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PRIMARY RESEARCH PAPER

The importance of protists as a food resource for *Astyanax lacustris* (Osteichthyes, Characiformes) larvae at different stages of development

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Abstract Investigating the main factors regulating the growth and survival of fish in early stages of development is essential for the understanding of their trophic relationships. Most fish larvae are active planktonic predators which feed on motile prey and their success in foraging is influenced by several factors, such as the availability of suitable prey. We aimed to investigate the role of ciliate protists as a food resource in the different stages of development of fish larvae of *Astyanax lacustris*, when presented to a natural prey assembly. Our results show, through an experimental approach, that ciliate protists are an important item in *A. lacustris* larvae diet, especially in the early stages of their development, becoming an additional resource in more advanced stages, when *A. lacustris* larvae change their food preference, selecting larger food items such as adult copepods.

Keywords Clearance rates · Ingestion rates · Protozoa · Ichthyoplankton · Larvae fish · Selectivity

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Introduction

In ecological studies, knowledge about the main factors regulating the growth and survival of fish in early stages of development is essential for the understanding of their trophic relationships as well as conservation of fishery resources (von Herbing et al., 2001; Santin et al., 2005; Picapedra et al., 2018). Omnivorous fish are dominant in lakes of tropical environments and their main cause of mortality occurs due to limited food resources (Leggett & Deblois, 1994) mainly during their initial development (von Herbing & Gallager, 2000). Studies have shown that

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less than 1% of fish larvae reach the juvenile stages in natural environments and survival rates are even lower for adulthood (Hjort, 1914; Sinclair, 1988).

Fish ontogeny can be characterized by a series of vulnerable periods (Figueiredo et al., 2007). After the hatching of eggs, the successful transition from endogenous feeding (vitelline sac consumption) to exogenous (dependent on external prey) determines the larvae survival, a phase known as the *critical period*, where the highest mortality rates are observed (Hjort, 1914; Kamler, 1992). Most fish larvae are active planktonic predators, which feed on motile prey, and their success in foraging is influenced by several factors, such as the development of organs necessary for feeding, mouth size of larvae, physical conditions of the environment, the escape response of prey, and, especially, the availability of suitable prey (Gallager et al., 1996; Sánchez-Velasco, 1998; Figueiredo et al., 2007; Lehtiniemi et al., 2007).

Many studies have shown that zooplanktonic organisms, mainly microcrustaceans, such as cladocerans and copepods, are the main prey responsible for the survival and growth of ichthyoplankton (Reiss et al., 2005; Dickmann et al., 2007; Lehtiniemi et al., 2007; Martin et al., 2012). However, in early stages of development (i.e. shortly after egg hatching and vitelline sac consumption), many species of fish are still rudimentary, with restricted swimming capacity, and are unable to catch fast-moving prey (von Herbing & Gallager, 2000; Makrakis et al., 2005; Montagnes et al., 2010). Thus, smaller, slower, more abundant organisms, such as ciliated protists, may allow the larvae to survive and grow until their swimming capacity and buccal opening allow them to feed on larger items (Buskey et al., 1993; Nagano et al., 2000; Figueiredo et al., 2007; Zingel et al., 2012, 2019). In addition, some larvae may have a low digestive capacity in their early stages of development, and protists are more easily assimilated than copepods and nauplii (Nagano et al., 2000; Figueiredo et al., 2007). As larvae develop, there is an increase in the capture of their prey and in the variety of food items (von Herbing & Gallager, 2000; Neves et al., 2015) expanding the trophic niche (Pepin & Penney, 1997). Larger and faster larvae are able to control their approach during prey hunting, decreasing the likelihood of detection (Kiørboe & Visser, 1999), and selecting more energetically advantageous organisms, either by quantity (larger size organisms) or energy quality (Stein, 1977).

Considering that the ecology of fish larvae is poorly studied when compared to that of adult individuals (Montagnes et al., 2010; Nunn et al., 2012; Zingel et al., 2019), investigate foraging strategies in the early stages of life (Lehtiniemi et al., 2007) and the interaction between the ichthyoplankton and its prey, can help to understand the survival and growth of fish larvae as well as functioning of the ecosystem (Cushing, 1990). The contribution of protists to the diet and growth of ichthyoplankton is still underestimated (Montagnes et al., 2010) and studies approaching this trophic link are still incipient, especially in freshwater environments. We aimed to experimentally investigate the relative importance of ciliate protists as a food item in the different stages of larval development of *Astyanax lacustris* (Lütken, 1875) in relation to other planktonic components (rotifers, nauplii, copepods, cladocerans). We chose this species because of their wide geographic distribution in South America (Nakatani et al., 2001; Sato et al., 2006) and importance as prey for top predator fish (Santin et al., 2005; Novakowski et al., 2007). We hypothesize that ciliates are an important food resource for larvae of *A. lacustris*, contributing fundamentally to their diet in the early stages of larval development and becoming an additional resource in the more advanced stages. Thus, we expected that (i) *A. lacustris* larvae would show a diet based mainly on ciliated protists in their pre-flexion stage (4 days after hatching), since they are still rudimentary, with limited swimming capacity and have a small mouth opening; (ii) at the flexion stage (13 days after hatching), their diet would be composed of both ciliates and zooplankton; and (iii) In the post-flexion stage (33 days after hatching) larvae would have a diet based mainly on larger food resources, such as adult copepods, and ciliates would be an additional resource at this stage.

Materials and methods

The larvae used in the experiments were cultivated in the laboratory through spawning induced by hormonal process. For this process 20 females and 20 adult males were separated, which remained fasted for 24 h before the procedure. The spawning was performed according to the protocol of Woynarovich and

Horváth (1983), in which carp pituitary extract (EHC) was applied in single doses in females (5.0 mg kg^{-1}) and males (2.5 mg kg^{-1}). Subsequently, the fish couples were placed in vats covered with a 70% shade blanket and maintained at 26 °C with thermostats. In cases where semi-natural spawning did not occur, manual oocyte extrusion was performed and fertilization was performed by the *dry* method.

Three induced spawning were performed in order to obtain the larvae at the developmental stages required for the experiment. After spawning, the larvae were kept in aquaria and fed with plankton by replacing 50% of the water in the aquariums with water from a container without fish twice a day. Nauplii of *Artemia* spp. were added to supplement the diet of larvae obtained in the first spawning, 15 days after egg hatching.

The community of plankton prey used in the experiment was obtained by collecting water from the subsurface of artificial lakes in a fishing village, located in the municipality of Marialva—PR, Brazil (23° 27′ 36.3″ S 51° 49′ 39.5″ W). The water was stored in gallons of 20 l and taken to the laboratory, where it was mixed in a single container (60 l) in order to homogenize the plankton. All larvae were acclimatized for 18 h in aquariums containing the water obtained from the container before the beginning of the experiments.

The physical and chemical variables of the water used were measured before the beginning of the experiment: water temperature 26.07 °C, pH 6.43, turbidity 15.0 (NTU), conductivity 245.0 µS cm^{-1} and dissolved oxygen 10.5 mg l^{-1}.

Feed experiment

Four treatments were established, each containing larvae at different stages of development: (i) pre-flexion larvae (4 days after hatching), (ii) larvae at the flexion stage (13 days after hatching) and (iii) larvae at the post-flexion stage (33 days after hatching), in addition to the control (in the absence of fish larvae). All treatments were performed in four replicates, except for the control that consisted of eight replicates, four for the initial time and four for the final time of the experiment.

The number of larvae per aquarium was standardized, but different for each stage of development, with 20 individuals being added for the pre-flexion stage treatment, 10 for flexion and 8 for post-flexion. All the larvae used were fasted for 8 h prior to the start of the experiments, being kept in the same water used in the experiment, previously filtered in a 5 µm mesh in order to remove their possible prey (big ciliate protists and zooplankton).

Transparent aquariums with circular border and capacity of 2 l were filled with 1.5 l of the water containing the natural prey assembly and kept at a constant temperature of 26 °C throughout the experiment with the use of thermostats. The volume of water in the aquarium was based in the previous study by Friedenberg et al. (2012). In addition, the aquariums were kept oxygenated during the experiment through the use of aerators. The experiment was conducted in the morning and lasted for 2 h, the incubation time being determined based on previous studies (Stoecker & Govoni, 1984; Ohman et al., 1991; Pryor & Epifanio, 1993).

The beginning of the experiment was marked with the simultaneous insertion of the larvae to the treatments. Samples were collected at the beginning of the experiment (t_i), where the control flasks were sampled for the initial estimate of prey abundance, and at the end of the experiment (t_f) when all the water contained in the aquaria was sampled and the larvae preserved. No larval mortality was found during the experiments. Besides that, due to the short time of the experiment (2 h) there were no changes in the physical and chemical variables of the water at the end of the experiment.

Laboratory analysis

At the end of the experiments, the larvae were placed in small flasks with water and anesthetized by adding benzocaine prepared in solution with alcohol (concentration of 100 g l^{-1}), until total loss of movement, the individuals remaining immersed in the solution for at least 10 min before being fixed in 10% formaldehyde. Measurements of standard length, total length, length of the oral cleft, length of the snout, diameter of the head, length of the head, height of the body, pre-anal myomers and post-anal myomers were obtained through the fixed larvae, according to Ahlstrom et al. (1976).

To estimate ciliate and zooplankton abundance, the water in each aquarium was concentrated at the beginning and end of the experiment in 100 ml with a 5 µm pore size mesh and fixed with 1% Lugol acetic solution. The ciliates were counted under inverted microscope (Olympus® CK40) by the sedimentation

of 10 ml of the sample in Utermöhl chambers (1958), and identified to the lowest taxonomic level possible (Foissner et al., 1999; Foissner & Berger, 1996). Zooplankton was quantified under a common optical microscope (Olympus® CX31) and identified to the lowest taxonomic level possible (Koste, 1978; Reid 1985; Elmoor-Loureiro, 2004).

Feeding rates of larvae and selectivity

To measure the feeding rates of the larvae we calculated the clearance rates (ml larva^{-1} h^{-1}), that were calculated using the changes in the total prey clearance in each treatment and the ingestion rates (cells ingested larva^{-1} h^{-1}) for each group of prey and in each treatment (Frost, 1972). The ingestion rate calculations are traditional measures of selective feeding and are based on changes in the abundance and/or biomass of prey at the end of the experiment in relation to their growth in the controls (absence of predators), being ideal to estimate the selection of food resources in experimental approaches (Frost, 1972; Marin et al., 1986; Rollwagen-Bollens & Penry, 2003; Friedenberg et al., 2012).

The clearance rates (C) (ml larva^{-1} h^{-1}) were calculated using the formula: $C = V * g/N$, where V = aquarium volume (ml), g = consumption coefficient and N = number of predators in the aquarium. The consumption coefficient (g) was calculated for each aquarium containing predators (larvae of *A. lacustris*), using the equation: $C_2' = C_1' * e^{(k-g)*(t_2-t_1)}$, where C_1' and C_2' are the abundance of predators in the aquariums (ind l^{-1}) at times t_1 and t_2. The constant cell growth coefficient (k) was calculated to estimate the growth of each group of prey every hour, using the formula: $C_2 = C_1 * e^{k(t_2-t_1)}$, where C_1 and C_2 are the abundances of prey (cells ml^{-1}) in the control aquariums at the beginning of the experiment (t_1) and at the end of the experiment (t_2).

The ingestion rates (I) (cells ingested larva^{-1} h^{-1}) were calculated using the formula: $I = \langle C \rangle * C$. The calculation of the average abundance of prey available $\langle C \rangle$ was also performed for each aquarium with the presence of a predator during the time of the experiment:

$$\langle C \rangle = C_1'[e^{\left(k-g\right)*(t_2-t_1)} - 1]/(t_2 - t_1) * (k - g).$$

In addition, we performed a second measure of food resource selection complementing the Ingestion rates, the electivity index (E^*) (Vanderploeg & Scavia, 1979a, b) was calculated. This measure of selectivity compares the proportion of a type of prey available in the medium, with the proportion of prey in the diet of the predator. The E^* index ranges from -1 to $+1$, where negative values indicate evasion to the given prey, zero corresponds to no selectivity, and positive values represent resource selection by consumers. In this study, we considered it as null selectivity when the standard error values of the analyzed groups presented positive and negative values.

To estimate E^*, a series of calculations were performed, following the approach described in Rollwagen-Bollens and Penry (2003). First, the number of individuals consumed by larvae (R_i) in each aquarium, for each type of prey, was determined as follows:

$$R_i = \frac{(N_{ic} + N_{fc})}{2} - N_{ft},$$

where i is the food item, N_{ic} is the average number of individuals present in each aquarium at the beginning of the experiment, N_{fc} is the average number of individuals present in each control tank at the end of the experiment and N_{ft} is the mean number of individuals present in each treatment at the end of the experiment.

The ratio of each type of prey in the diet (r_i) and the available items in the (n_i) medium was calculated as follows:

$$r_i = \frac{R_i}{\sum_{j=1}^{m} R_j},$$

where m is the number of types of prey, ΣR_j is the sum of the number of individuals consumed by the larvae for each type of prey (number of ciliates + number of rotifers + number of copepods + number of cladocerans).

$$n_i = \frac{N_{ic}}{\sum_{j=1}^{m} N_{jc}},$$

where ΣN_{jc} is the sum of the average number of individuals present in each control tank at the end of the experiment for each type of prey (number of ciliates + number of rotifers + number of nauplii + number of copepods + number of cladocerans).

Subsequently, the electivity was calculated for each type of prey, according to the formula below:

$$E_i^* = \frac{\frac{r_i}{n_i} - \frac{1}{m}}{\frac{r_i}{n_i} + \frac{1}{m}}.$$

Data analysis

Nonmetric multidimensional scaling (NMDS) was performed to summarize morphometric parameters of each larval development stage, based on Bray–Curtis distance. Significant differences were tested through a permutational multivariate analysis of variance (PERMANOVA) determined by 999 permutations with subsequent pairwise comparisons for significant results.

To determine significant differences in the abundance of each prey group (ciliates, rotifers, nauplii, copepods and cladocerans) at the end of the experiment, we performed one-way ANOVA test ($P < 0.05$) between the control and each treatment. Subsequently, clearance and ingestion rates were calculated considering only the groups of prey that showed significant differences in abundance at the end of the experiment.

Differences in clearance rates among treatments were tested using one-way ANOVA and Fisher's post hoc test (Fisher LSD). Differences in ingestion rates for each type of prey among treatments were tested in the same way (ANOVA one way and post hoc Fisher LSD), however, when the normality and homogeneity assumptions (evaluated using Levene's test) were not met, we used Kruskal–Wallis test (1952) and Dunn's test (1964). In addition, based on the ingestion rates, the relative importance of each prey group (% prey abundance consumed per hour) was calculated from the sum of the total clearance rates per larvae, for each treatment. Statistical analyses were performed using program R (R Core Team, 2013).

Results

The values of the morphometric parameters (Table ESM_1) of the larvae increased proportionally with larval development, that is, as the larvae developed, they had larger body sizes, larger mouth cleft, larger eye diameter, etc. (PERMANOVA: Pseudo-$F = 591.02$, $P = 0.001$; pairwise test: pre-flexion × flexion: $F = 358.44$, $P = 0.003$; pre-flexion × post-flexion: $F = 576.52$, $P = 0.003$; flexion × post-flexion: $F = 241.28$, $P = 0.003$) (Figure ESM_1). The mean abundance of each type of prey analyzed at the end of the experiments (after 2 h), for each treatment, are summarized in Supplementary Information Table ESM_2, showing the reduction or increase in prey abundance in each treatment compared to the control, showing the consumption of different prey categories. The composition of ciliates prey and mean and size were included as Supplementary Information Table ESM_3.

All prey communities, with the single exception of rotifers, were found to have significantly different prey abundances between the treatment and the control at the end of the experiment (Table 1). In our study, rotifers were not included in the clearance and ingestion rates analyses because their abundance was not significantly different between controls and treatments, suggesting that these organisms are not a relevant food item in the diet of *A. lacustris* larvae.

The clearance rates (ml larva^{-1} h^{-1}) were significantly different between treatments (ANOVA, $F = 16.6118$; $P = 0.003$), being significantly higher in the treatment with post-flexion larvae than in those with flexion and pre-flexion larvae (Fig. 1). In addition, clearance rates in treatment with flexion larvae were significantly higher than in pre-flexion (Fig. 1). The increase in clearance rates according to the ontogeny of the larvae suggests a consequent increase in the amount of food resources ingested with the development of the larvae, with the post-flexion larvae having clearance rates approximately 57% higher than the pre-flexion larvae and 26% larger than the flexion larvae. The flexion larvae had clearance rates approximately 30% higher than the pre-flexion larvae.

In the pre-flexion treatment, the positive E^* electivity values for ciliates, cladocerans and copepods indicate a selectivity of these available food resources at this stage of development. The nauplii, for this treatment, presented neutral values in relation to the selectivity of the larvae (Fig. 2). The absence of selectivity of nauplii by *A. lacustris* larvae was repeated in the treatment with larvae in flexion. However, this absence of selectivity also extended to the ciliate and cladoceran groups. In this treatment, only copepods showed positive E^* electivity values

Table 1 One-way ANOVA results for prey abundances between each treatment and control

One-way ANOVA	df	SS	MS	*F* value	Pr (> *F*)
Pre-flexion treatment					
Ciliates					
Treatments	1	888,578	888,578	25.53	0.002*
Residuals	6	208,824	34,804		
Rotifers					
Treatments	1	1,205	1,205	0.22	0.653
Residuals	6	32,235	5,373		
Nauplii					
Treatments	1	136,417	136,417	21.97	0.003*
Residuals	6	37,254	6,209		
Copepods					
Treatments	1	212,011	212,011	39.09	< 0.001*
Residuals	6	32,542	5,424		
Cladocerans					
Treatments	1	3,984	3,984	6.80	0.040*
Residuals	6	3,513	586		
Flexion treatment					
Ciliates					
Treatments	1	571,006	571,006	15.94	0.007*
Residuals	6	214,970	35,828		
Rotifers					
Treatments	1	54	54	0.002	0.968
Residuals	6	181,982	30,330		
Nauplii					
Treatments	1	108,266	108,266	15.53	0.007*
Residuals	6	41,839	6,973		
Copepods					
Treatments	1	195,781	195,781	31.99	0.002*
Residuals	6	30,596	6,119		
Cladocerans					
Treatments	1	3,918	3,918	46.71	< 0.001*
Residuals	6	503	84		
Post-flexion treatment					
Ciliates					
Treatments	1	686,517	686,517	13.78	0.009*
Residuals	6	299,025	49,838		
Rotifers					
Treatments	1	6,741	6,741	0.255	0.631
Residuals	6	158,374	26,396		
Nauplii					
Treatments	1	152,904	152,904	23.95	0.002*
Residuals	6	38,307	6,385		
Copepods					
Treatments	1	305,762	305,762	55.04	< 0.001*
Residuals	6	33,334	5,556		

Table 1 continued

One-way ANOVA	df	SS	MS	*F* value	Pr (> *F*)
Cladocerans					
Treatments	1	9,376	9,376	67.34	< 0.001*
Residuals	6	835	139		

df Degrees of freedom, *SS* sum of squares, *MS* mean squares

Asterisks at *P* values indicate significance at $P < 0.05$

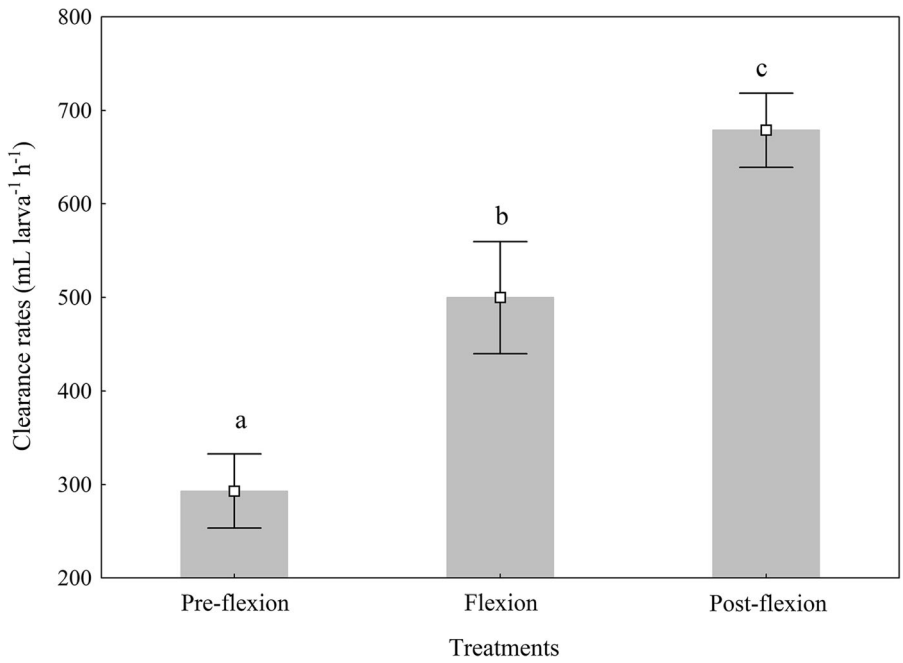

Fig. 1 Clearance rates (ml $larva^{-1}$ h^{-1}) for each stage of larval development of *A. lacustris*. Bars represent the standard errors and letters in columns indicate statistical significance between the treatments. Treatments not sharing a letter differ significantly at $P < 0.05$ (Fisher HSD)

(Fig. 2). For the treatment with post-flexion larvae, the *E** values demonstrate an increased preference for larger prey (nauplii, cladocerans and copepods), as well as selection against ciliated prey (Fig. 2).

The ingestion rates (ind $larva^{-1}$ h^{-1}) were similar among treatments for some prey groups, with nauplii and cladocerans being similarly ingested, regardless of larval development stage (Fig. 3; Table 2). For ciliates and copepods, larvae showed significant differences in the ingestion rates between the treatments [ciliates: Kruskal–Wallis: $H(3) = 4.6222$; $P = 0.049$, copepods: Table 2]. Since ciliates were consumed approximately 24% less in the post-flexion treatment than in pre-flexion (Dunn's test: $P = 0.02$) and 14% less in flexion than in pre-flexion (Dunn's test: $P = 0.04$). Copepod ingestion rates were approximately 18% lower in pre-flexion larvae treatment than flexion and approximately 33% less than post-flexion treatments (Fig. 3; Table 2).

Thus, the relative importance of each prey group, based on their ingestion, has changed according to the development of larvae, with the largest contribution of smaller-sized prey (ciliates and nauplii) found for pre-flexion larvae and a gradual decrease toward more developed larvae (Fig. 4). Larger body preys (copepods and cladocerans) showed an inverse pattern, with

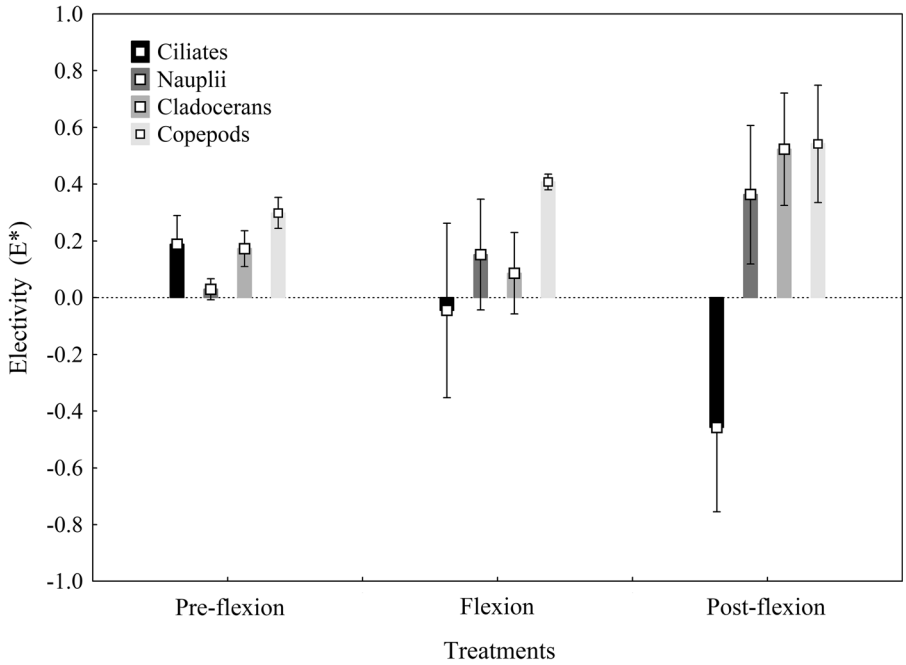

Fig. 2 Electivity indices (E^*) for each type of prey in each treatment. Bars represent the standard errors. Positive values up to + 1 representing increasing preference, zero suggests neutral preference (when the standard error values of the analyzed groups presented positive and negative values were considered it as null selectivity) and negative values down to − 1 representing increasing avoidance

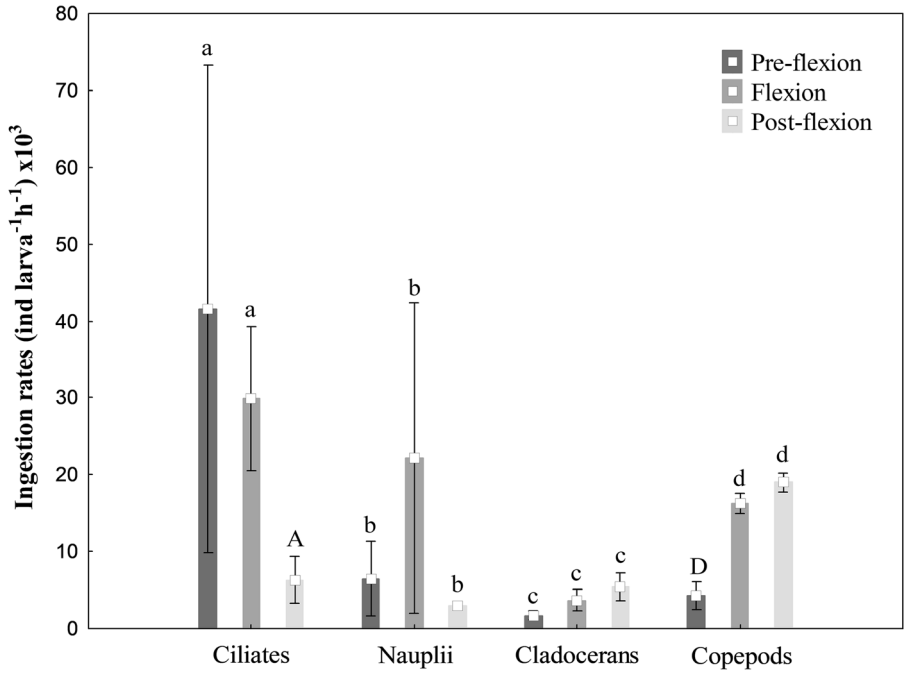

Fig. 3 Ingestion rates (ind $larva^{-1}$ h^{-1}) for each type of prey in each treatment. Bars represent the standard errors and letters in columns indicate statistical significance between the treatments, for each prey separately. Equal letters show statistical differences at $P < 0.05$ (Fisher HSD) through upper- and lower-case characters

Table 2 One-way ANOVA and Fisher LSD test results for ingestion rates of prey between the different treatments

One-way ANOVA	df	SS	MS	*F* value	Pr (> *F*)
Nauplii I					
Treatments	2	6.2830	3.1415	0.7276	0.5212
Residuals	6	2.5903	4.1373		
Cladocerans I					
Treatments	2	19,970	99,854	1.74660	0.2524
Residuals	6	34,302	57,170		
Copepods I					
Treatments	2	3.7074	1.8537	28.5597	< 0.001*
Residuals	6	3.8944	6.4907		
Fisher LSD					Pr (>|*t*|)
Copepods I					
Pre-flexion–flexion					0.0011*
Pre-flexion–post-flexion					< 0.001*
Flexion–post-flexion					0.2364

df Degrees of freedom, *SS* sum of squares, *MS* mean squares

Asterisks at *P* values indicate significance at $P < 0.05$

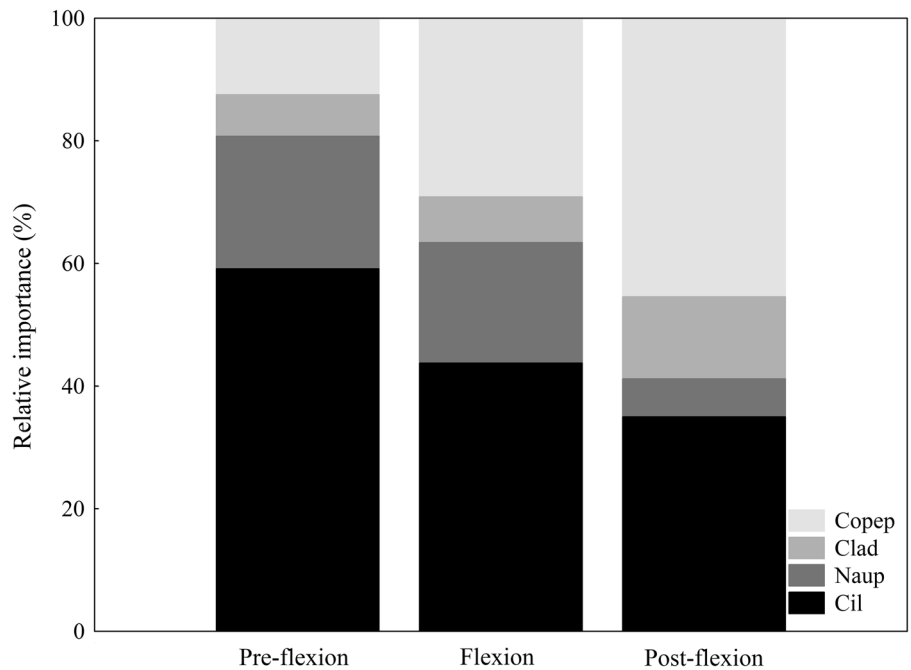

Fig. 4 Relative importance (%) of each type of prey in each treatment. *Copep* copepods, *Clad* cladocerans, *Naup* nauplii, *Cil* ciliates

an increase in their relative importance with larval development (Fig. 4).

Discussion

As hypothesized, our results indicate that the ciliate protists constituted an important food source for the larvae of *A. lacustris*, mainly in the early stages of their development. In addition, as predicted, these larvae altered their food preference as they developed, starting to ingest organisms with larger body sizes. Fish larvae are usually diurnal and visual predators that select their prey in order to maximize energy gains per unit of time (von Herbing et al., 2001), which may influence their growth and survival (Mayer & Wahl,

1997). We observed a significant increase in the diameter of the eye as the larvae developed from pre-flexion to post-flexion, which is directly related to their visual ability (Nakatani et al., 2001). However, their ability to vision is not the only important feature to detect a potential prey, but also the particularities of the prey itself, such as size, abundance, color, movement patterns and speed of swimming (Buskey et al., 1993; Mayer and Wahl 1997; Figueiredo et al., 2007; Makrakis et al., 2008; Zingel et al., 2012).

The clearance rates of *A. lacustris* larvae increased significantly as they developed, suggesting that larvae of larger body sizes have a greater energetic demand and ingest a larger number of prey. Other studies have found a relationship between an increase in the body size of fish larvae and an increase in the number of prey consumed (Reiss et al., 2005; Dickmann et al., 2007) which may occur because larvae improved their swimming skills and, consequently, increase the approach and capture prey over time (von Herbing & Gallager, 2000). In addition, the size of the buccal cleft becomes larger at the late stages, allowing the ingestion of a wider variety of prey types as well as larger prey (Nunn et al., 2012).

The results showed that the *A. lacustris* larvae selected ciliated protists in their pre-flexion stage and that the relative importance found for ciliates decreased with the larval development, reinforcing its importance as the first food for younger larvae, corroborating our first prediction. The importance of ciliates for the survival and growth of different species of fish larvae in early stages of development has been well documented in several geographic regions (Nagano et al., 2000; von Herbing & Gallager, 2000; Figueiredo et al., 2005; Zingel et al., 2012), however, studies in freshwater environments are still very scarce. When evaluating the ingestion rate and relative importance of ciliates in the diet of *A. lacustris*, we fill a gap pointed out by Montagnes et al. (2010), which discusses the need to quantify the relative contribution of protists to the diet of fish larvae. Thus, our results provide subsidies for future studies in freshwater ecosystems.

Other studies have also pointed out ciliates as an important food resource for young fish larvae. For example, Herbing et al. (2001) showed that Atlantic cod larvae (*Gadus morhua* Linnaeus, 1758) consume exclusively ciliates in their early stages, obtaining sufficient energy to survive and develop until they are able to ingest larger prey. In a recent study, Zingel et al. (2019) also shown that ciliates represent are essential food for first-feeding fish larvae of different species, in freshwater and marine environments. The authors found that 63% of the total carbon biomass ingested by larval freshwater fish was ciliates, a value almost 2 times higher than that found for the marine environment, which was 36%. Ciliates consumption may increase the survival rates of newly hatched larvae, since they are rich in fatty acids and omega-3 (Gallager et al., 1996; von Herbing & Gallager, 2000), however, their contribution has been underestimated for decades, since studies using digestive tract analysis of larvae could not find significant amounts of protists due to their rapid digestion (Meeren & Naess, 1993). Only loricated ciliates were recorded in the stomachs and intestines of fish larvae, representing a small portion of the community of protists in freshwater environments (Foissner & Berger, 1996), leading to the incorrect assumption that their contribution is insignificant (Friedenberg et al., 2012). Despite their small size and high transparency, ciliates are highly abundant in aquatic environments and have a low swimming speed and predictable movements when compared to other zooplankton organisms, characteristics that reduce the energy expenses with their persecution, besides facilitating their capture (von Herbing & Gallager, 2000). Newly hatched fish larvae are usually rudimentary, have a restricted swimming capacity and small buccal clefts, and protists are a prey of adequate size to allow their growth and survival (Nagano et al., 2000; von Herbing et al., 2001; Makrakis et al., 2008; Zingel et al., 2019).

The trophic relationship between ciliates and fish larvae also represents a link between planktonic food webs and higher trophic levels (McQueen et al., 1986), since fish larvae are very important in the diet of larger fish (Santin et al., 2005; Novakowski et al., 2007). Studies about this trophic link can also help to clarify important questions about the role of these protists in aquatic food webs. In tropical ecosystems, due to the predominance of smaller primary producers, it has been suggested that the food webs are stretched, with the insertion of intermediate trophic levels (Sarmento, 2012). Thus, studies have pointed out that the transfer of matter and energy from microorganisms does not reach higher trophic levels due to the losses that occur at these intermediate levels, with the importance of microorganisms often being restricted to nutrient

cycling (Fenchel, 2008). However, if ciliates can be preyed upon, digested and absorbed by fish larvae, this energy that was lost can directly reach the upper trophic levels, raising the importance of microbial food webs within tropical aquatic ecosystems.

Besides their importance in aquatic food webs, the link between fish larvae and ciliates may represent a great alternative for maintenance and survival of fish stocks in the field of aquaculture (Montagnes et al., 2010). Success in fish production depends largely on the availability of adequate food for the first feeding of the larvae (Das et al., 2012). Therefore, in order to achieve maximum growth and survival of fish larvae, and consequently, high profitability, the nutritional components of the food provided must contain a high content of nutrients (Das et al., 2012). Ciliates are promising candidates for mass production, as they have high nutritional value, can be grown easily and economically and still reduce the amount of organic matter in the cultivation system by consuming fine organic particles (Rhodes & Phelps, 2008; Das et al., 2012). Thus, further investigation of which species are preferentially consumed by larvae would be of great interest, since most ciliate species are considered cosmopolitan (Finlay & Fenchel, 2004) and, therefore, can serve as food resources for fish larvae in different ecosystems around the world.

Regarding other planktonic groups, although nauplii and cladocerans had similar ingestion rates among treatments, their relative importance shows a tendency for nauplii to be more relevant in the feeding of rudimentary larvae, whereas the consumption of cladocerans seems to be more important for more developed larvae, suggesting that these organisms may represent intermediate resources between the small ciliates and the large copepods, as expected in our second prediction. Many studies point to nauplii as the first food resource for fish larvae (Last, 1978; Munk & Kiorboe, 1985; Dickmann et al., 2007). These organisms show greater average sizes than ciliates, besides being more visible due to their coloration (Zaret, 1980). However, nauplii superior swimming velocities when compared to protists, makes it difficult for rudimentary larvae to capture them (von Herbing & Gallager, 2000), resulting in their reduced consumption, compared to ciliates, for pre-flexion larvae, as we observed. Gallager et al. (1996) showed that 4-to-5-day old cod larvae preferred to consume ciliates as their first food because they could not efficiently capture nauplii, beginning their ingestion after reaching approximately 8 days of age.

Although *A. lacustris* selected larger organisms such as microcrustaceans since the earliest stages, the results of ingestion rates showed that the largest components, adult copepods, were consumed in greater quantity in the flexion and post-flexion stages, corroborating our third prediction. Larger prey are known to be more advantageous energetically (Mayer & Wahl, 1997). However, despite the energetic advantages, larger prey shows higher fugitive responses, making rapid movements and sudden changes of direction, thus reducing the likelihood of their capture (Buskey et al., 1993). Among the largest zooplanktonic components, some studies have shown that copepods have a greater chance to escape than cladocerans (Zaret, 1980), but they also show a higher energy return, since they have a caloric density slightly larger than a cladocera of the same size (Cumminns & Wuycheck, 1971). Picapedra et al. (2018) also found predominance in the consumption of copepods by *Plagioscion squamosissimus* (Heckel, 1840) larvae, in flexion and post-flexion stages, due to an improvement in the swimming capacity of the larvae, visual acuity and maxillary development. Therefore, it is believed that in our study, more developed larvae were more successful in capturing and ingesting their apparently preferred prey, the copepods. An increase in the relative importance of copepods in more developed stages of fish larvae may be related to their ability to efficiently search for and capture larger and faster prey species (Mayer & Wahl, 1997; Picapedra et al., 2018).

Conclusions

Our results bring advances in the understanding of the feeding dynamics of *A. lacustris* larvae, and indicate that ciliate protists are an important item in their diet, especially in the early stages of their development, becoming an additional resource in more advanced stages, when larger bodied zooplankton, mainly copepods, are more consumed. Furthermore, we show that the preference of *A. lacustris* larvae changes along with its ontogeny and encourage the development of new research that consider the different development stages of fish larvae, to avoid biased results. Future studies should focus not only on fish species with

commercial interest, as has been the case, but also on those of relevant ecological importance and biodiversity conservation, especially in freshwater environments in Neotropical regions, where these data are yet incipient. Finally, our results not only demonstrate that ciliates are important for the diet of the initial stages of fish larvae, but also provide future perspectives and evidence that any change in the structure of the ciliate community, resulting from anthropogenic or environmental changes, can directly influence larvae survival rates, generating a cascading effect.

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Data availability The data used in this study will be made available by the authors.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted [National Council for Control of Animal Experimentation CONCEA and the Ethics Committee on Animal Use of the State University of Maringá (CEUA/UEM) under Protocol Number 3087210916].

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