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BIOLOGY

Co-feeding period on the survival and growth of larvae (*Botia lohachata*) during the feeding transition

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ABSTRACT: This study evaluated the effect of the co-feeding (C) period on the growth and survival of larvae of the yo-yo loach *Botia lohachata* ornamental species during the weaning period from live food (LF) to inert food (IF). The breeders were subjected to induced spawning using crude pituitary homogenate. A total of 2,400 larvae were used with an average initial weight of 0.39 ± 0.09 mg and an average total length of 4.08 ± 0.21 mm, from a single breeding pair. The live food was newly hatched nauplii of *Artemia franciscana,* and the inert diet was a commercial ration with 55% crude protein and two particle sizes (142–350 µm and 350–500 µm). As the first food, all larvae received *A. franciscana* during the first six days, except for the fasting (NC) and inert food (IF) groups. The following co-feeding strategies were employed: C_4 = 4 days of co-feeding, C_8 = 8 days of co-feeding, C_{12} = 12 days of co-feeding, and LF = *Artemia* during the entire experimental period. The longest period with live food promoted better larval growth rates. The specific growth rate of larvae fed live food during the entire period (LF) was higher than that of the other groups, followed by the co-feeding strategies. There was no effect (P > 0.01) on larval survival in the LF, C_{12}, C_8, and C4 groups, which ranged from 41% to 53% after 30 days. Treatment R resulted in high mortality and low growth, suggesting that live food should be the first food source for *B. lohachata* larvae.
Key words: Cobitidae, ornamental fish, fish larvae, inert food, live food.

Co-feeding na sobrevivência e crescimento de larvas de botia yoyo *Botia lohachata* durante a transição alimentar

RESUMO: Este estudo teve como objetivo avaliar o efeito do período de co-alimentação (C) no crescimento e sobrevivência de larvas da espécie ornamental *Botia lohachata* durante o período de desmame de alimento vivo (LF) para alimento inerte (IF). Os reprodutores foram submetidos à desova induzida com homogeneizado de hipófise. Foram utilizadas 2.400 larvas com peso inicial médio de 0,39 ± 0,09 mg e comprimento total médio de 4,08 ± 0,21 mm, de um único casal reprodutor. O alimento vivo foi náuplios recém-eclodidos de *Artemia franciscana*, e a dieta inerte foi uma ração comercial com 55% de proteína bruta e dois tamanhos de partículas (142–350 µm e 350–500 µm). Como primeiro alimento, todas as larvas receberam *A. franciscana* durante os primeiros seis dias, exceto os grupos jejum (NC) e alimento inerte (IF). As seguintes estratégias de co-alimentação foram empregadas: C4 = 4 dias de co-alimentação, C8 = 8 dias de co-alimentação, C12 = 12 dias de co-alimentação e LF = Artemia durante todo o período experimental. O maior período com alimento vivo promoveu melhores taxas de crescimento larval. A taxa de crescimento específico das larvas alimentadas com ração viva durante todo o período (LF) foi maior que a dos demais grupos, seguida das estratégias de co-alimentação. Não houve efeito (P > 0,01) na sobrevivência larval nos grupos LF, C12, C8 e C4, que variou de 41% a 53% após 30 dias. O tratamento IN resultou em alta mortalidade e baixo crescimento, sugerindo que o alimento vivo deve ser a primeira fonte de alimento para as larvas de *B. lohachata.*
Palavras-chave: Cobitidae, peixes ornamentais, larvas de peixes, alimentos inertes, alimentos vivos, desmame.

INTRODUCTION

The yoyo loach belongs to the Cobitidae family and is a freshwater fish, occurring in tropical climates (24–30 °C). It is endemic to Pakistan, India, Bangladesh, and Nepal (FISHBASE, 2022), and is economically important because it is widely used for the ornamentation of lakes and aquariums in several countries. The larviculture stage is one of the most critical stages in the fish production chain. At this stage, the newly hatched larvae are dependent on yolk reserves, and when the yolk sac is exhausted they need to ingest exogenous food to satisfy their increasing energy demands (MENOSSI et al. 2012; FOSSE et al., 2018). For most species that have been studied, the digestive tract of larvae shortly after hatching

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appears histologically as a straight, undifferentiated tube and must undergo further development and differentiation before exogenous food is provided so that it can be efficiently ingested and digested (LAZO et al., 2011) Depending on the size of the larva and the specialization of its digestive tract at the beginning of exogenous feeding, live food is essential to ensure the growth and survival of many fish species (JOMORI et al., 2008; MENOSSI et al. 2012). The supply of *Artemia* nauplii as live food during the early stages of development has already been successfully studied for larvae of other freshwater fish species, such as beta *Betta splendens* (FOSSE et al., 2013), tambacu *Colossoma macropomum* × *Piaractus mesopotamicus* (LOMBARDI & GOMES, 2008), the black catfish *Rhinelepis aspera* (LUZ & SANTOS, 2010), and common carp *Cyprinus carpio* (FOSSE et al., 2018). Although efficient, the supply of live food over a long period can create significant economic barriers to the commercial production of juveniles (ROCHA et al., 2008; JOMORI et al., 2005). Strategies for the early replacement of live food have been tested for several species, with the objective of determining the period of supply that does not negatively influence the growth and survival of larvae.

Strategies for replacing live food with inert food can be implemented abruptly, completely replacing live food with inert food, or gradually, starting with a period of joint feeding and then reducing the amount of live food and gradually increasing the supply of inert feed, until the larvae are exclusively supplied with inert feed (FOSSE et al., 2018). This join feeding method is called a co-feeding strategy, and growth rates are similar to or higher than those achieved with an exclusive supply of brine shrimp (STEJSKAL et al., 2021; ŁĄCZYŃSKA et al., 2016). The co-feeding strategy involves early adaptation by the larva to capture, ingest, digest, and absorb nutrients from the inert food, thereby avoiding low growth and high mortality rates resulting from starvation which can occur when there is a sudden transition between two diets that are physically and chemically different (JUCHNO et al., 2016).

Therefore, the objective of this study evaluated the influence of the co-feeding period on the growth and survival of larvae of the ornamental species *Botia lohachata* during the transition from live to inert food.

MATERIALS AND METHODS

Larvae were obtained by submitting matrices to induce reproduction using crude carp pituitary homogenate. Subsequently, the gametes were extruded, fertilization was performed dry, and the eggs were stored in a funnel-type incubator (WOYNAROVICH & HORVATH, 1989). All larvae used in the experiment were obtained from a single breeding pair.

On the fourth day after hatching (4 DAH), the larvae exhibited accentuated swimming activity in the horizontal direction and the following characteristics: reduced yolk sac, open anus, and open mouth with movement. A pâté with brine shrimp and dry feed was offered, and it was possible to visually verify the ingestion and food in the digestive tract, proving that the larvae were able to consume exogenous food. At the beginning of the experiment, the 4 – DAH larvae of *B. lohachata* had average initial weights of 0.39±0.09 mg and average initial total lengths of 4.08 ± 0.21 mm, when exogenous feeding was initiated. They were counted individually and distributed at a density of 10 larvae L-1 of water, resulting in a total of 2,400 larvae used in the experiment.

An intensive hatchery system consisting of 24 experimental units with a useful volume of 10 L each and an independent water inlet and outlet, was used. Each unit had continuous circulation with a mechanical, biological, and ultraviolet filter system, in addition to temperature control using a heater equipped with a thermostat. The flow was regulated to allow for 14 water renewals per day in the experimental units.

During an experimental period of 30 days, the loach larvae were subjected to six treatments, with four replications per treatment, as outlined in figure 1.

Description of treatments

LF= supply of live food throughout the 30–day experimental period; C_{12} = 6 days of live food + 12 days of co-feeding (live food and inert food) + 12 days of inert food; C_8 = 6 days of live food + 8 days of co-feeding + 16 days of inert food; C_4 = 6 days of live food + 4 days of co-feeding + 20 days of inert food; IF= exclusive supply of inert food from the first exogenous feeding; NC= larvae subjected to fasting as a negative control.

Newly hatched *Artemia franciscana* nauplii (22–24 h) were used as live food. The daily amount of nauplii provided from the first to the sixth day was 150 nauplii/larva/day. From the seventh day, 300 nauplii/larva/day were offered. In the co-feeding treatments, the amount of nauplii was gradually reduced in the last three days to 75%, 50%, and 25% of the total offered, respectively. In the LF group,

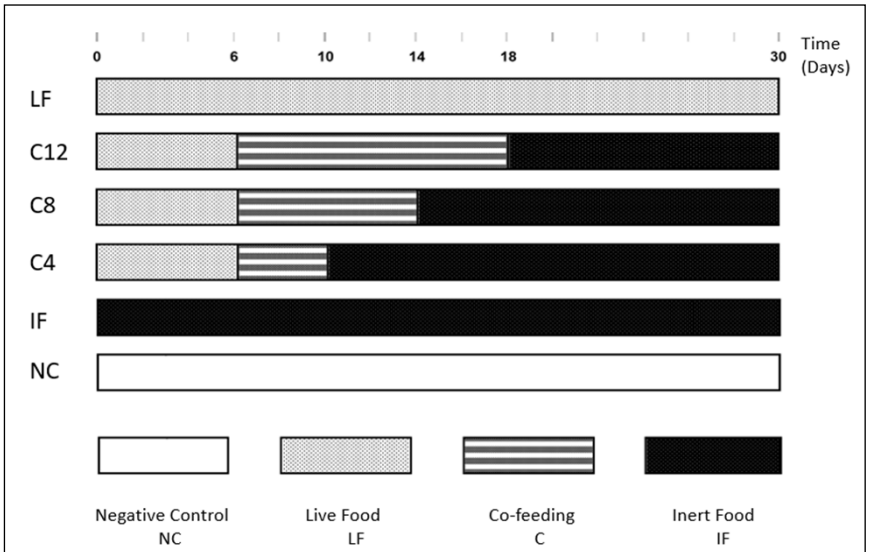

Figure 1 - Food supply and food transition scheme for yoyo loach *Botia lohachata* larvae. LF: live food; C12: 12 days of co-feeding + 12 days of inert food; C8: 8 days of co-feeding + 16 days of inert food; C4: 4 days of co-feeding + 20 days of inert food; IF: exclusive supply of inert food from the first exogenous feeding; NC: larvae subjected to fasting as a negative control.

after the eighteenth day, the amount was increased to 600 nauplii/day/larva. To quantify the nauplii, they were siphoned from the incubator, concentrated on sieves, and stored in a graduated beaker, containing 2 L of saline water under aeration. To count the nauplii, three 1 ml samples were collected and diluted 10 times. Thereafter, 1 ml subsamples were collected and quantified under a stereoscopic microscope. Based on the mean value obtained, the total number of available nauplii was calculated and consequently, the volume required for each treatment.

Extruded commercial feed was used as the inert food, sold in powder form, as indicated for the initial stages of carnivorous and omnivorous tropical fish. The commercial feed contained guaranteed levels of protein (min): 55.0 %; ethereal extract (min.): 10 %; raw fiber (max.): 5.0 %, calcium (max.):2.0 %, phosphorus (min.): 1.0 %, ash (max.): 10.0 %, and vitamin C: 500,0 mg.

The feed was passed through different sieves to obtain two particle size classes (142–350 µm and 350–500 µm). The smaller sized feed particles (142–350 µm) were offered until the eighteenth day, which was then replaced by the larger sized (350–500 µm) feed particles.

For food management, live food was provided first, followed by inert food for approximately 5 min. Inert food was provided ad libitum for the treatment groups that exclusively received inert food. For the other groups, both live food and inert food was provided three times a day, always at 8:00 am, 12:00 pm, and 5:00 pm.

An artificial light program was not used and the experiment followed the natural photoperiod with approximately 13 h of light. The oxygen levels, temperature, and pH of the water were monitored daily at 09:00 am. Oxygen levels and temperature were monitored with an oximeter and pH was measured with a pH meter, both instruments were digital and had two-place accuracy. The total ammonia (mg L-1) was measured at the beginning and end of the experiment.

The water quality variables measured daily were similar in the six treatments, with mean temperature values of 28.8 ± 0.1 °C, pH 6.9 ± 0.1, and dissolved oxygen 6.3 ± 0.2 mg L-1. Non-ionized ammonia was monitored weekly and remained below 0.01 mg L-1 during the experimental period.

The drainage system screens, filter wool, and supply system hose were cleaned every 5 days. The experimental units were siphoned daily at 2:00 pm to remove feces, food remains, and dead larvae. Mortality was recorded immediately after the first treatment and during the cleaning of the experimental units; in both cases, dead larvae were removed with the aid of a pipette.

To assess growth and survival, an initial biometric analysis was performed, followed by four intermediate biometrics (n = 5 larvae) at the beginning and end of each co-feeding period, and a final one was performed at the end of the experiment (n = 10 larvae for each experimental unit). For the intermediate biometrics, the larvae were only removed from the first repetition of each treatment. Only data from three replicates were included in the analysis of final survival and growth. The total length and wet weight of the larvae were measured. From the biometric data, the following parameters were calculated: weight/length ratio, weight gain = final weight – initial weight, specific growth rate (SGR) = (ln final weight – ln initial weight) × 100/time interval (days), and survival (number of final larvae / (number of initial larvae – no. larvae collected for biometrics) × 100), for each experimental unit.

After measuring weight and length in all biometrics, the five larvae were fixed in Carson's formalin for histological analysis, whereby the larvae were embedded in paraffin blocks. Five micrometer thick cuts were obtained using a LEICA semi-automatic microtome, using steel knives. Subsequently, the sections were stained using the hematoxylin-eosin (HE) method.

The experiment was conducted using a completely randomized design, and the data obtained through biometric measurements, presented as mean ± standard deviation, were subjected to parametric analysis of variance (ANOVA), considering the results of the repetition. For results that showed significant differences between treatments ($P < 0.05$), the means were compared using Tukey's test. Survival and SGR data for weight and length were transformed into $y = \arcsin \sqrt{x}/100$, where x is the survival value in percentage. All analyses were performed using SAS software (version 9.0).

RESULTS AND DISCUSSION

The survival rates of the larvae in treated groups during the observed periods are presented in table 1. The larval survival after 30 days ranged from 41% to 53%. The different co-feeding periods did not influence the survival of *B. lohachata* larvae. There was no significant difference ($P > 0.01$) in the survival of larvae subjected to treatments LF, $C_{12,}$ $C_{8,}$ and $C_{4,}$ in all periods.

There was high mortality in the initial period (4 – 10 DAH) for all treatments. The recorded mortality could not be attributed to a possible effect of the treatments, because the high mortality rates of larvae due to starvation (group NC; negative control) were concentrated from 10 DAH. Previous studies only found high mortality in pacu larvae after 18 DAH when they were subjected to fasting and fed exclusively with an inert diet (LEITÃO et al., 2011; MENOSSI et al., 2012).

In the larvae older than 10 DAH, after 6 days of the experiment, the survival rates in the treatments using brine shrimp were above 82.5%; however, in the other treatments, even after the initial period of 6 days, there was high mortality. However, there was

Table 1 - Survival behavior of yoyo loach larvae during the experimental period.

Periods	----------Treatments----------					
	LF [8]	C_{12} [9]	C_8 [10]	C_4 [11]	IF [12]	NC [13]
04 DAH [1]	100	100	100	100	100	100
10 DAH [2]	53.7 ± 12.9 a	48.7 ± 2.5 a	51.0 ± 6.2 a	49.7 ± 5.7 a	23.0 ± 4.4 a	51.0 ± 17.8 a
14 DAH [3]	53.3 ± 12.3 a	48.7 ± 2.5 a	51.0 ± 6.2 a	49.3 ± 5.9 a	20.7 ± 4.0 b	9.3 ± 2. b
18 DAH[43]	53.3 ± 12.3 a	48.3 ± 3.1 a	50.7 ± 5.9 a	49.0 ± 6.1 a	15.3 ± 6.7 b	6.3 ± 1.2 b
22 DAH [5]	53.3 ± 12.3 a	48.3 ± 3.1 a	50.3 ± 5.5 a	47.3 ± 4.2 a	10.3 ± 4.9 b	5.0 ± 2.0 b
28 DAH [6]	53.3 ± 12.3 a	48.0 ± 3.6 a	49.3 ± 6.4 a	43.3 ± 3.5 a	8.0 ± 3.6 b	3.4 ± 2.9 b
33 DAH [7]	53.3 ± 12.3 a	46.7 ± 4.0 a	44.0 ± 7.0 a	41.0 ± 5.6 a	5.3 ± 5.0 b	1.7 ± 2.9 b
----------Statistical Analysis of Final Means----------						
10 to 33 DAH	99.3 a	95.9 a	86. 3 a	82.5 a	23 b	3.3 c

Day after hatching: [1] 04 day, [2] 10 day; [3] 14 days; [4] 18 day; [5] 22 days; [6] 28 days; [7] 33 days; 8; [9]live food; [10] 6 days of live food + 12 days of co-feeding (live food and inert food) + 12 days of inert food; [10] 6 days of live food + 8 days of co-feeding + 16 days of inert food; [11] 6 days of live food + 4 days of co-feeding + 20 days of inert food; [12] exclusive supply of inert food from the first exogenous feeding; [13] larvae subjected to fasting as a negative control; different superscript letters in the same column are differentiated to 0.05 significance by Tukey's test.

no improvement in this variable. In the fasting group, less than 4 % of the larvae that were viable after 10 days of the experiment were alive on day 33. There was no significant effect ($P > 0.01$) on the survival of larvae treated with live food, including the shortest period of co-feeding. Normally, early suppression of live food affects the growth and survival of altricial larvae (LEITÃO et al., 2011).

High mortality in larvae from 4 – 10 DAH; Larvae surviving until the end of treatments IF and NC, where no food was offered in the latter, demonstrated a strong ability to survive on a probable bacterial film formed at the bottom of the experimental unit (daily cleaning), combined with the cannibalization of dead larvae before they were removed. The larvae in these two groups also swam upside down in the experimental units, apparently looking for food, and exhibited negative phototaxis, being attracted to dark objects. This is a descriptive analysis of the results and further studies are needed to better understand the biology of this poorly-studied species. The statistical analysis between the means of survival data, from the tenth day after hatching, can be seen in table 1.

The LF treatment larvae had significantly higher ($P < 0.01$) mean values for all performance variables analyzed at the end of the experiment (Table 2), followed by those subjected to co-feeding. The specific growth rates in terms of weight and length of larvae subjected to C_{12} and C_8 did not differ from each other and were superior to those of C_4. MENOSSI et al. (2012) verified the best growth and survival performance, as well as faster organogenesis of the digestive system, among pacu larvae fed exclusively with live food after 23 days of experimentation.

Although, the weight and length averages of the larvae subjected to treatment IF did not differ from those of treatment NC (fasted larvae), their respective specific growth rates indicate the superiority of larvae in the IF treatment group, both in weight and length (Table 2). The results of the weight and length variables (Figure 2), obtained through the intermediate biometric measurements during the experiment, confirmed the superiority of LF and treatments involving co-feeding.

The results obtained from the analysis of the histological slides indicated that at the end of the experiment, the larvae that received brine shrimp in their food had better zootechnical performance and organogenesis of their digestive system, which was notably more developed because intestinal villi had formed and there was a thickening of the muscular layer of the intestines, than the larvae of the fasting treatment, which did not present either villi or a muscular layer in the intestine, and of the diet treatment, in which the villi were beginning to form and did not present with continuous intestinal musculature after 30 days of the experiment (Figure 3).

In figure 3, it can be seen that in NC, where the larvae were fasted as a negative control, there was no formation of intestinal villi and an absence of intestinal musculature layers. In IF, where the exclusive supply of inert food took place, the formation of intestinal villi was initiated and a thin muscular layer could be seen when the first exogenous feeding began. In treatments C_4 (6 days of live food + 4 days of co-feeding + 20 days of inert food); C_8 (6 days of live food + 8 days of co-feeding + 16 days of inert food); C_{12} (6 days of live food + 12 days of co-feeding (live food and inert food) + 12 days of inert food) and LF (supply of live food during the entire experimental period), it was possible to see the formation of intestinal villi and the muscular layer of the thick intestine in several intestinal segments.

Table 2 - Zootechnical performance of yoyo loach *Botia lohachata* submitted to different feeding protocols during 30 days of larviculture.

Treatments	weight (mg)	SGR [7] (weight) (% day-1)	Lenght (mm)	SGR [7] (Length) (% day-1)
LF [1]	192.8 ± 57.6 [a]	20.5 ± 0.9 [a]	25.4 ± 2.7 [a]	6.1 ± 0.3 [a]
C_{12} [2]	122.1 ± 41.8 [b]	18.9 ± 1.3 [b]	20.4 ± 2.5 [b]	5.3 ± 0.4 [b]
C_8 [3]	89.2 ± 23.7 [c]	18.0 ± 0.9 [b]	18.5 ± 1.7 [b]	5.0 ± 0.3 [b]
C_4 [4]	55.6 ± 17.5 [d]	16.4 ± 1.0 [c]	15.6 ± 1.7 [c]	4.5 ± 0.4 [c]
IF [5]	19.0 ± 8.9 [d]	12.7 ± 1.3 [d]	11.3 ± 1.64 [d]	3.4 ± 0.5 [d]
NC [6]	7.7 ± 5.6 [d]	8.9 ± 3.0 [e]	8.8 ± 2.4 [d]	2.5 ± 0.9 [e]

[1] live food; [2] 6 days of live food + 12 days of co-feeding (live food and inert food) + 12 days of inert food; [3] 6 days of live food + 8 days of co-feeding + 16 days of inert food; [4] 6 days of live food + 4 days of co-feeding + 20 days of inert food; [5] exclusive supply of inert food from the first exogenous feeding; [6] larvae subjected to fasting as a negative control; [7] specific growth rate; different superscript letters in the same column are differentiated from each other; different superscript letters in the same column are differentiated to 0.05 significance by Tukey's test.

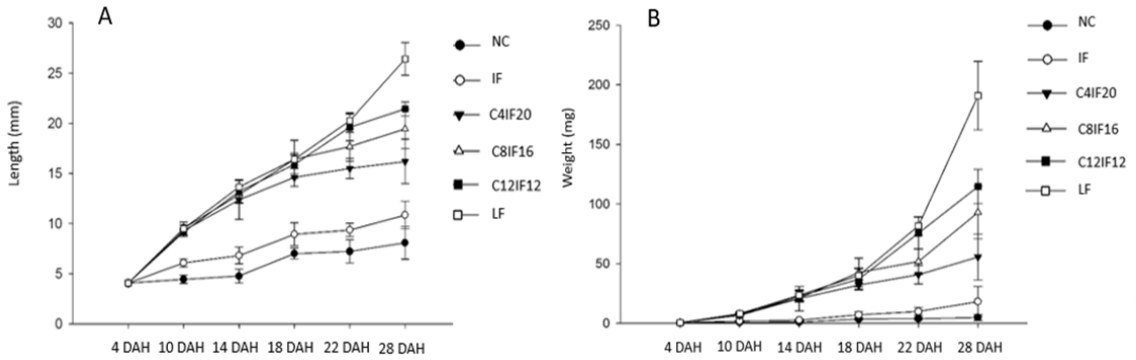

Figure 2 - (A) Weight (mg) and (B) length (mm) of yoyo loach *B. lohachata* larvae submitted to different feeding protocols, obtained in the intermediate biometrics. NC: larvae subjected to fasting as a negative control; IF: exclusive supply of inert food from the first exogenous feeding; C4IF20: 4 days of co-feeding + 20 days of inert food; C8IF16: 8 days of co-feeding + 16 days of inert food; C12IF12: 12 days of co-feeding (live food and inert food) + 12 days of inert food; LF: live food.

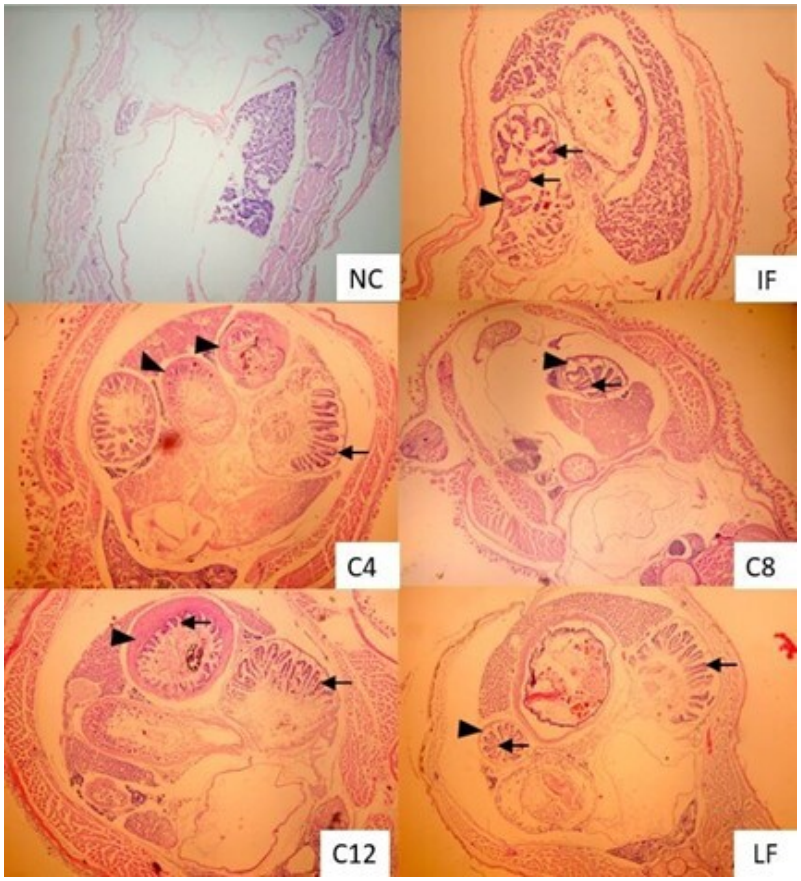

Figure 3 - Photomicrograph of histological sections of intestine of yoyo loach *Botia lohachata* larvae 33 days after hatching (DAE), indicating intestinal villi (arrow) and the muscular layer (arrowhead). NC: larvae subjected to fasting as a negative control; IF: exclusive supply of inert food from the first exogenous feeding; C4: 4 days of co-feeding + 20 days of inert food; C8: 8 days of co-feeding + 16 days of inert food; C12: 12 days of co-feeding + 12days of inert food; LF: live food.

As observed for *B. lohachata*, there are reports of ontogenic delay in the digestive system of larvae of different species when there was a suppression of live food in their initial feeding (JOMORI, 2005; ENGROLA et al., 2009; MENOSSI et al., 2012). Developed intestinal villi become important because they increase the absorption of food provided to individuals (JUNQUEIRA & CARNEIRO, 2013), thus allowing them to have greater zootechnical performance.

CONCLUSION

The *Botia lohachata* larvae need a longer period of live food supply before undergoing a food transition from live to inert food to avoid losses in growth rates in weight and length. The early replacement of live food with shorter co-feeding periods, has a greater impact on growth than on the survival of the species. The co-feeding strategy that included live food was able to stimulate the development of intestinal structures, which were more developed in fish fed brine shrimp from the beginning of the experimental period. Further studies should be conducted with greater diversification in terms of co-feeding days to obtain a more efficient hatchery protocol.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

The research project was approved by the bioethics committee in animal experimentation, of Universidade Estadual do Norte Fluminense Darcy Ribeiro, protocol nº 529.

AUTHORS’ CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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