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Stock Density, Feeding, and Water Salinity for Larval Rearing of Yellowtail Tetra (*Astyanax altiparanae*)

Densidad de población, alimentación y salinidad del agua para la cría de larvas de tetra cola amarilla (*Astyanax altiparanae*)

Rafaela Manchin Bertolini[1,2]
Nivaldo Ferreira do Nascimento[1,3]
Lucia Suárez López[5]
Gabriel Marra Schade[1,2]
Juliana Dias Bastos Xavier[1,4]
Emy Yano[1]
Jose Augusto Senhorini[1,2]
Matheus Pereira dos Santos[1,4]
George Shigueki Yasui[1,2]

[1]Laboratory of Fish Biotechnology, National Center for Research and Conservation of Continental Fish, Chico Mendes Institute of Biodiversity Conservation, Rodovia Pref. Euberto Nemésio Pereira de Godoy, Pirassununga, SP 13630- 970, Brazil.
[2]Institute of Bioscience, São Paulo State University, Rua Prof. Doutor Antônio Celso Wagner Zanin, s/no., Botucatu, SP 18618–689, Brazil.
[3]Academic Unit, Federal Rural University of Pernambuco (UFRPE), Serra Talhada, Brazil.
[4](UFRRJ) – Federal Rural University of Rio de Janeiro. Rodovia BR 465, Km 07, s/n Zona Rural, Seropédica - RJ, 23890-000, Brazil.
[5]Scientific Research Center and of Higher Education of Ensenada (CICESE), Department of Aquaculture, Ensenada, Baja California 22860, México.

Corresponding author: Rafaela Manchin Bertolini. National Center for Research and Conservation of Continental Aquatic Biodiversity (CEPTA). Rodovia SP-201 (Pref. Euberto Nemésio Pereira de Godoy), km 6,5, Caixa Postal 64, CEP 13.641-001- Pirassununga – SP, Brazil.email: rafaelambertolini@hotmail.com

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Abstract
One of the critical points in fish rearing is larviculture, a phase that requires suitable food for first feeding. This study aimed to evaluate the effect of three variables on the larviculture of the yellowtail tetra *Astyanax altiparanae*: 1) type of *Artemia franciscana* nauplii used for feeding (Fresh, frozen, and freeze-dried); 2) salinity (0.2%, 0.4%, and 0.6% NaCl); and 3) stocking density (50, 100, 150, and 200 larvae/L^{-1}). Growth and survival were assessed over 10 days. On day 10, larvae fed Artemia nauplii (fresh) had a higher length and survival rate compared to other treatments ($P < 0.05$). Water at 0.4% and 0.6% NaCl showed significantly lower survival ($P < 0.05$) and no survival, respectively. Lower density (50 larvae L^{-1}) showed the greatest length (7.73 ± 0.08 mm), and the highest density (200 larvae L^{-1}) presented the lowest length (5.86 ± 0.10 mm). These results are innovative for this species, opening up new possibilities for larviculture in both laboratory and aquaculture settings.
Keywords: *Artemia* sp, Characin, first feeding, fish, larviculture.

Resumen
Uno de los puntos críticos en la crianza de peces es la larvicultura, una fase que requiere alimento adecuado para la primera alimentación. El objetivo de este estudio fue evaluar el efecto de tres variables en la larvicultura del tetra de cola amarilla *Astyanax altiparanae*: tipo de nauplios de *Artemia franciscana* utilizados para la alimentación (frescos, congelados y liofilizados); 2) salinidad (0.2%, 0.4% y 0.6% NaCl); y 3) densidad de cultivo (50, 100, 150 y 200larvas/L^{-1}). El crecimiento y la supervivencia se evaluaron durante 10 días. Al día 10, las larvas alimentadas con nauplios de artemia frescos presentaron mayor longitud y tasa de supervivencia en comparación con los demás tratamientos ($P< 0.05$). El agua con 0.4% y 0.6% NaCl mostró una supervivencia significativamente menor ($P < 0.05$) y nula, respectivamente. La menor densidad (50 larvas L^{-1}) presento la mayor longitud (7.73 ± 0.08 mm), mientras que la mayor densidad (200 larvas L^{-1}) presento la menor longitud (5.86 ± 0.10 mm). Estos resultados son innovadores para esta especie, abriendo nuevas posibilidades para la larvicultura con fines de laboratorio y acuicultura.
Palabras clave: *Artemia sp*, Characin, primera alimentación, peces, larvicultura.

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Introduction

The yellowtail tetra, *Astyanax altiparanae* (Garutti and Britski, 2000), is a small characin fish with potential in both aquaculture production and academic research. This species exhibits intertidal spawning, reproducing several times throughout the year (Orsi *et al.* 2004; Porto-Foresti *et al.* 2010), and tolerates environments with limiting conditions, such as low water quality and high stocking densities (Garutti & Britski 2000). As a result, *A. altiparanae* has been used as a model organism for academic research for the last several years *(Yasui et al.* 2022). In previous studies, a protocol was developed for *in vitro* fertilization (Yasui *et al.* 2015), which enables studies on chromosome and gamete manipulation (Ferreira Do Nascimento *et al.* 2017a; do Nascimento *et al.*, 2020). Such protocol was used to describe the gamete ultrastructure and the embryonic development in detail (dos Santos *et al.* 2016), induce triploid fish (Adamov *et al.* 2016; do Nascimento *et al.* 2017b), as well as for the labeling of primordial germ cells (PGCs) and transplantation of PGCs (Coelho *et al.* 2023; Rosero *et al.* 2023). However, further investigation is needed to optimize the rearing period to evaluate other parameters in adult fish.

Undoubtedly, one of the critical points in fish rearing is larviculture, which is a phase that requires suitable food for first feeding. In this scenario, several fish species require live food, such as Artemia sp. nauplii, primarily because formulated diets are ineffective (Aguilera *et al.* 2012). The larvae of *A. altiparanae*, on the other hand, recognize and ingest this type of food, but this feeding regime does not guarantee growth and survival (Bertolini *et al.* 2018). Despite these results, this capacity to ingest dry food could be considered as a basis for testing other types of feed, such as frozen or lyophilized *Artemia* sp. nauplii.

The *Artemia* sp. nauplii have been extensively used for several fish species, mainly due to their good acceptance and high nutritional value (Bertolini *et al.* 2018; Jomori *et al.* 2003; Luz and Portella, 2005; Madkour *et al.* 2023). However, since *Artemia* sp. nauplii originate from hypersaline environments, their survival in freshwater is limited, potentially reducing their viability and negatively impacting water quality (Beux and Zaniboni Filho, 2006; Jomori *et al.* 2012). Therefore, the use of sodium chloride (NaCl) is an interesting alternative, as saline water extends the lifetime of *Artemia* sp. nauplii, increasing food intake and growth of the fish larvae (Luz *et al.* 2008). In addition, NaCl can be used for the prevention and control of parasites, such as *Ichthyophthirius multifiliis* (de O. Garcia *et al.* 2007), *Epistylis sp. (Rodrigues et al., 2019), and trichodinid sp.* (Naas *et al.*, 2024).

Other key factors that have been studied in fish larviculture are the stocking density, which may affect survival (Campagnolo and Nuñer 2006; Santos *et al.* 2021), growth (Bolasina *et al.* 2006; Reis *et al.* 2021), feeding (Reis *et al.* 2021; Wendelaar Bonga 1997), and larval behavior (Andrade *et al.* 2004; Santana *et al.* 2020). Very high stocking densities reduce water quality because of increased nitrogenous waste excretion (Jobling 1994; Reis *et al.* 2021), leading to stress and illness (Gil Barcellos *et al.* 2000; Iguchi *et al.* 2003; King *et al.* 2007). On the other hand, very low stocking densities result in underutilization of the space, thereby reducing the production capacity (Baldisserotto 2002).

Therefore, the present study was conducted to evaluate three important variables in the optimization of yellowtail tetra *Astyanax altiparanae* larviculture: different types of food, salinity, and stocking density.

Materials and methods

All the procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of CEPTA (CEUA #02031.000033/2015-11).

Origin of broodstock and breeding

Adult yellowtail tetras (*A. altiparanae*) used in this study were obtained from the Mogi Guassu River (21.925706 ° S, 47.369496 ° W), São Paulo, Brazil. The fish were maintained in 1,000 m² earthen ponds and were fed twice a day with commercial pellets containing 40%–45% of crude protein. Endogenous food (plankton) was also available in the ponds. Spawning was induced in females (SL ≈ 9 ± 0.39 cm) and males (SL ≈ 6 ± 1.25 cm) using a previously developed protocol in the same laboratory (Yasui et al., 2015). The fish were anesthetized in a menthol solution (100 mg L^{-1}) and then injected with a single dose of carp pituitary gland (3 mg kg^{-1} for males and females). The fish were then separated into three couples, which resulted in three different batches of fry (replicates). For each experiment, larvae from these three different couples were used. After semi-natural spawning, the fertilized eggs were siphoned using a plastic hose.

The fertilized eggs were then incubated in a floating apparatus with a 100 µm nylon mesh bottom. The mesh

was placed in 380L aquariums with constant aeration until hatching. Dead fish were removed at 6-h intervals.

Experiment 1: first feeding

A one-factor completely randomized design was used. Three treatments with *Artemia* sp. nauplii were used: T1 = Fresh (control); T2 = Frozen; and T3 = Freeze-dried for 10 days. After yolk absorption, larvae considered normal (dos Santos *et al.*, 2016) were distributed in circular, transparent acrylic containers (each containing 100 larvae) with a diameter of 150 mm and a water volume of 1 L. A total of 9 containers (three replicates per treatment) were used. Each container was placed in a biochemical oxygen demand (BOD) incubator with the temperature set at 26°C and 12 h of light per day. Food was provided twice a day (at 8 am and 4 pm). For maintenance of water quality, two partial daily changes of 50% of the total volume were performed, approximately one hour after feeding.

For food preparation, the *Artemia* sp. nauplii in T1 were hatched daily. For this, the cysts were incubated in 1 L of artificial seawater (30%) in a conical container. Light and aeration were continuously provided by a fluorescent lamp and an aquarium air pump, respectively. Under those conditions, cysts hatched within approximately 24 hours.

For treatments T2 and T3, the cysts were hatched under the same conditions described above. Subsequently, the *Artemia* sp. nauplii were concentrated, transferred to 1.5 mL microtubes, and frozen at -20°C (T2). For T3, the microtubes were frozen and, subsequently, subjected to lyophilization.

Experiment 2: Salinity

Three different salinities were tested using a one-factor completely randomized design: 0.2%, 0.4%, and 0.6% NaCl (Êxodo Científica), in addition to a control at 0% for 10 days. After yolk absorption, larvae considered normal were distributed as described above. A total of 16 containers were used, resulting in four replicates per treatment. Each container was placed in a biochemical oxygen demand (BOD) incubator with the temperature set at 26°C and 12 h of light per day. Salinity was evaluated in the best food regime obtained in Experiment 1 (Fresh Artemia sp. nauplii) and was provided twice daily (at 8:00 a.m. and 5:00 p.m.). For maintenance of water quality, two partial daily changes of 50% of the total volume were performed, approximately one hour after feeding.

Experiment 3: Stocking Density

A one-factor completely randomized design was used for testing four stocking densities: 50, 100, 150, and 200 larvae L^{-1} for 10 days. After yolk absorption, larvae were distributed as described previously. A total of 16 containers were used, resulting in four replicates per treatment. Stocking density was evaluated using the best food regime and salinity obtained in Experiments 1 (Fresh *Artemia* sp. nauplii) and 2 (0.2% NaCl), respectively. The same previously standardized conditions were maintained in this experiment, such as controlled temperature, 12 hours of light per day, a feeding regime (twice a day), and partial water changes.

Growth and survival assessment

The same procedures for growth evaluation were performed in all experiments. Length (mm) was measured daily for 20 individuals randomly captured in each container. First, the fish were placed in a 50 mm Petri dish containing 29.3 mM 2-phenoxyethanol as an anesthetic to facilitate handling (Carvalho *et al.*, 2022). Subsequently, the larvae were observed under a stereomicroscope (Nikon SMZ-1500, Tokyo, Japan) with a CCD camera (Nikon DS-Fi, Tokyo, Japan). Then, digital images were taken using the NIS-Elements software (Nikon, Tokyo, Japan), and the total length was measured from these images. Growth performance was measured for 10 days, and at this moment, the survival rate from each container was also calculated.

Statistics

Data were obtained from three replicates and are presented as mean ± standard error (SE). Prior to analysis, data were tested for normality and homogeneity of variances using the Shapiro–Wilk and Levene's tests, respectively. When assumptions were met, data were analyzed using one-way ANOVA followed by Tukey's multiple range test for post hoc comparisons. Survival rates, expressed as percentages, were first subjected to arcsine square root transformation to stabilize variances and approximate normal distribution, as recommended by Zar (2000). Comparisons of survival among treatments were then conducted using one-way ANOVA, followed by Tukey's test. All statistical

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analyses were performed using STATISTICA software (version 7.0, StatSoft, USA), with a significance level of 0.05.

Results

Larval growth and survival at different feeding

At the beginning of the experiment, no difference was observed between treatments ($P > 0.05$). From day two until day five, larvae with frozen and freeze-dried feed were similar ($P > 0.05$) and had significantly lower length (mm) than the fresh *Artemia sp.* nauplii treatment ($P < 0.05$). From the sixth day onwards, the freeze-dried treatment had no survival (0%), and larvae fed fresh *Artemia* sp. nauplii were longer (6.07 ± 0.06 mm at day 10) than the frozen treatment (4.40 ± 0.15 mm at day 10). Data on length and survival are detailed in Table 1.

Larval growth and survival at different salinities

At the beginning of the experiment (day 0), no significant differences in length were observed among treatments ($P > 0.05$), with initial sizes ranging from 3.65 ± 0.03 mm to 3.83 ± 0.13mm. From day 1 to 3, lower survival rates were observed for larvae maintained at a salinity of 0.6%, and there were no survivors by day 4. However, no significant difference ($P > 0.05$) was observed throughout the experiment for the control and the salinity levels of 0.2% and 0.4%. Growth was similar among treatments, with final lengths of 6.63 ± 0.57 mm, 6.49 ± 0.62 mm, and 7.01 ± 0.48 mm, respectively. At day 10, the control and 0.2% salinity treatment showed no difference in survival rate (%). However, water at 0.4% salinity showed significantly lower survival (24.33 ± 15.96%) compared to the other treatments (P> 0.01). Data on length and survival at different salinities are detailed in Table 2.

Larval growth and survival at different densities

At the beginning of the experiment, on days 0, 1, 2, and 4, there was no difference in length among all treatments ($P > 0.05$). However, all other days of the experiment showed that the lowest density (50 larvae L-1) presented the greatest length (7.73 ± 0.08 mm), and the highest density (200 larvae L^{-1}) presented the shortest length (5.86 ± 0.11 mm). No difference was observed for survival rates ($P > 0.05$), and all treatments showed high values. Data on length and survival at different densities are detailed in Table 3.

Table 1. Growth performance (mm) of yellowtail tetra *Astyanax altiparanae* fed with fresh, frozen, and freeze-dried *Artemia* sp. nauplii for 10 days. At 3 days post-hatching, fish were maintained in 1-L acrylic tanks (100 fish per tank) at 26°C.

Days	Treatments			*P - value*
	Fresh (mm)	Frozen (mm)	Freeze-dried (mm)	
0	3.63 ± 0.05[a]	3.48 ± 0.03[a]	3.56 ± 0.02[a]	$P > 0.05$
1	3.76 ± 0.01[a]	3.59 ± 0.02[b]	3.62 ± 0.01[b]	$P < 0.01$
2	3.85 ± 0.03[a]	3.50 ± 0.02[b]	3.61 ± 0.04[b]	$P < 0.05$
3	3.97 ± 0.10[a]	3.71 ± 0.04[b]	3.48 ± 0.06[b]	$P < 0.05$
4	4.17 ± 0.08[a]	3.87 ± 0.04[b]	3.74 ± 0.13[b]	$P < 0.05$
5	4.48 ± 0.06[a]	3.81 ± 0.19[b]	3.63 ± 0.03[b]	$P < 0.05$
6	5.08 ± 0.12[a]	3.90 ± 0.09[b]	0.00 ± 0.00[c]	$P < 0.05$
7	5.31 ± 0.03[a]	4.20 ± 0.11[b]	0.00 ± 0.00[c]	$P < 0.01$
8	5.44 ± 0.04[a]	4.18 ± 0.13[b]	0.00 ± 0.00[c]	$P < 0.01$
9	5.85 ± 0.09[a]	4.25 ± 0.17[b]	0.00 ± 0.00[c]	$P < 0.01$
10	6.07 ± 0.06[a]	4.40 ± 0.15[b]	0.00 ± 0.00[c]	$P < 0.01$
Survival Rate (%)	38.33 ± 4.48[a]	12.67 ± 5.78[a]	0.00 ± 0.00[c]	$P < 0.01$

Data are shown as mean ± standard error, and each data point was generated from triplicate samples from different spawning events. *The p-values shown in the table refer to the* ANOVA analysis. Identical lowercase letters within columns denote non-significant differences as determined by the Tukey multiple range test ($p = 0.05$).

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Table 2. Growth performance (mm) of yellowtail tetra *Astyanax altiparanae* fed with fresh *Artemia* sp. nauplii and kept at three different saline concentrations (0, 0.2, 0.4, and 0.6% NaCl) for 10 days. At 3 days post hatching, fish were maintained in 1-L acrylic tanks (100 fish per tank) at 26°C.

	Treatments				
Days	**Control (mm)**	**0.2% NaCl (mm)**	**0.4% NaCl (mm)**	**0.6% NaCl (mm)**	***P - value***
0	3.80 ± 0.08^{a}	3.83 ± 0.13^{a}	3.75 ± 0.18^{a}	3.65 ± 0.03^{a}	*P > 0.05*
1	4.19 ± 0.20^{a}	4.24 ± 0.28^{a}	3.80 ± 0.28^{a}	1.19 ± 0.11^{b}	*P< 0.05*
2	4.50 ± 0.27^{a}	4.53 ± 0.40^{a}	4.05 ± 0.43^{ab}	1.26 ± 0.12^{b}	*P< 0.05*
3	4.95 ± 0.40^{a}	5.10 ± 0.27^{a}	4.33 ± 0.51^{ab}	1.20 ± 0.12^{b}	*P< 0.05*
4	5.34 ± 0.55^{a}	5.39 ± 0.31^{a}	4.65 ± 0.71^{a}	0.00 ± 0.00^{c}	*P> 0.05*
5	5.71 ± 0.29^{a}	5.54 ± 0.33^{a}	5.05 ± 0.50^{a}	0.00 ± 0.00^{c}	*P> 0.05*
6	5.75 ± 0.37^{a}	5.66 ± 0.52^{a}	5.25 ± 0.43^{a}	0.00 ± 0.00^{c}	*P> 0.05*
7	5.79 ± 0.44^{a}	5.91 ± 0.32^{a}	5.77 ± 0.25^{a}	0.00 ± 0.00^{c}	*P> 0.05*
8	6.03 ± 0.23^{a}	5.88 ± 0.46^{a}	6.15 ± 0.24^{a}	0.00 ± 0.00^{c}	*P> 0.05*
9	6.32 ± 0.64^{a}	6.25 ± 0.59^{a}	6.52 ± 0.31^{a}	0.00 ± 0.00^{c}	*P> 0.05*
10	6.63 ± 0.57^{a}	6.49 ± 0.62^{a}	7.01 ± 0.48^{a}	0.00 ± 0.00^{c}	*P> 0.05*
Survival Rate (%)	70.67 ± 6.94^{a}	70.33 ± 3.18^{a}	24.33 ± 15.96^{b}	0.00 ± 0.00^{c}	*P< 0.01*

Data are shown as mean ± standard error, and each data point was generated from triplicate samples from different spawning events. *The p-values shown in the table refer to the* ANOVA analysis. Identical lowercase letters within columns denote non-significant differences as determined by the Tukey multiple range test (*p = 0.05*).

Table 3. Growth performance (mm) of yellowtail tetra *Astyanax altiparanae* fed with fresh *Artemia* sp. nauplii and kept at four different densities (50, 100, 150, and 200 L^{-1} larvae) at 0.2% NaCl for 10 days.

	Treatments				
Days	**50 (mm)**	**100 (mm)**	**150 (mm)**	**200 (mm)**	***P - value***
1	3.98 ± 0.53^{a}	3.97 ± 0.56^{a}	3.96 ± 0.48^{a}	3.93 ± 0.88^{a}	*P > 0.05*
2	4.26 ± 0.86^{a}	4.19 ± 0.67^{a}	4.18 ± 0.66^{a}	4.11 ± 0.55^{a}	*P> 0.05*
3	4.68 ± 0.78^{a}	4.50 ± 0.55^{ab}	4.47 ± 0.44^{ab}	4.36 ± 0.50^{b}	*P< 0.05*
4	5.14 ± 0.28^{a}	4.81 ± 0.16^{a}	4.77 ± 0.16^{a}	4.54 ± 0.11^{a}	*P> 0.05*
5	5.49 ± 0.30^{a}	5.16 ± 0.11^{a}	4.92 ± 0.12^{a}	5.06 ± 0.36^{a}	*P> 0.05*
6	5.95 ± 0.17^{a}	5.58 ± 0.15^{ab}	5.19 ± 0.09^{b}	5.03 ± 0.12^{b}	*P< 0.01*
7	6.49 ± 0.04^{a}	5.84 ± 0.04^{b}	5.52 ± 0.07^{bc}	5.31 ± 0.12^{c}	*P< 0.01*
8	6.87 ± 0.04^{a}	6.12 ± 0.09^{b}	5.79 ± 0.13^{bc}	5.46 ± 0.17^{bc}	*P< 0.01*
9	7.35 ± 0.08^{a}	6.45 ± 0.07^{b}	6.04 ± 0.05^{bc}	5.78 ± 0.15^{c}	*P< 0.01*
10	7.73 ± 0.07^{a}	6.57 ± 0.08^{b}	6.20 ± 0.03^{c}	5.86 ± 0.11^{c}	*P< 0.01*
Survival Rate (%)	97.33 ± 1.33	98.67 ± 1.33	98.22 ± 0.97	95.67 ± 0.17	*P> 0.05*

Data are shown as mean ± standard error, and each data point was generated from triplicate samples from different spawning events. *The p-values shown in the table refer to the* ANOVA analysis. Identical lowercase letters within columns denote non-significant differences as determined by the Tukey multiple range test (*p = 0.05*).

Discusion

In this study, it was observed that the use of fresh *Artemia* sp. nauplii, low salinity (0.2% NaCl), and stocking densities of 50 and 100 larvae L^{-1} provided better results in the initial feeding of *Astyanax altiparanae* larvae.

The *A. altiparanae* larvae, unlike those from other fish species (Aguilera *et al.* 2012), can ingest a formulated diet, even resulting in no survival or reduced growth (Bertolini et al., 2018; Piotrowska *et al.* 2021). In this study, similar results were observed for the *A. altiparanae* larva feed, either frozen or freeze-dried *Artemia* sp. nauplii, when compared with the fresh feed treatment. Additionally, live foods provide digestive enzymes (Kolkovski, 2001) that facilitate their absorption, and these are probably reduced in frozen and freeze-dried *Artemia* sp. nauplii.

The larviculture of fish in low-salinity water had several advantages, such as disease prevention and control (e.g., the parasite *Ichthyophthirius multifiliis* and Trichodina *sp.*) (Tavares-Dias 2022), decrease of nitrogen compounds (Colt 2006), and increasing lifetime and availability of *Artemia* sp. nauplii (Jomori *et al.* 2012). In freshwater, the reduced lifetime of *Artemia* sp. nauplii can decrease ingestion, and the larvae may not consider dead nauplii as prey, which can have a rapid impact on water quality (Fernando Beux and Zaniboni-Filho, 2008; Jomori *et al.*, 2012). Thus, dead *Artemia* sp. nauplii must be removed frequently, and low salinity water is an interesting alternative. The use of NaCl in larviculture yields positive results for several species, including *Colossoma macropomum*, *Astronotus ocellatus*, *Brycon amazonicus,* and *Leporinus microcephalus* (Jomori *et al.* 2013).

However, NaCl concentration must be standardized because elevated salinity can lead to larval mortality (Fabregat *et al.* 2017; Tavares-Dias 2022). In the present study, NaCl concentration higher than 0.2% negatively affected the analyzed parameters. Consequently, the use of low-salinity water has been suggested to increase the survival rate and promote greater growth (Ferreira *et al.* 2023; Jomori *et al.* 2013).

Stocking density can also affect survival and growth during larviculture. Higher densities may lead to reduced growth and high mortality due to competition for space, food, and accumulation of metabolic waste. On the other hand, very low densities may not be enough to achieve the demand for larvae (Lu *et al.* 2017; Marshall *et al.* 2014). In the present study, stocking densities did not affect the survival. However, the final growth was reduced when higher densities were used.

Conclusion

In conclusion, the larviculture of yellowtail tetra *A. altiparanae* was optimized using the following conditions: fresh *Artemia* sp. nauplii, water with 0.2% NaCl, and a stocking density of 50 to 100 L^{-1} larvae. These results are innovative for this species, opening new possibilities for larviculture in both laboratory and aquaculture settings.

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