoxylin-Eosin and Giemsa stain. We have

demonstrated that the decrease in water levels in reservoirs because of the lack of rain significantly increased the prevalence of parasites. The protozoan *Trichodina* sp. was the main biological indicator found in the gills of the fish caught in the lakes of Ibirapuera and Recreational Fishing. In Biritiba Dam showed high prevalence of free-living amoebas in fish feces after drought. Therefore, the detection parasites in fish can be recommended as an indicator of climate change with serious consequences for water quality and environmental health.

INTRODUCTION

The presence of parasites within the environment often becomes evident after a massive infestation causing clinical signs or leading to mortality of the infected hosts. Such a situation can be combined with biotic or abiotic changes in the environment (1), in the application of fish parasites as environmental indicators. Examination of parasite may reflect alterations in food web structure and function that result from the many ecological disturbances to host distributions, water levels, eutrophication, stratification, ice cover, acidification, oceanic currents, ultraviolet radiation, extreme weather, and are predicted to accompany climatic change (2). In general, responses of hosts and their communities vary depending on the type and intensity of the stressor, the parasite life cycle and exposure time (3). Pollution and stress are often associated with a reduction in parasitic species or under eutrophic conditions increase the number of parasites in the presence host site (3).

The most common groups of parasites examined to date in response to environmental stress are ectoparasites (4,5), including trichodinids (6) and monogeneans (7). These parasites are transmitted directly and reproduce rapidly. Generally, their populations proliferate under conditions stressful to their hosts (8), a response that is more in line with the concept of ecosystem health equalling the absence of disease. The gills are the most delicate structures of the teleost body and their vulnerability is thus considerable because their external location and necessarily intimate contact with the water means. Was demonstrated the relationship between *Trichodina* sp. infection on *Merlangius merlangus* and organic pollution, measured as levels of nitrite, nitrate and phosphate in the surrounding environment (9). These relationships suggest the usefulness of fish parasites as biological indicators for ecosystem change.

The parasite-host-environment relationship has been, over the years, the basis of epidemiology and the study of diseases. It is known that climate change interfere with the survival of the pathogen and the occurrence of disease, but this close relationship identification is difficult. In this respect, the presence of biological indicators of environmental changes that act as tracers such changes is critical. Parasites may be indicators of climate change, which is expected to affect the structure and species composition of entire ecosystems (10). We analyzed the occurrence of parasites in freshwater fish collected before and after a long period of drought and showed that the prevalence of parasites, in particular protozoa, increased significantly after the drought, showing that these pathogens are an indicator of climate change.

MATERIAL AND METHODS

Study area. Freshwater fish samples were taken in three different reservoirs of water: Biritiba Dam, Ibirapuera Lake and Recreational Fishing Lake. The Biritiba Dam is located on Biritiba-Mirim city (23°36’10,14284”; 46°05’26,24162”) and has 102,439.866 meters perimeter and area of 1,494.0472 hectars. It is part of the Alto Tiete Producer System, which is administered by Sabesp for the capture, storage and treatment of water for the metropolitan region of São Paulo. Ibirapuera park has an area of 1,584,000 m^2 and is located in the central area of the Metropolitan Region of São Paulo. The 3 lakes Ibirapuera Park are powered by Sapateiro

Stream, which is part of the basin of the Pinheiros River, where a consolidated urbanization area. The pollution stream is due to the excess of organic matter from wastewater that is discharged in the same. The recreational had two tanks of 32 m each and number of fish reaching approximately 1,500 kg per tank and the water supply was made by artesian well.

Collection and transport of fish. Fish were collected in two periods of observation. Part of the specimens (n = 63) were collected before the biggest dry season in 80 years in the state of São Paulo (August 2013 to March 2014) and the other part of the specimens (n = 87) after dry period (April 2014 to January 2015), in which there was a considerable reduction in water levels of the reservoirs studied. The specimens were captured with the use of fishing equipment. We collected 50 specimens of fish in each selected area. The animals were transported alive to the Paulista University Research Laboratory in plastic boxes with habitat own water and maintaining the aeration with oxygen pump. Fish were housed in tanks at 24 ± 1ºC in the aquatic facility of the Paulista University – UNIP. All procedures were approved by the Ethics Committee of the University (number 220/2014).

Collection of biometric data and identification of parasites. Prior to necropsy, fish were anaesthetized by immersion in a 150 mg/L solution of tricaine methane sulfonate (MS222 Sigma-Aldrich, St. Louis, Misouri, USA) until paralysis of the operculum (11). Were collected biometric data of each fish by checking your body mass and measure. Then, the entire outer body surface was examined using stereoscopic binocular microscope to check for damage or loss coat (epidermis and scales) such observations for determining the existence of parasites. Gills areas were scraped to collect material for observation under a light microscope. The intestinal contents were collected and stained with Lugol's iodine solution to 10% for the identification of possible intestinal parasites. In the case of presence of parasites, they were placed between slide and cover slip, fixed with methanol and subjected to Giemsa staining and Ziehl Nielsen. Parasites were identified using morphological parameters (12).

Necropsy and histopathology. Necropsy was performed with incisions in the ventral region, with two transverse and one longitudinal, exposing the viscera and organs to meet lesions like cysts, swellings and lacerations. The body, fins, mouth, eyes and inner capping of each fish were examined for possible parasites. Subsequently, the gills, skin, liver, spleen and intestines were removed and fixed in Davidson's solution for 8 hours and kept in 70% alcohol. These materials were then routinely prepared for histological analysis embedding in paraffin and stained with Hematoxylin-Eosin and Giemsa stain. In addition to our analysis, randomly selected fish samples before and after the drought and we count the number of parasites in histological sections of gills (10 microscopic fields with 100x magnification). The mean values before and after dry were analyzed statistically.

Statistical analysis. For comparisons between quantitative variables, the two-tailed test and Mann-Whitney test was used, the value of $P < 0.1$ was considered significant in SSPSS 15:00 software.

RESULTS

Collected species and biometric data. We collected 150 specimens of fish that included: *Oreochromis niloticus* (n = 81)*; Hypostomus affinis* (n = 4)*; Astyanax bimaculatus* (n = 40), *Crenicichla lenticulata* (n = 9) e *Geophagus brasiliensis* (n = 15). Table 1 shows the distribution of animals according to species, collection site and biometric data. The analysis of biometric data showed that the drought did not affect them significantly (date not shown).

Prevalence of parasites as environmental indicators. During the study period there was the biggest drought in 80 years in São Paulo state, Brazil. We realize the fish collected before and after the drought and we have demonstrated that the decrease in water levels of the dams studied due to the lack of rain dramatically altered the parasite burden of the evaluated fishes

These changes were especially observed in the reservoirs maintained by the natural flow of rain, as the Ibirapuera Lake and Biritiba Dam. In the Recreational Fishing Lake, where the amount of water in the reservoirs was maintained very close artificial form of the ideal, yet there was a reduction of water levels during the study period (Figure 1, Table 2). Randomly we count the number of parasites in the gills of fish before (4.08±2.05) and after (2.33±1.35) the drought, however there was no statistically significant difference in that respect.

Before the drought, the fishes surveyed in Ibirapuera Lake were free of parasites, but after the dry period, all fishes had *Trichodina* sp. (Figure 1) in their gills (30/30), only part of the animals had monogeneans (7/30) and a fish with skin lesion had the presence of *Epistylis* sp. (Table 3). Despite of the significant increase of parasitism in fish, those responsible for the park's fauna control reported no increased mortality of fish during this period.

In the Biritiba Dam, before the drought were observed non identified Trematodes eggs (Figure 2) in 5 of 20 fishes, but after the dry season all fishes surveyed had parasites, especially not identified *Amoebae* (20/30), *Entamoeba* sp. (20/30) and *Balantidium* sp. (4/30) in intestinal contents; were also observed *Psiconoodinium* sp. (2/30) and Monogenea (7/30) in the gills (Table 2, 3). But unlike what was observed in fishes from Ibirapuera Lake, the parasites were present in the examined intestinal content and not in the gills (Table 3).

In Recreational Fishing Lake, the prevalence of parasites was high in two periods evaluated, but we observed a significant increase in prevalence after the drought in fresh examination of the gills (Table 2). Before dry, it was observed *Trichodina* sp. (18/23), *Myxobolus* sp. (3/23), *Henneguya shaharini* (9/23) and *Ichthyophthirius multifiliis* (2/23). After the drought, we find *Trichodina* sp. (27/27), *Henneguya shaharini* (4/27) and Monogenea (2/27). There was especially the gills involvement that had large amounts of

Trichodina sp., which were constituted in biological markers of climate change that occurred during the study period (Table 3).

Histopathological lesions and parasites. The most significant lesions were observed in the gills, which revealed secondary lamellae fusion as a result of hyperplasia and degenerative action caused by presence of parasites (Figure 2). In same cases associated with mucus cell hyperplasia and inflammatory infiltrate. Kidney, spleen and liver another lesion pattern can be described as areas of necrosis, keratinized cell wall and hyperchromatic cells. The liver had severe steatosis and inflammatory infiltrate with a predominance of melanomacrophages.

Ciliates. *Trichodina* sp. appeared to be a saucer-shaped, hemispheric, dumbbell-shaped, sac-like or flattened cylindrical organism in cross-section of the gills between secondary lamellae (Figure 2); in fresh examination we observe the characteristic ciliary movement. The specie *I. multifiliis* was large in size (approximately 0.1 to 1.0 mm) and had a horse-shaped macronucleus. *Piscinoodinium* sp. in wet mount examination of the skin or gills reveals numerous, oval, opaque non-motile trophonts (about 9-12 X 40-90 µm), only 2 fish Biritiba Dam had this parasite on the gills. *Balantidium* sp. was oviform in shape, about 40 to 140 x 25 to 115 µm in size and uniformly covered with longitudinal ciliary rows.

Amoebae. *Entamoeba* sp. cysts were observed in the intestinal contents from Biritiba Dam. They showed a nucleus with small, round central nucleolus and small peripheral granules of chromatin (Figure 2). Other not identified *Amoebae* had different characteristics, which prevented identification by the methods used.

Myxosporidia. Two species of myxosporidia were found in the gills - *Henneguya* sp. and *Myxobolus* sp.. Cysts of *Henneguya shaharini* were located in secondary lamellae and free spores were seen in fresh examination (Figure 2). Cysts of *Myxobolus* sp. were observed inside the muscle associated with gill arch (Figure 2).

Monogenea. In gills, Monogenea Gyrodactylidae with elongated bodies (approximately 1 mm long) present haptor later with hooks smears was identified in the histological sections of the skin and gills.

DISCUSSION

The relationship between a healthy ecosystem and parasites on hosts inhabiting that ecosystem draws considerable attention in recent years (13, 14, 15). Parasites are really important components of any ecosystem, playing a key role in population dynamics and community structure and can provide important information about the stress of the environment, the food web structure and biodiversity (3,14,15). The impact of parasitism extends beyond the host as an individual or with its population; evidence suggests that parasites play an important role in structuring ecological communities (3,16,17). While there can be no doubt that particular fish parasite species and metrics describe the environmental conditions in the aquatic realm (impact/ecosystem bioindicators), the use of fish parasites as biological indicators of climate change is more unclear (15). In this study we demonstrated that parasitic protozoa can be used as biological indicators of climatic environmental changes, since there was a significant increase in the prevalence of parasites on the gills or fish feces after the most relevant drought in the last years in São Paulo, Brazil. We demonstrated that reducing the water level of the reservoir has increased the parasitic burden of fish, showing deterioration in water quality and damage to environmental health, so we reinforced that the parasites are an important indicator of environmental health.

Trichodinids are considered ectocommensals feeding on the skin surface and gill epithelium. Its mode of attachment on body surface and gill filaments may favor other infections (18). We found that trichodinids was the most dominant parasite in the gills and can be considered as an climate change indicator, since in all fish surveyed there was a considerable increase in the prevalence of this ciliated with the aggravation of water crisis that occurred during the study period, due to lack of rain. In Ibirapuera Lake, we observed a significant change since before the drought fish had no parasites and dry after all the fish had *Trichodina* sp. in their gills. There are many studies suggesting that *Trichodina* sp. could be an appropriate epibiont as a biological indicator of pollution, if a site is polluted organically (eutrophication), it means that there are more bacteria and more trichodinids. Mobiline peritrichous ciliates were used experimentally as biological indicators of water quality (19). The trichodinid prevalence and density being related with the bacterial biomass in the environment and can be a biological indicator to compare polluted and unpolluted areas (20). However, our results indicated an increase in parasitic levels in fish related to climate change, which is probably also related to water quality, what still has been little demonstrated by epidemiological studies as we realize.

Oreochromis sp. (tilápia) is known as one of the most important fish in the world pisciculture, thus justified their presence in different collections carried out this work. Its origin is African and has many physiological attributes that justify its popularity; among them we can highlight tolerance to climate variations in temperature, adaptability to different salinity, oxygenation and overpopulation, and their resistance to diseases and infections (18). For this reason it is the most abundance species in Ibirapuera Lake, but despite its natural resistance, climate change favored the increased prevalence of infection *Trichodina* sp. in fish after drought, emphasizing that to ensure the health of animal species is need the preservation of environmental health. On the other hand, because of *Oreochromis* sp. (tilápia) be considered as a fish highly resistant to extreme weather conditions, capture and analysis parasitological study facing climate change analysis is indicated completely, since the presence of parasites is indicative of environmental health.

The same was observed in Recreational Fishing Lake after the drought, and *Trichodina* sp. was the most prevalent ectoparasite. The Recreational Fishing Lake has different conditions from the other two observed reservoir. Being a privately owned aimed at recreational fishing, the reservoir conditions are artificially controlled to maintain constant water level and diseased fish are taken weekly. Nevertheless, the levels of their reservoirs also decreased after the drought and were reported by the owner increase of death in fish. Data that contribute to strengthening the observed higher prevalence of *Trichodina* sp. the gills as an indicator of climate change.

In the Biritiba Dam, which is a water supply reservoir for several cities in the state of São Paulo, the biological indicators of climate change observed were free-living amoebas (FLA), characterized as *Amoeba* sp. and *Entamoeba* sp., found in the feces of animals studied only after drought. FLA infecting freshwater and marine fish includes those described thus far as agents of fish diseases, associated with other disease conditions and isolated from organs of asymptomatic fish. FLA are widespread in nature and are normal inhabitants of freshwater microbial ecosystems (21,22). The FLA may feed on various microorganisms present in the aquatic biofilm, some of which may have an impact on human and animal health. The interaction of FLA with microorganisms can bring benefits to microorganisms which prevent intracellular digestion and escape phagocytosis, getting protected from environmental adversity (23). From a public health perspective, amoebae, and notably amoebal cysts, can be highly resistant to various physical and chemical stresses and can thus protect any intracellular microorganism from deleterious environmental conditions that would normally kill them (23,24). For this reason, the meeting FLA was important in the water reservoir that supplies several cities, since these protozoa as well as biological indicators of climate change can be carriers of pathogens to humans. *Astyanax* sp. are species of small and opportunistic as to food, with a wide variety of food composition and are considered generally as omnivores, with preference for insects and plants, thereby promoting various forms of endoparasites transmission. The rapid development, easy to adapt to feed and to thermal shock, the tetra has great potential for fish farming. Abiotic factors affect the abundance and the prevalence of parasites, among the main abiotic factors can be mentioned depth, habitat, ecological disturbances, pollution, community composition and temperature of hosts is one of the most important factors in relation parasite-host-environment. Our results suggest that *Astyanax* sp. fish has the greatest potential to study parasites such as climate change indicators for dry before they were negative for parasites after dry and were positive.

Parasites of the phylum Myxozoa (myxosporean) are commonly found infecting fish in natural environment and to create fresh or marine water systems around the world and some species are pathogenic to their hosts (18). Thus, in some situations, a particular parasite higher index may provide information on a specific form of environmental stress. Myxoporean the community's spottail shiners in the St Lawrence River were connected to urban wastewater Montreal city (25). They found that the prevalence of these parasites have increased in the area in May with organic pollution. We found myxoporean in fish created in Recreational Fishing Lake before the dry season and did not observe positive after drought. Therefore the conditions evaluated in this study, this group was not an indicator of environmental change. Although the number of animals infected by myxoporean has been down, it occurred in recreational fisheries, where parasitic prevalence was high. It is likely that the drought degraded environmental conditions may have been a limiting factor for the survival of these protozoa, so their absence can also be an indicator of environmental changes, reinforcing the idea that healthy environments provide healthy parasitic populations.

The fresh examination of gill smears and bowel removed stool revealed higher levels of parasitism than the histological analysis of tissues, especially because we have found free-living amoebae in the intestinal content, not associated with specific pathological lesions, so fresh examination was better for diagnosing parasitism.

In conclusion, the results of this study suggest that monitoring of the prevalence of parasites in fish by analyzing smears fresh of gills and fecal examination can be used as a biological indicator of the climate change. It is an easy and inexpensive method, which may be annexed water tanks. The incorporation of parasitology into environmental assessments and any biotic inventories should be encouraged strongly.

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Table 1. Average biometrics collected fish in Ibirapuera Lake, Biritiba Dam and Recreational Fishing considering weight and average length of examined species.

Local	Sample	Fish number	Average weight g (± SD) Before	Average length cm (± SD) Before
Ibirapuera Lake	*Oreochromis niloticus*	46	619.1 (± 291.4)	36.3 (± 0.8)
	Hypostomus affinis	4	934.7 (± 387.4)	60 (± 1.6)
Biritiba Dam	*Astianax bimaculatus*	40	30.7 (± 8.8)	13.2 (± 0.3)
	Crenicichla lenticulata	10	245.2 (± 103.7)	37.8(± 0.38)
Recreational Fishing	*Oreochromis niloticus*	35	100.8 (± 42.6)	15.6 (± 0.45)
	Geophagus brasiliensis	15	20.5 (± 4.4)	11.9 (± 0.8)

Table 2. Parasite prevalence found by fresh examination or histopathological analysis before and after the dry period in Recreational Fishing Lake, Ibirapuera Lake and Biritiba Dam.

Local	**Parasite by fresh examination***		**Parasites by histopathology****	
	Phase one (before drought) Positive number/total (%)	Phase two (after drought) Positive number/total (%)	Phase one (before drought) Positive number/total (%)	Phase two (after drought) Positive number/total (%)
Ibirapuera lake	0/20 (0.0)	30/30 (100)[a]	0/20 (0.0)	30/30 (100)[a]
Biritiba Dam	5/20 (25.0)	30/30 (100)[a]	0/20 (0.0)	7/30 (23.4)[a]
Recreational fishing lake	18/23 (78.2)	27/27 (100)[a]	20/23 (87.0)	27/27 (100)
Total	23/63 (36.5)	87/87 (100)[a]	20/63 (31.8)	64/87 (73.5)[a]

*Fresh examination: it included the smear of gills and direct examination of intestinal contents.

**Histopathology: included the search of parasites in the gills and intestines.

a. Statistically significant difference P <0.1, Mann-Whitney test.

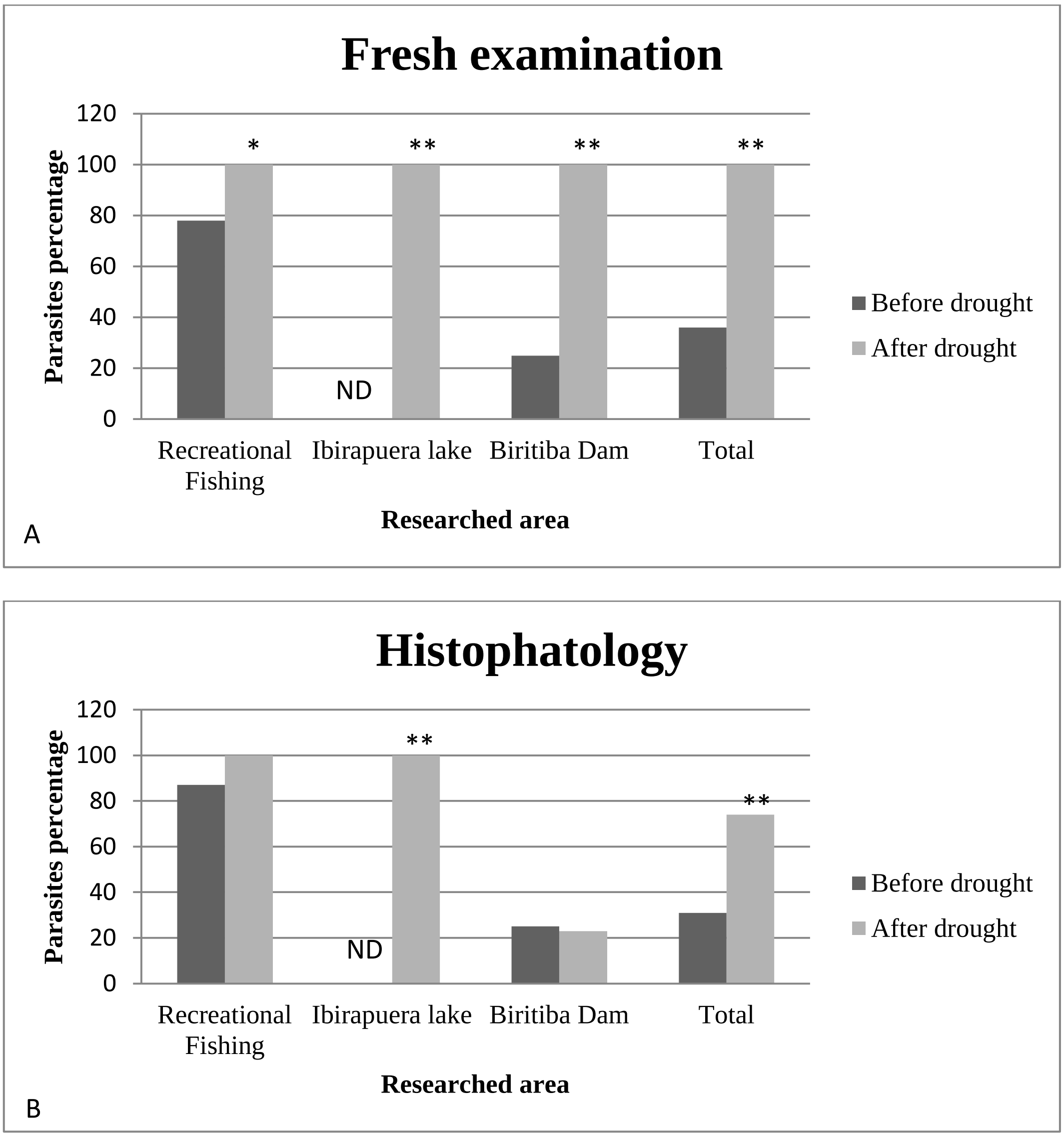

Figure 1. Parasites percentage observed in fresh examination (A) and by histopathology (B) of fish before and after dry period in all studied areas.

ND – Not identified

** Statistically significant difference P<0.1, Mann-Whitney test.

Table 3. Parasites found in fishes of the Recreational Fishing Lake, Ibirapuera Lake and Biritiba Dam before and after drought.

	Parasites	**Positive number before drought/total (%)**	**Positive number after drought/total (%)**	**Sample**
Ibirapuera Lake	*Trichodina* sp.	-	30/30 (100)	Gill
	Monogenea	-	7/30 (23.3)	Gill
	Epistylis sp.	-	1/30 (3.3)	Skin lesion
Biritiba Dam	*Amoebae sp.*	-	20/30 (66.7)	Intestinal contents
	Entamoeba sp.	-	20/30 (66.7)	Intestinal contents
	Balantidium sp.	-	4/30 (13.3)	Intestinal contents
	Piscinoodinium sp.	-	2/30 (6.6)	Gill
	Trematodes eggs	5/20 (25)	2/30 (6.6)	Intestinal contents
	Monogenea	-	7/30 (23.3)	Gill
Recreational Fishing Lake	*Trichodina* sp.	18/23 (78.2)	27/27 (100)	Gill
	Myxobolus sp.	3/23 (13)	-	Gill
	Henneguya sp.	9/23 (39.1)	4/27 (14.8)	Gill
	Ichthyophthirius multifiliis	2/23 (8.7)	-	Gill
	Monogenea	-	2/27 (7.4)	Gill

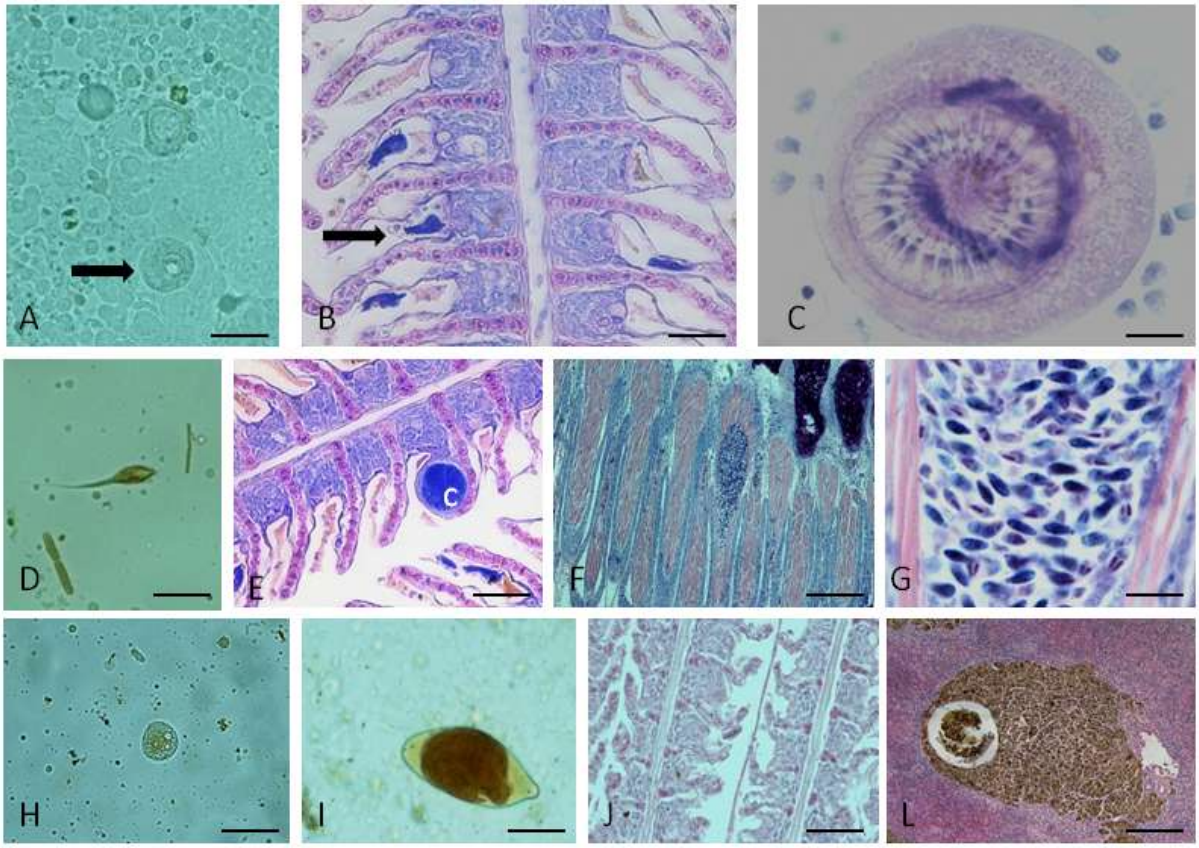

Figure 2. A- *Trichodina* sp. on the gill of *Oreochromis niloticus* from Ibirapuera Lake by fresh examination (Scale bar=40 µm). B- Cross-section of gill showing *Trichodina* sp. between the secondary lamellae (Giemsa stain, Scale bar=40 µm). C- Detail of *Trichodina* sp. (Giemsa stain, Scale bar=5 µm). D- Typical *Henneguya* sp. myxospore isolated from gills of *Geophagus brasiliensis* (Scale bar=10 µm). E- Cross-section of gill showing *Henneguya* sp. cyst (c) (Scale bar = 50 µm). F- Large cyst of *Myxobolus* sp. in the muscle associated with the branchial arch of *Geophagus brasiliensis* (Giemsa stain, Scale bar=150 µm). G- Detail of *Myxobolus* sp spore (Giemsa stain, Scale bar=20 µm). H- Amoeba cysts by fresh examination (Scale bar=20 µm). I- Trematodes eggs (Scale bar=20 µm). J- Cross-section of gill showing hyperplasia and secondary lamellae fusion (HE, Scale bar=150 µm). L- Cross-section of spleen showing inflammatory infiltrate with a predominance of melanomacrophages (HE, Scale bar=150 µm).