



ECOSYSTEMS

The influence of environmental factors on *Clinostomum* sp. (Digenea) infection in the fish *Cichlasoma paranaense* (Kullander, 1983) in Central Brazil

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Abstract: This study analyzed the parasitism by *Clinostomum* sp. metacercariae of the neotropical fish *Cichlasoma paranaense* (Kullander 1983) and environmental variables' influence on their host-parasite relationship in Central Brazil. Fish were collected in five impoundments (I) from three towns: Itauçú (n = 2), Inhumas (n = 1), and Goiânia (n = 2), Goiás State, Brazil. Ninety-five fish were analyzed, weighted and length measured. Afterward, the presence of metacercariae in the gills, muscles, fins, and body cavities was investigated. The metacercariae were fixed in AFA solution, kept in 70% alcohol, and stained with carmine. The infection prevalence revealed that 0.64, 0.56 and 0.91% of fish was infected. The abundance was 4.14, 3.22 and 5.88, with a mean intensity of 6.44, 5.80 and 6.42 per impoundment (I 13, I 15 and I 16), respectively. The frequency of metacercariae was higher in fish collected in Goiânia. High frequency of parasites was observed in the gills, muscles, and fins. The limnological variables did not present direct interference in the parasitism. The fish' standard length influenced the number of metacercariae positively but did not differ to the relative condition coefficient.

Key words: Digenean, host fish, host-parasite relationship, limnological variables, metacercariae.

INTRODUCTION

Trematode parasites of the genus *Clinostomum* (Leidy 1856) are widespread and are described considering different approaches such as ecological, epidemiological, evolutionary, genetic, molecular, morphological, taxonomic, and others (Bonett et al. 2011, Caffara et al. 2014, 2017, Calhoun et al. 2019, Chai & Jung 2019, Nicola et al. 2020, Rizvi et al. 2020). Even so, the dynamic of their host-parasite relationship is not totally revealed (Calhoun et al. 2019).

Clinostomum sp. (Digenea) is a parasite that causes a zoonosis known as clinostomiasis (Lee et al. 2017). Some species require two or more

hosts during their life cycle (Kohn et al. 2013). In *Clinostomum complanatum*, the cycle begins when piscivorous birds, such as cormorants, socós and herons, containing the adult parasites housed in the digestive system, eliminate embryonic eggs in the water through the feces. These eggs reach susceptible aquatic mollusks, generating cercariae released into the water, which penetrate the fish where they become metacercariae. The cycle is completed when birds ingest fish infected with these encysted larval forms in the dermis, muscles, gills, or intestine (Bullard & Overstreet 2008, Pavanelli et al. 2015). This trematode can affect the economy

by causing damages that may compromise fish meat quality and human health (Sutili et al. 2014). Although *Clinostomum* sp. metacercariae have already been found in several Brazilian freshwater fish and fish farms, there are no evidence of human infection with *Clinostomum* sp. in Brazil (Pavanelli et al. 2013).

In eutrophic aquatic or anthropogenic impacted environments, parasite fauna and fish health may be affected, causing an increase or decrease in the abundance and/or richness of the parasites in the fish host (Silva-Souza et al. 2003). Thus, one way of assessing “the health of the environment” is by studying the parasitic fauna in fish, which responds differently to environmental variations (Sures 2003).

Despite a large number of freshwater fish species in Brazil, there are only a few researches of parasitism by the larvae of Clinostomidae in fish (Silva-Souza & Ludwig 2005). This paper was the first to describe *Clinostomum* sp. presence in eyes, gills, muscle, fins, and body cavities of the fish *Cichlasoma paranaense* (Kullander 1983), which is a cichlid fish (Cichliformes: Cichlidae) that may be employed as a food source in the Upper Paraná River Basin, Goiás State, Central Brazil. Hereby, it was determined the metacercariae prevalence, abundance, and mean intensity in this fish species. Besides, we assessed the environmental variables and their relationship with the parasite life cycle. This study is relevant for understanding the biology, biodiversity, and host relation of these parasites in South America.

MATERIALS AND METHODS

Ethical approval and Informed Consent

Animal care and handling were carried out following institutional guidelines according to Brazilian laws by a Permanent License for Collections of Zoological Material (number

34144-1) by the Biodiversity Authorization and Information System – SISBIO.

Sampling protocols

Cichlasoma paranaense (n = 90) were sampled from three impoundments in the rural area in Itauçú and Inhumas (identified as impoundment I07, I12 and I13) and sixty fish from two impoundments in the rural area in Goiânia (I15 and I16), Goiás State, Brazil (Figure 1). Fish were collected between 30 May and 6 June 2017 (dry season) using fishnets (5 mm, 20 mm, and 25 mm). The animals were anesthetized with clove oil solution (90 mg L⁻¹) for 10 minutes (Simões & Gomes 2009). They were subsequently stored in cooler boxes containing ice and kept in a freezer (- 14 °C). Then, total weight (W), standard length (SL) and total length (TL) were measured.

Analysis of parasitism

The encysted metacercariae were removed from the gills, muscles, fins, and intestine using a stereomicroscope (LEICA MZ 9.5, Wetzlar, Germany). The metacercariae were extracted from the cysts using small needles. The parasites were then mounted with a coverslip to flatten the specimens. The fixative solution Alcohol-Formaldehyde-Acetic Acid - AFA (85 mL of ethanol, 25 mL of formaldehyde, and 5 mL of acetic acid) was added drop by drop to the edge of the coverslip to fix the parasites for 90 minutes then transferred to 70% alcohol. Subsequently, the metacercariae were stained with carmine (Eiras et al. 2006). The parasites were observed on an optical microscope to perform morphological descriptions. The taxonomic identification of the metacercariae was based on previous study (Thatcher 2006). The prevalence, abundance and mean intensity host were determined according to Bush et al. (1997).

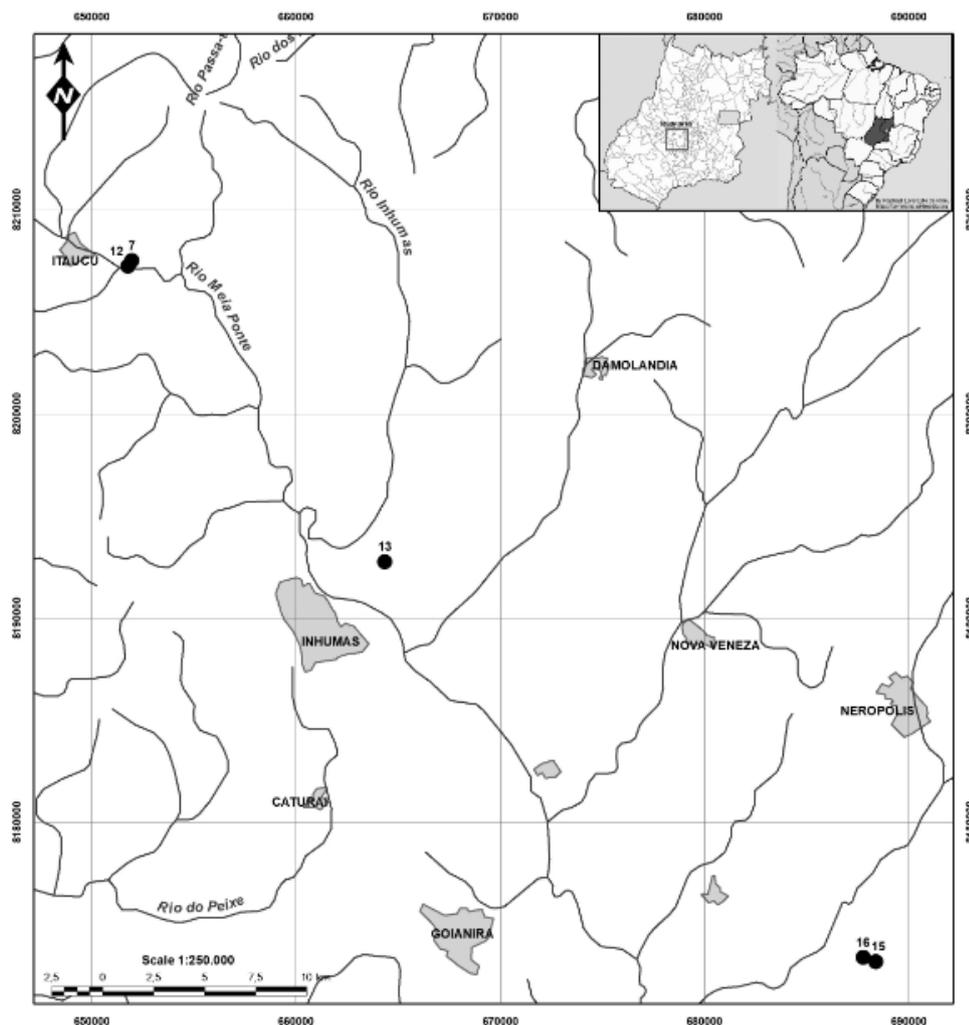


Figure 1. Map of the location of Itauçu, Inhumas and Goiânia, Goiás, Brazil.

Environmental characterization

To obtain a characterization of the local environment measurements of each impoundment, ecological variables associated with native vegetation were achieved, such as percentage of total vegetation coverage inside the impoundments (*Cint*) and on the impoundment edge (*Cmar*), and percentage of the soil with forest vegetation (*Sflo*) within a radius of 500 meters around the impoundment. In addition, we have measured limnological variables: temperature (Temp, °C), dissolved oxygen (OD, %), conductivity (Cond, $\mu\text{s}/\text{cm}$) and pH using a multiparameter probe model HI9829.

Statistical analysis

A Principal Component Analysis (PCA) was used to describe the local structure of the impoundments. The main components' selection with the greatest contribution to be used as a predictor variable was performed by using the Broken-Stick method (Jackson 1993). The entire procedure was performed using the "prcomp function" of the "stats" package in the R software (R Core Team 2018).

The Shapiro-Wilk test was employed to assess the normality of the data. Due to the heterogeneous structure and no normality of the data, mainly non-parametric analyses were used to test the proposed hypothesis.

The variation in the data was reduced using the logarithmic transformation ($\text{Log} + 1$). The Kruskal-Wallis (H) test was used to verify the differences in metacercariae prevalence between the impoundments and between the organs analyzed in the fish. Post-hoc Bonferroni tests were employed to verify differences between pairs of ponds and organs independently. This test was used to avoid type I error and reduce false positives. The relationship between the prevalence of metacercariae and the limnological variables of the impoundments was verified by Spearman's correlation analyses.

Initially, the SL and W values were projected on scatter plots considering SL as an independent variable and W as a dependent one to assess the body fish condition. The adjustment of the W/SL ratio curve ($W = a \cdot \text{SL}^b$) and the estimation of the values of a (linear coefficient or intercept), b (slope/gradient) and R^2 (coefficient of determination) were obtained using the potential regression (Jobling 2008). The slope (b) generated by the regression was used as an indicator of the type of growth and interpreted as follows: (b) = 3, the growth was isometric; (b) > 3, the growth was positive allometric; and when (b) < 3, the growth was of the negative allometric type. The values of a and b were subsequently used to calculate the Relative Condition Factor (Kr) (Le Cren 1951, Froese 2006).

Since the biometric variables (W, SL, and TL) of the fish were highly correlated ($r > 0.88$), we decided to use only the SL in the following analyses. The SL values were standardized using the logarithmic transformation. Differences in SL and Kr between the impoundments were verified using Kruskal-Wallis Analysis of Variance, as previously described. The relationship between Kr and the lake's limnological variables was verified by Spearman's correlation analyses. Simple linear regressions were performed to

evaluate the influence of SL on the parasitic load (number of metacercariae) and the effect of the parasitic load on Kr.

RESULTS

In this study, 55 fish (57.89%) were parasitized by *Clinostomum* sp. metacercariae. The parasitic load varied from 1 to 32 (6.16 ± 5.90) metacercariae by parasitized fish. The number of metacercariae per fish differed between impoundment (H = 14.08; $p < 0.01$) and was higher in I16 (mean = 5.88 ± 5.75) compared to I15 (mean = 3.22 ± 6.51 ; $p < 0.01$) (Figure 2a). Impoundment I13 (mean = 4.14 ± 5.87) did not differ from the others ($p > 0.05$). Fish collected in the I07 and I12 impoundments were not parasitized. No significant correlation was observed between environmental variables (vegetation structure and limnology) and the prevalence of metacercariae ($p < 0.05$).

Regarding organ parasitism (Figure 2b), it was observed that the number of metacercariae differed (H = 48.39; $p < 0.01$; gl = 2), with the highest parasitic load occurring, respectively, in the gills, muscles and fins ($p < 0.05$) in relation to the other organs. The SL varied between sampled impoundments (H = 12.98; $p = 0.01$; gl = 4) (Figure 3a), and positively influenced the number of metacercariae present in the individuals ($R^2 = 0.36$; $p = 0.03$; gl = 93) (Figure 3b).

The weight-length ratio indicated negative allometric growth when all specimens were considered ($y = 0.1033x^{2.6604}$), with a lower evaluation in parasitized specimens ($y = 0.1995x^{2.3224}$) compared to non-parasitized specimens ($y = 0.082x^{2.7867}$) (Figure 3a and b). In fact, Kr varied between the sampled impoundments (H = 25.72; $p < 0.01$; gl = 4) (Figure 3c) and was negatively correlated with the temperature variables ($r = -0.87$; $p = 0.05$; gl = 4) (Figure 3d) and pH ($r = -0.87$; $p = 0.05$; gl = 4) (Figure 3e); however, Kr was not significantly

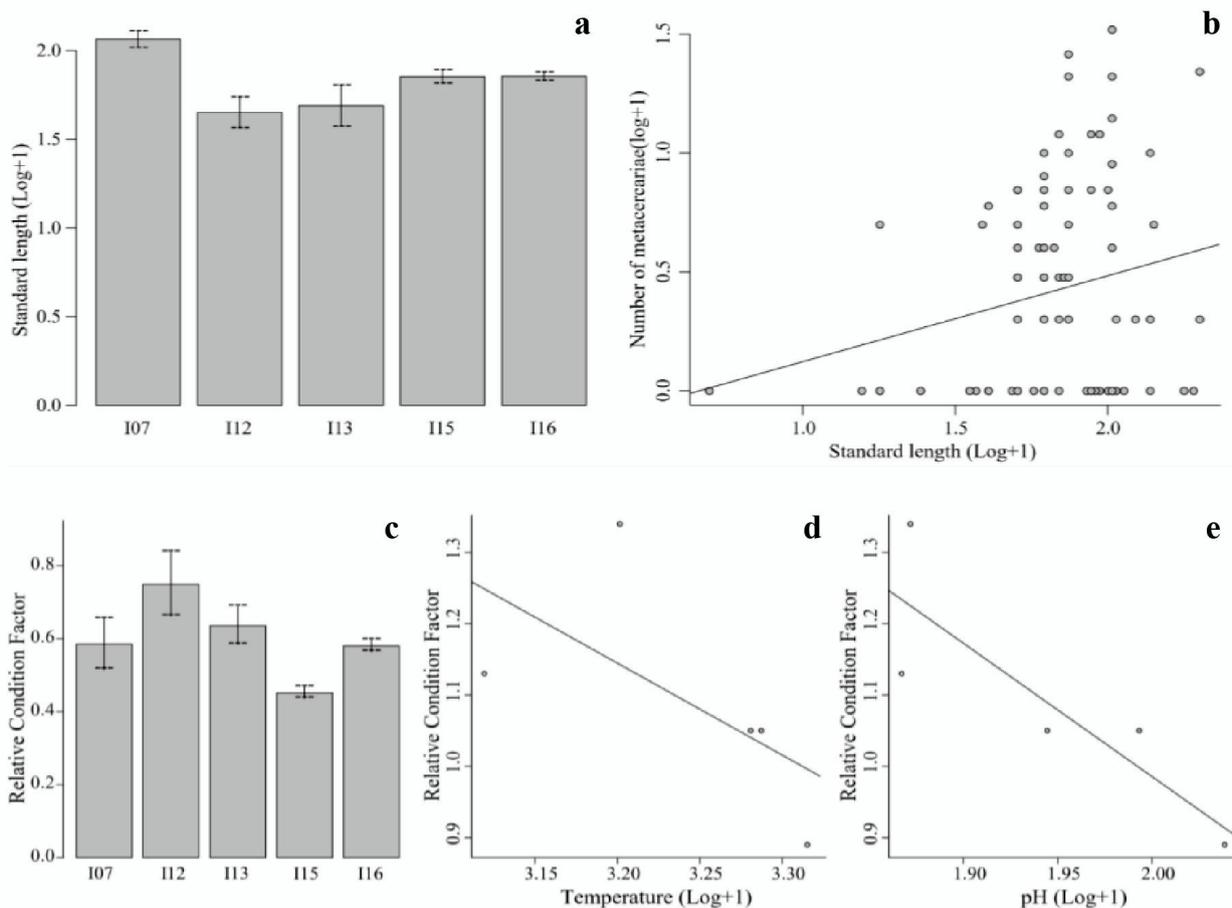


Figure 3. Variation (a) and the influence of standard length on the number of metacercariae (b). Variation (c) and influence of temperature (d) and pH (e) on the relative condition factor of *Cichlasoma paranaense* in impoundments sampled in Itauçu (I07, I12), Inhumas (I13) and Goiânia (I15 and I16), Goiás, Brazil.

metacercariae have already been found in several fish species in Brazil and other countries in South America (Bozza & Hahn 2010, Eiras et al. 2010, Pavanelli et al. 2013, Sohn et al. 2019). In the Nakdong-Gang River in Korea, the prevalence of the *Clinostomum* spp. metacercariae in fish was 19.3 to 97.7%, and the parasite intensity varied between 1.5 to 27.0. These data reinforce the use of different fish species as the second intermediate hosts of *Clinostomum* sp. Besides, it was noted that there is endemism of *Clinostomum* metacercariae in different hosts and the same area (Sohn et al. 2019). *C. paranaense* is a fish species with an omnivorous feeding habit, being able to feed on small

insects, worms, crustaceans, and secondarily on vegetable material. This result shows that their feed habits may increase the chances to become an intermediate host of several parasites, including *Clinostomum* sp. (Bozza & Hahn 2010).

Nicola et al. (2020) showed a possible preference of *Clinostomum* sp. for *Loricariichthys platymetopon* (Loricariidae) due to a prevalence of 77.7% and an average intensity of 87 parasites per fish. The authors indicate that the fish physiological adaptations favor its predation by birds, being interpreted as an evolutionary run and success of the parasite in manipulating its host.

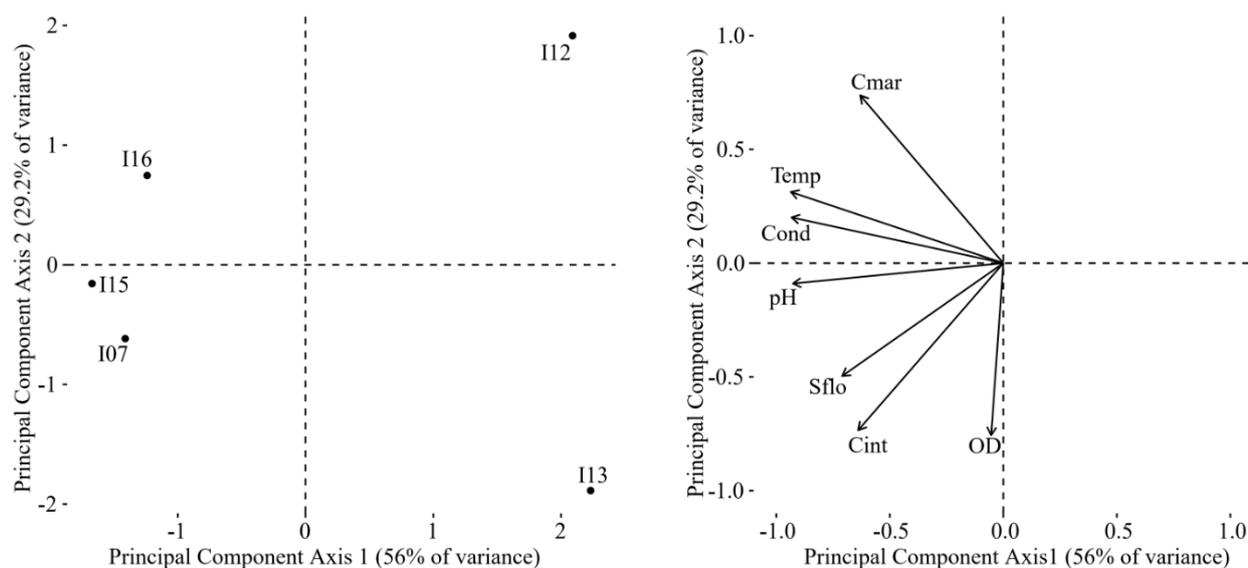


Figure 4. Biplot of the Principal Component Analysis (PCA) generated from the environmental variables obtained from five impoundments in Itauçu (I07, I12), Inhumas (I13) and Goiânia (I15 and I16), Goiás, Brazil. Environmental variables: Cint = percentage of vegetation cover –inland-, Cmar = percentage of vegetation cover –at the edge-, Sflo = percentage of soil with forest vegetation within a radius of 500 meters, Temp = temperature, Cond = conductivity, OD = dissolved oxygen.

When evaluating parasitism by organs, the gills, muscles and fins showed higher prevalence, respectively. *Clinostomum* metacercariae have been found in the muscles, fins, operculum, visceral cavity and mouth of several species of fish from the Paraná River (Dias et al. 2003), as well as, in the oral cavity, eyes and skin of cichlid fish (Santos et al. 2012). The parasitological survey of 262 wild cichlids fish sampled from in Lake Kinneret (Israel) presented metacercariae of *C. phalacrocoracis* in 18 fingerlings as reported by Caffara et al. (2014). Also, *Clinostomum* spp. metacercariae were found in the anterior part of the abdominal cavity, muscle, and gill arches.

The metacercariae of *Clinostomum* sp. encysted in the fins of *L. platymetopon* resulted in degeneration and destruction of muscle fibers; as a consequence, movements have become slower (Eiras et al. 1999). Fish with high parasitic intensity are lethargic when compared to hosts with low intensity of infection, resulting in impaired swimming ability (Nicola et al. 2020), mobility, search for food and ability to escape

from predators (Montes et al. 2020). In addition, metacercaria infections can impaired growth, which affects the development and survival of the fish, also causes morphological changes, making the animal look non-appreciable for human consumption and therefore requiring the disposal of these individuals (Echi et al. 2012, Pavanelli et al. 2015). The histopathological analysis results showed that the muscle of the cichlid *Crenicichla vittata* (Heckel 1840) was the most affected tissue caused by the presence of the metacercariae. The *Clinostomum* metacercaria into the fascia of the muscle tissue showed abundant leukocyte infiltration, desquamation cells, connective tissue with lymphocytic infiltration, abundant cellular and pigment granules (Montes et al. 2020).

We observed that the standard length of the animal influenced the number of metacercariae per fish. A positive correlation between the prevalence of parasite species and host length can be attributed to behavioral or trophic changes over the life of endoparasites

(Kalantan et al. 1987). Literature showed varied responses in the relationship between fish length and parasitism, which can present a positive correlation as observed in *Oreochromis mossambicus* parasitized by *Clinostomum* metacercariae (Mutengu & Mhlanga 2018) and *Semaprochilodus insignis* parasitized by monogeneans and nematodes in Central Amazonia (Silva et al. 2011); negative correlation verified in *Oreochromis niloticus* parasitized by the monogenea *Cichlidogyrus sclerosus* in fish farms in the State of São Paulo/Brazil (Lizama et al. 2007). The absence of correlation was also observed in *Plagioscion squamosissimus* parasitized by *Diplostomum* sp. in the lower Rio Claro (Silva et al. 2018) and *Leporinus lacustris* and its ectoparasites in the upper Paraná (Guidelli et al. 2006).

In addition, the condition factor (K_r) is a data widely used in analyses of the parasite's influence on fish welfare (Le Cren 1951). In all cases, the negative allometric growth pattern contrasts with the expected positive allometric growth pattern for the studied species (Froese & Pauly 2018). It indicates that regional or inherent factors of the impoundment (e.g., resource availability, negative biotic interactions) may have caused such a growth pattern. Thus, the results showed that the variations in K_r of the analyzed fish occurred independently of parasitism by *Clinostomum* sp. By means of K_r, it is possible to assess the animals' welfare by considering the relationship between expected weight, observed weight and body size of the fish, as well as the existence of variations caused by environmental changes, food scarcity, or by parasitism (Le Cren 1951).

Parasitism in fish may be related to several aspects, such as geographic factors, age, trophic link or host diet, seasons, and environmental factors, such as water chemical composition and also characteristics of the native habitat

(Pavanelli et al. 2013). Therefore, we also assessed the environmental variables and their relationship with this parasite's presence in some impoundment in Central Brazil. Our findings contribute to the understanding of the biology, biodiversity, and host preferences of these parasites.

However, despite the inconsistency in the relationship with the limnological variables, possibly due to the low number of explored impoundments, the results show evidence that justifies such variation. For example, the temperature is a relevant factor in longevity, infectivity, and rate of development of larval stages and adult stage of trematode parasites, in addition to inducing behavioral changes and host abundance (Souza et al. 2008). The increase in temperature due to climatic changes influences the life cycle of *C. complanatum* from the evaporation of water that results puddles of cold mud and a reduction in the volume of water in the aquatic environment. These phenomena make the habitat favourable for intermediate host snails and increase the chances of the parasite infecting fish (Rizvi et al. 2020). In our study, ponds that presented higher temperatures (I15 and I16) had fish with a higher parasitic load of *Clinostomum* sp. In addition, other factors (e.g., frequency of birds in each pond) may also have influenced parasitism in *C. paranaense* and should be considered in the design of future studies. The host immune system's responses are another important factor that influences the parasite-host relationship (Mignatti et al. 2016).

Regarding the maintenance of the cycle of parasites of the genus *Clinostomum*, freshwater snails act as the first intermediate host. Studies of the *C. complanatum* cycle in the Paraná River floodplain pointed to the snail *Biomphalaria peregrina* as the first host, several species of fish as the second intermediate host and some

species of water birds as definitive hosts (Dias et al. 2003). Shells of gastropod mollusks, similar to those of *B. peregrina*, were observed in I16 during the fish collection, reinforcing the maintenance of the parasite cycle of this genus on site. Due to the complexity of the life cycle of digenetic parasites, new studies are needed to help understand the contributions of the environment, the parasite and the hosts to infection's success.

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Y.R.R.S. assisted in performing experiments, analyzed the data and wrote the manuscript. A.B.L. assisted in performing experiments. W.P.R. assisted in fish sampling, performed data analysis and interpretation. M.B.C. assisted in fish sampling and performing experiments. T.L.R. contributed to the design and implementation of the research and revised the manuscript for intellectual content. J.Y.S. assisted in performing experiments and stained the metacercariae. M.I.G.M. contributed to the writing and translation of the manuscript. L.D.S. designed the study, analyzed the data, and supervised the project.

