

Original article

Ontogenetic development of tetra *Astyanax lacustris* (Characiformes: Characidae)

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Ontogenetic development of the tetra *Astyanax lacustris* was studied under laboratory conditions. Larvae obtained by induced reproduction were maintained individually in tissue-culture plates, at 25°C. Daily observations and morphometric measurements were performed. Larvae hatched with a total length (TL) of 3.02 ± 0.34 mm (average \pm standard deviation) without pigmentation and 0.06 ± 0.02 mm³ of yolk reserves. The yolk-sac larval period lasted 26 h post-hatching (hph). During this period, eye pigmentation started, the digestive tract emerged, the anus opened, and the animals began to show steady movements. Inflation of the swimming bladder initiates the preflexion stage, which extended until 230 hph. During this stage, the transition from endogenous to exogenous feeding was observed, with the yolk reserve being completely absorbed after 74 hph (TL: 4.17 ± 0.36 mm). Up to 86 hph it was possible to observe the first food ingested within the digestive tract. This period was followed by the flexion stage, with the folding of the notochord tip and development of the caudal fin (11–13 days post-hatching). Finally, during the last larval developmental stage (postflexion), the segmentation of the fin rays was completed, and the emergence of scales was observed (TL: 5.97 ± 0.65 mm). The larval ontogenetic development of *A. lacustris* was completed after 22 post-hatching days (dph).

Keywords: *Astyanax lacustris*, Larval morphometry, Notochord, Ontogeny, Swim bladder.

O desenvolvimento ontogenético do lambari *Astyanax lacustris* foi estudado em condições ambientais controladas. Larvas obtidas através de reprodução induzida, foram mantidas individualmente em placas de cultivo celular, a 25°C. Diariamente foram realizadas observações e medições morfométricas. As larvas eclodiram com $3,02 \pm 0,34$ mm (média \pm desvio padrão) de comprimento total (CT); sem pigmentation e com $0,06 \pm 0,02$ mm³ de reserva vitelínica. O período larval vitelino estendeu-se durante as primeiras 26 horas pós-eclosão (hpe) e durante este período teve início o processo de pigmentation dos olhos, o surgimento do tubo digestório, abertura anal e os animais passaram a apresentar movimentação constante. Com o inflamento da bexiga natatória tem início o estágio de pré-flexão, que se estendeu por até 230 hpe. Durante este estágio, foi observada a transição entre a alimentação endógena e a exógena, sendo a reserva vitelínica totalmente absorvida após 74 hpe (com os animais atingindo $4,17 \pm 0,36$ mm de CT) e em até 86 hpe foi possível observar a primeira alimentação no trato digestório. Após esse período, teve início o estágio de flexão da parte final da notocorda e da estruturação da nadadeira caudal (11 e 13 dias após a eclosão). Por fim, no último estágio de desenvolvimento larval (pós-flexão), foi observada a segmentação completa dos raios das nadadeiras e o início do aparecimento das escamas (CT: $5,97 \pm 0,65$ mm). O desenvolvimento ontogenético larval de *A. lacustris* foi concluído após 22 dias pós-eclosão (dpe).

Palavras-chave: *Astyanax lacustris*, Bexiga natatória, Morfometria larval, Notocorda, Ontogenia.

Introduction

Ordinarily, embryos and larvae are the most susceptible life stages to changes in environmental conditions, and these stages can get affected even by events of low intensity and short duration (Ramos *et al.*, 2015). Thus, studies of post-hatching fish development are often used to assess the effects of a variety of xenobiotics, such as heavy metals

(Sfakianakis *et al.*, 2015), silver (Barrera, 2013), and oil derivatives (Lema *et al.*, 2007; Ingvarsdóttir *et al.*, 2012), on the quality of aquatic environment (McKim, 1977; Meier *et al.*, 2010).

During the early stages of ontogenetic development, several metamorphic processes lead to the differentiation of specific structures, including the definition of food and reproductive characters of each species. These processes,

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combined with possible physiological limitations of the larvae, can cause high mortality rates within the first hours of life (Nakatani *et al.*, 2001; Russo *et al.*, 2014).

The exclusive use of morphometric indicators, such as length and weight, does not necessarily reflect the relations that have occurred during larval development (Maciel *et al.*, 2010). According to Gisbert (1999) and Kupren *et al.* (2014) the different morphometric changes associated with the allometric or isometric development of each region of the body, as well as larval movement and feeding, are parameters that allow better analysis of the initial ontogeny of fish, providing further accurate information on the normal development of the species.

Astyanax lacustris (Lütken, 1875), achieves 10 to 15 cm length and 60 grams in weight, and it is found in small streams to large rivers in watersheds of the neotropical environments in South America (Porto-Foresti *et al.*, 2005). The species has been increasingly used as a bioindicator in environmental studies (Ostrensky *et al.*, 2003; Nogueira *et al.*, 2009; Dal Pont, 2012; Siqueira-Silva *et al.*, 2015), due to its great trophic plasticity and diverse environmental compartments occupation (Peretti, Andrian, 2008). It has also been used as a model species in laboratory tests (Almeida, 2007; Vicente, 2014; Ostrensky *et al.*, 2015) among other reasons, for presenting short life cycle, small size and ease of reproduction and handling under controlled conditions (Garutti, 2003).

This study sought to describe the morphological processes of the tetra *Astyanax lacustris* by evaluating and individually identifying larvae transformations and reporting specific events occurring from hatching to the end of the postflexion period. This study may contribute to the use of this species as a model organism in several laboratory studies and assays.

Material and Methods

Broodstock management and hormonal induction. The specimens of *A. lacustris* used for the induced reproduction process were selected based on their secondary sexual characters. We selected only the males that presented spicules on their anal fin, based on the texture of the spicules, and selected females that presented red urogenital pore and bulging belly.

Mature individuals, deprived of food for 24 h, were removed from the breeding tank using a hand net and anaesthetized, to accordance with the method proposed by Vicente (2014), by using 35 $\mu\text{L L}^{-1}$ clove essential oil solution (10%). After morphometric evaluation, three females and nine males, with 24.1 ± 1.4 g and 5.2 ± 2.0 g (mean weight \pm standard deviation), and 9.9 ± 1.0 cm and 8.4 ± 0.3 cm (total length \pm standard deviation) respectively, were selected.

Selected broodfishes were housed in a final maturation system, consisting of a 100-L polyethylene tank connected to mechanical and biological filters through a submerged pump (SB-2000, Sarlobetter, Brazil), with constant aeration and temperature of 25°C.

For hormonal induction, we used crude carp pituitary extract (CCPE), which was administered in a single dose of 5.0 mg/kg for females and 1.0 mg/kg for males. The accumulated thermal unit (ATU) was calculated from the time of hormone administration and ranged between 190 and 200 hours-degree (HD).

Spawns. After hormonal induction, all broodfishes were placed in a tank-grid of 25 \times 15 \times 15 cm (length, width, height), made from a woven-nylon net of 3 mm and a mesh of 1.5 \times 1.5 cm, which was maintained in a 100-L polyethylene tank, at a controlled temperature of 25°C using a 300 watts heater (BOYU, Taiwan). The tank was also connected to an external 60 L physical and biological filtration system. The animals were kept in the tank-grid until ovulation and subsequent fertilization of oocytes.

Immediately after spawning, the eggs were removed by siphoning, retained on a 0.5-mm-mesh sieve and washed with tap water. Then, they were collected with a Pasteur pipette, and observed using an optical microscope Leica® DMLS fitted with a digital camera Dino-Eye (AM-423X, Taiwan). Eggs were then transferred into eight tissue-culture plates, each with six wells and a lid (Kasvi K12-006, China). Each well had a total volume of 17 ml and contained one fertilized egg. The plates were kept in vertical incubators B.O.D. (ADAMO, Brazil), and maintained with 12 h light/12 h dark photoperiods at 25°C.

Morphology and morphometric measurements were obtained using a stereomicroscope Leica® MZ6, with a magnification of 0.63 \times and 0.8 \times , fitted with a Dino-Eye camera (AM-423X, Taiwan). Larval development and the number of heartbeats were recorded daily using the optical microscope Leica® DMLS. Differentiation of the various stages of larval development was performed as described by Nakatani *et al.* (2001) (Tab. 1.), with some modifications. The evolutionary timeline was described in hours-degree (HD), accumulated thermal unit (ATU), hours post-induction (hpi), minutes post-hatching (mph), hours post-hatching (hph), and days post-hatching (dph).

Tab. 1. Description of *Astyanax lacustris* developmental stages, from hatching until the end of the initial period of development.

Stage	Description
Yolk-sac larval	From hatching until the beginning of the development of the digestive tract, which is characterised by the opening of the oral and anal cavities and the appearance of the swim bladder;
Preflexion	From the appearance of the swim bladder until the beginning of the distal folding of the notochord. Also featured by the beginning of the segmentation of the pectoral and anal fin rays;
Flexion	Development of the dorsal and caudal fin rays, and total folding of the distal tip of the notochord;
Postflexion	Characterised by the total segmentation of the anal, dorsal, and caudal fin rays, and the beginning of the development of scales.

Larval morphology and morphometry. To perform the morphological analysis and morphometric measurements the tissue-culture plates were individually removed from the B.O.D. incubator. The water from each well was then reduced to 3/4 of its original volume using a Pasteur pipette, and then the animals were individually observed in the wells using the stereomicroscope.

General observations were performed at regular intervals of 60 min from hatching to full absorption of the yolk reserves; every 12 h from total absorption of the yolk reserves until complete folding of the notochord; and every 24 h from this point until the end of the postflexion stage.

When 50% of the animals had completely absorbed their yolk reserves, all animals of the experiment were fed with 0,1 g the microencapsulated ration (American B.P., USA), containing 46% protein, weighed on a precision scale (Shimadzu-AUY220, Japan), and then dissolved in 40 ml of water. By using a Pasteur pipette, one drop of this suspension was given to each larva every day at 18:00h.

The animals were examined under an optical microscope (4X), and had their morphological variables recorded based on the development of internal and external structures. We established the developmental stage by taking into consideration the time at which all other remaining animals completed the development of this structure.

Morphometric analyses involved the measurement of the following parameters: i) Total length (TL), measured as notochord length in preflexion and flexion larvae, and as standard length in postflexion larvae; ii) body height (BH); iii) head length (HL); iv) head height (HH); v) eye diameter (ED); vi) yolk-sac length (YL); vii) yolk-sac height (YH); and viii) yolk reserve volume (V), calculated according to the equation:

$$V (mm^3) = \frac{(\pi \cdot YL \cdot YH^2)}{6}$$

At the end of each observation, the original volume (*i.e.* 17 ml) of water in each well was refilled, and the plates were again covered with the lid, re-housed in the incubator, and maintained until the following period of observation. To ensure temperature control, the room where the tests were conducted was also maintained at 25°C.

Water quality. The water used for daily renewal of the culture wells was collected from a polyethylene water tank (1000 L) linked to a biological and mechanic air-lift-type filter, being then filtered in a bag (45 µm), chlorinated, and neutralized with sodium thiosulfate. Before use, the water was kept in an incubator in order to reach 25°C.

Due to the low volumes, the siphoned water from the wells was concentrated in a beaker and analyzed as a single sample. The water collected from the wells and the water used for refills were both daily analyzed for the following physicochemical parameters: pH, using a digital pH meter (AZ-86505, Taiwan); dissolved oxygen (DO) and oxygen saturation percentage (%SO₂), using a digital oximeter (YSI Pro20, USA); alkalinity

and carbon dioxide (CO₂) concentration, measured by titration with 0.02N sodium hydroxide solution (APHA 2005); and nitrogen concentration in the form of total ammonia [N-AT = NH₃ + NH₄⁺], obtained using the indophenol method (APHA 2005) and the nitrite method (APHA 1995). Colorimetric analyses were performed on a bench spectrophotometer (Spectronic 20, Genesys, USA).

Statistical analysis. A database was created and organized using the Excel[®] and Access[®], Microsoft Office Professional Plus 2013. Water quality data was analyzed using the Shapiro–Wilk normality test, followed by the Mann–Whitney *U* test. Biometric data were compared using descriptive statistics. Survival was analysed by the Kaplan–Meier method. All analyses were performed using the Statsoft Statistica^{MR} version 12.0[®].

Results

Yolk-sac stage (0 - 26 hph). After hatching, the head the larvae of *A. lacustris* is attached to the anterior region of the yolk-sac, resembling a distended posture, and the larvae have no fins. Some larvae hatch without pigmentation, whereas others present pigmentation at the edge of the yolk-sac. At this developmental stage, all larvae have an embryonic membrane and a rudimentary digestive tract with no anal opening. During the first hours after hatching, pigmentation of the eyes is initiated together with great development of the embryonic fin membrane.

The head is observed in three different positions during the first hours of larval development. Initially, it is attached to the yolk-sac, separated by the perivitelline space. About 10-12 hph, the head assumes a semi-terminal position and followed by a terminal position 20 h later. During this stage, the mouth also undergoes significant transformation, moving from a ventral to a terminal position (Fig. 1).

The yolk-sac stage was the one with the shortest duration (between 18 and 26 hph). This stage ends when the swim bladder, located above the digestive tract and below the notochordal segment, becomes functional.

The size of the post-hatching larvae ranged from 2.28 to 3.31 mm. Two hph, we observed the beginning of pigmentation of the yolk-sac frontal region and the eyes. Between 5 and 7 hph, the TL was 2.99 ± 0.31 mm (mean ± standard deviation), and it was possible to identify the digestive tract, with a straight morphology, and the anal opening.

Preflexion (27 - 230 hph). After inflating with air, the swim bladder, along with the other swim structures (caudal and pectoral fins), supported a steady larval movement. At this stage, we observed an increased pigmentation particularly in the eyes, which were completely dark at 32 hph.

The development of the four gill arches rapidly occurred a few hours later (37 hph). The movement of the operculum, the constant movements of the pectoral fins, and the blood flow were now synchronized with the heartbeats (Fig. 2).

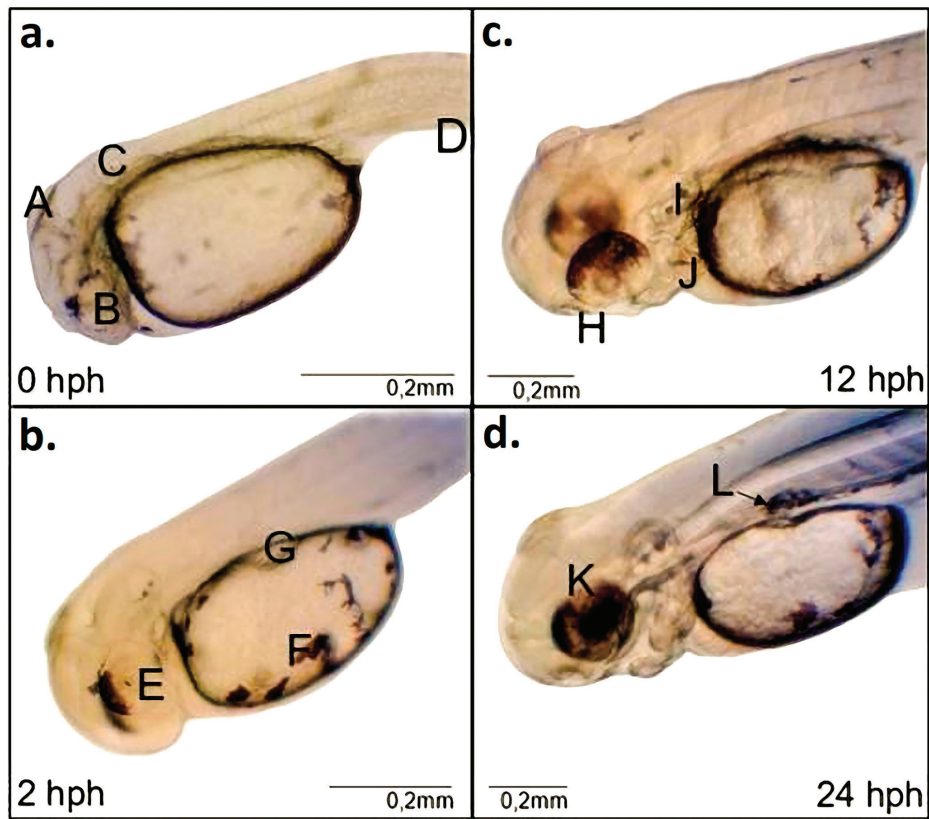


Fig. 1. Development of *Astyanax lacustris* during the yolk-sac larval stage: **a.** newly hatched larva, with adhesive gland (A), eyes with little pigmentation (B), the optic vesicle (C), and no anal opening (D); **b.** increased pigmentation of the eye (E), increased pigmentation of the yolk reserves (F), and button of the pectoral fin (G); **c.** mouth in ventral position (H), further development of the optic vesicle (I), and pigmentation of the heart (J); **d.** pigmented eyes (K) and swim bladder (L).

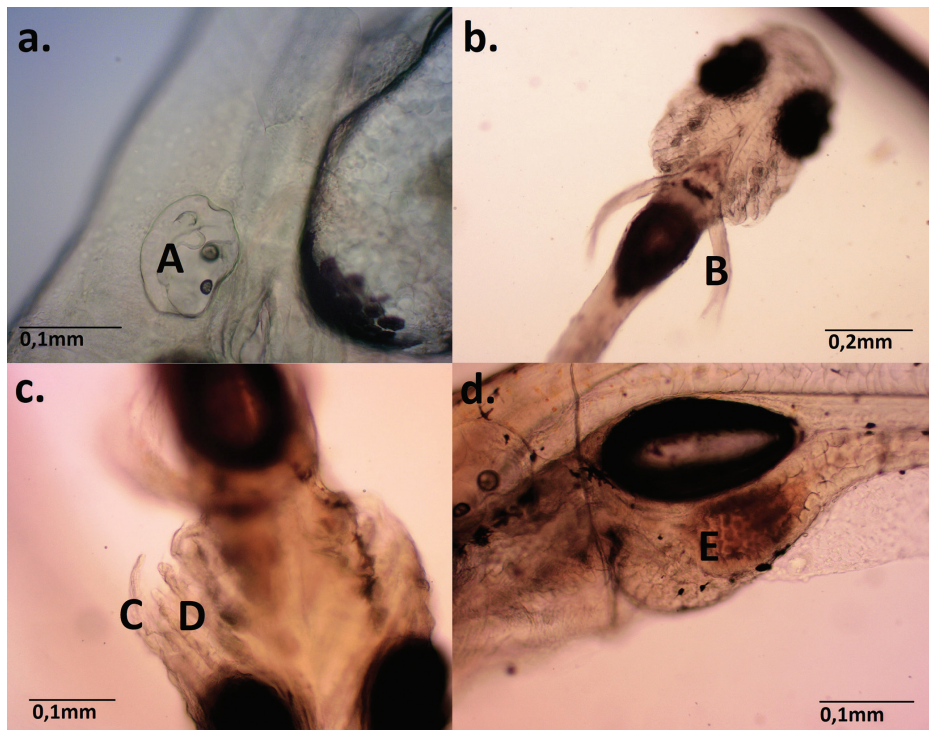


Fig. 2. Development of *Astyanax lacustris* during the preflexion stage: **a.** optical vesicle (A); **b.** fins pectoral (B); **c.** operculum (C), gill arches (D); **d.** food in the digestive tract (D).

This was the most critical time for the survival of the animals because abrupt morpho-physiological changes occurred in some larvae (Tab. 2), especially in those whose yolk reserves had been exhausted (74 hph) and still had no food in the digestive tract (86 hph).

During this stage, there was an increase in body pigmentation. The animals began to show a greater amount of dendritic chromatophores, which were mostly distributed above the notochord, and punctate chromatophores, mainly distributed in the upper region of the head. The key event for the transition to the following developmental stage was the beginning of the folding of the notochord distal part. More precisely, the folding of the tip of the caudal fin.

Flexion (231 - 314 hph). During the flexion stage, complete pigmentation of the eye (retina) was observed. At the beginning of the flexion stage, on average 30 myomeres were noted (Fig. 3a). Bodies were more pigmented (dark brown) when compared with the previous stage. During this period, the rays of the anal fin became structured, concurrent with the folding of the distal tip of the notochord, forming a 45° angle (Fig. 3b).

When folding of the notochord was completed, 7-11 (minimum–maximum) rays were observed in the upper region of the caudal fin, and 8-18 rays in the lower region.

Tab. 2. Chronology of the main morphological events from hatching until complete absorption of the yolk reserves of the tetra *Astyanax lacustris*. *Time expressed as minimum–maximum observed; **Values are expressed as means and standard deviation.

Time* (mpf)	Timeline		Larval Stage	Size** (mm)	Observed morphological event
	hph	ATU			
80 - 120	1 - 2	25 - 50	Yolk-sac larval	2.99 ± 0.32	Beginning of eye pigmentation
120 - 240	3 - 4	74 - 100	Yolk-sac larval	2.99 ± 0.24	Beginning of blood pigmentation
300 - 360	5 - 6	125 - 150	Yolk-sac larval	3.00 ± 0.24	Formation of the digestive tract
300 - 420	5 - 7	125 - 175	Yolk-sac larval	3.00 ± 0.24	Opening of the anal cavity
360 - 420	6 - 7	150 - 175	Yolk-sac larval	3.00 ± 0.24	Observation of a gill arch
480 - 600	8 - 10	200 - 250	Yolk-sac larval	3.01 ± 0.32	Head detached from yolk-sac
660 - 720	11 - 12	275 - 300	Yolk-sac larval	3.03 ± 0.31	Head in semiterminal position
1140 - 1200	19 - 20	475 - 500	Yolk-sac larval	3.05 ± 0.31	Mouth in terminal position
1380 - 1440	23 - 24	575 - 600	Yolk-sac larval	3.07 ± 0.31	Development of the pectoral fins
1080 - 1560	18 - 26	450 - 650	Yolk-sac larval	3.04 ± 0.28	Swim bladder inflated
1200 - 1560	20 - 26	500 - 650	Yolk-sac larval	3.05 ± 0.29	Development of the mandible
1800 - 1920	30 - 32	750 - 800	Preflexion	3.11 ± 0.29	Head in terminal position
1860 - 1920	31 - 32	775 - 800	Preflexion	3.12 ± 0.30	Eye pupil fully pigmented
1920 - 1980	32 - 33	800 - 825	Preflexion	3.13 ± 0.30	Completed formation of gill arches
2280 - 2460	38 - 41	950 - 1025	Preflexion	3.29 ± 0.29	Bundling of the intestine
2580 - 2700	43 - 45	1075 - 1125	Preflexion	3.42 ± 0.30	Appearance of the pharynx and oesophagus
3300 - 4440	55 - 74	1375 - 1850	Preflexion	3.92 ± 0.32	Complete absorption of yolk reserves
3720 - 5160	62 - 86	1550 - 2150	Preflexion	4.02 ± 0.30	First food in the digestive tract
5160 - 5880	86 - 98	2150 - 2450	Preflexion	4.16 ± 0.33	Disappearance of the adhesive gland

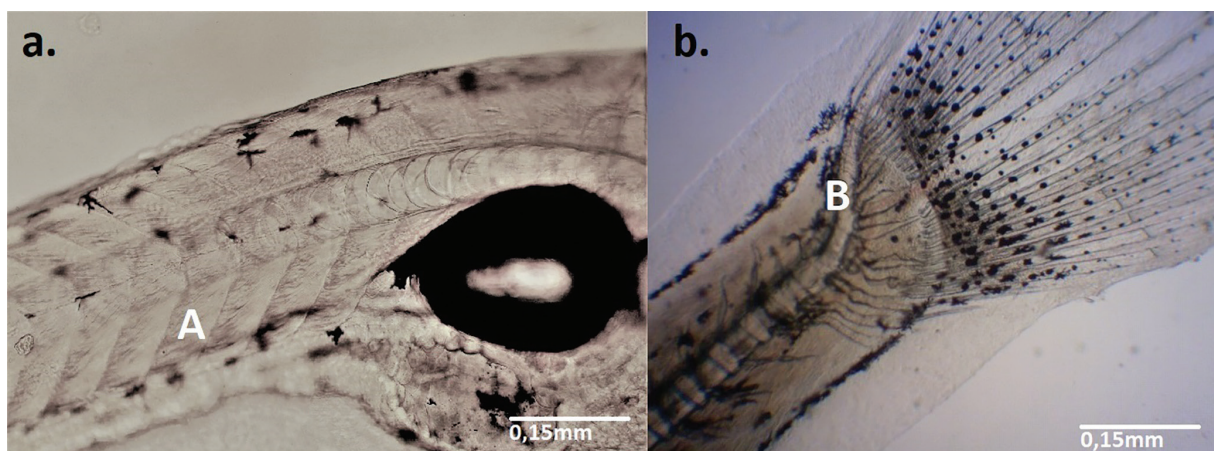


Fig. 3. Individual of *Astyanax lacustris* in the flexion stage: **a.** myomeres (A); **b.** flexion of the notochord (B).

Postflexion (315 - 542 dph). During the postflexion stage, increased pigmentation of the lateral region of the head and torso was noted. In the operculum, several chromatophores were observed. The swim bladder presented two compartments, an anterior and a posterior. By using *in vivo* microscopy, the presence of food in the intestinal folds could be observed. This visual identification of food was only possible at the beginning of the postflexion stage because during this stage the body becomes opaque; thus, after this stage, the differentiation of internal organs is no longer possible.

The first evidence of scales appeared at 17 dph (Fig. 4). Initially, they were concentrated in the ventral region, along with the swim bladder extension and the structures of the digestive tract and operculum. No myomeres could be observed at this stage.

Water quality in the experimental system. During yolk-sac larval development, there was no significant difference in the parameters measured to assess the quality of the water between maintenance (well) and water renewal ($p > 0.05$)

(as shown in Tab. 3). During the preflexion stage, there was a tendency to decrease of DO concentration, %SO₂ and alkalinity compared to those of the renewal water. The pH showed slight acidification compared to the maintenance water during the flexion and postflexion stages. Regarding the levels of total ammonia, nitrite, and CO₂, a significant increase was observed in the siphoned water from the tissue-culture wells. During the larval stages, there was an increase trend in the concentration of ammonia, carbon dioxide, and alkalinity. Other parameters did not show any correlation with larval development.

Survival. We noted two critical moments for the survival (Fig. 5) during larval ontogeny (Fig. 6). The first occurred during the feeding transition period, causing 28% mortality between 4 and 5 dph. The second occurred at the beginning of the notochord folding (between 10 and 11 dph, during which the survival rate was 36%. From 18 dph onwards, there were no more deaths, and the final survival rate was 8%.

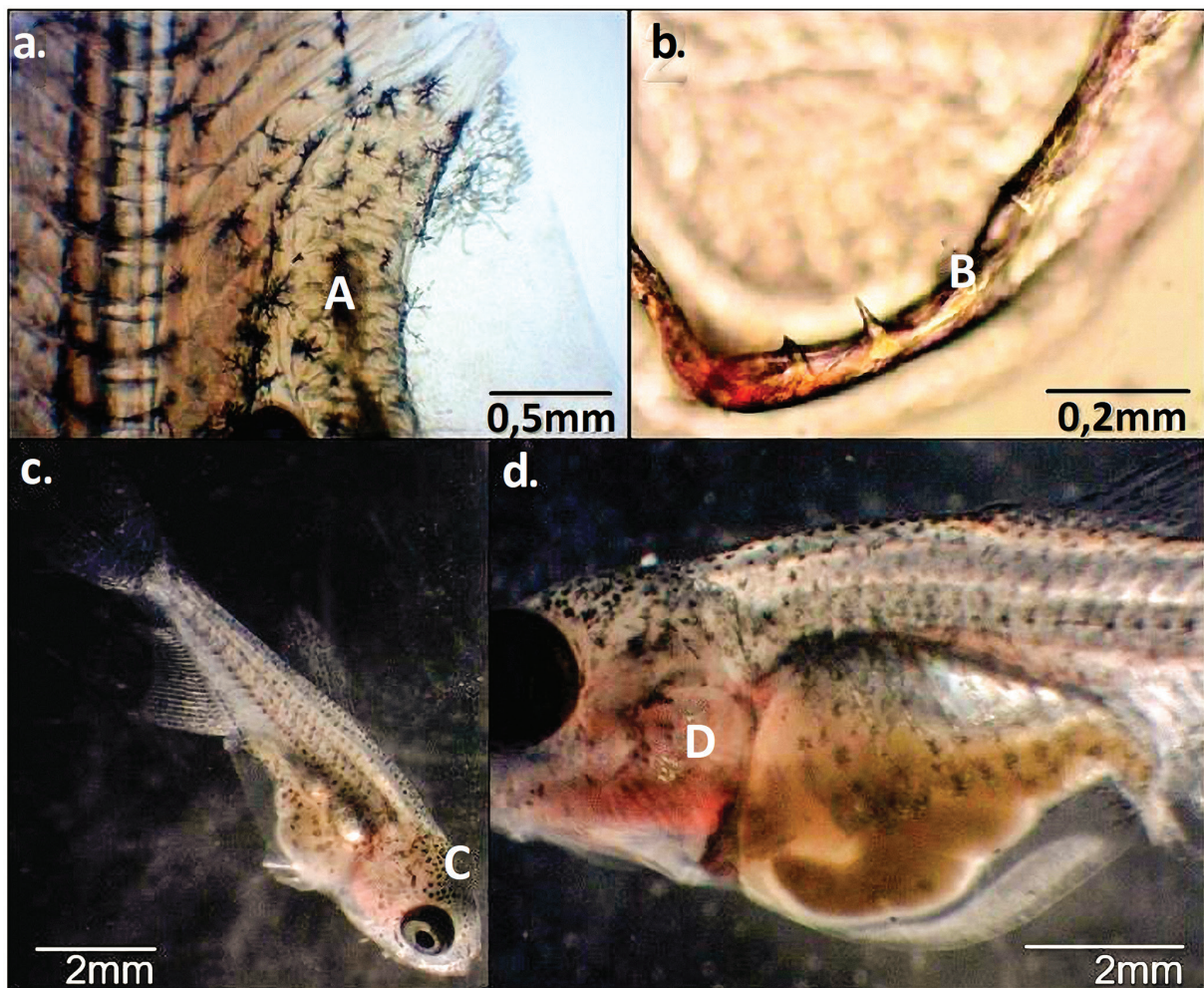


Fig. 4. Specimens of *Astyanax lacustris* in the postflexion stage: **a.** presence of food in the digestive tract (A); **b.** teeth (B); **c.** punctate pigmentation on the dorsal region of the head (C); and **d.** scales (D).

Tab. 3. Water quality parameters of the tissue culture plates system measured at each developmental stage of *Astyanax lacustris*, before and after water renewal. * Values are expressed as medians and ranges (min–max). Lowercase letters (superscript) indicate significant differences ($p < 0.05$) of water quality parameters between times of analysis (*i.e.* measured before and after water renewal), in each of the developmental stages. Capital letters (superscript) indicate significant differences ($p < 0.05$) of water quality parameters between stages: YX: Indicates significant differences between water quality parameters used for renewal of culture plates (Before); AB: Indicates significant differences between the water quality parameters within the culture plates along each stage (After).

Stage		Parameter						
		pH	OD (mg.L ⁻¹)	OD (% Sat.)	N-AT (mg.L ⁻¹)	N-NO ₂ ⁻ (mg.L ⁻¹)	CO ₂ (mg.L ⁻¹ CaCO ₃)	Alcalinity (mg.L ⁻¹)
Yolk-sac larval	Renewal	7,38	6,61	77,80	0,02	0,01	0,44	50,60
	Maintenance	7,63	5,71	58,90	0,05	0,09	0,45	50,20
Preflexion	Renewal	7,52 (7,25-7,82)	6,71 ^{a*} (6,02-6,92)	85,90 ^a (72,10-90,50)	0,03 ^{aY} (0,02-0,04)	0,01 ^a (0,01-0,01)	0,50 ^a (0,27-0,57)	68,00 ^{aY} (55,60-82,80)
	Maintenance	7,34 (5,58-7,68)	3,54 ^b (3,25-5,89)	47,90 ^b (44,60-63,70)	0,43 ^{bA} (0,06-0,48)	0,15 ^b (0,06-0,19)	1,04 ^{bA} (0,62-1,18)	27,80 ^{bA} (14,40-46,50)
Flexion	Renewal	7,46 ^a (7,34-7,68)	6,41 ^a (6,31-6,52)	83,60 ^a (80,20-84,50)	0,02 ^{aXY} (0,02-0,03)	0,01 ^a (0,01-0,02)	0,42 ^a (0,31-0,56)	45,80 ^{aXY} (44,00-58,00)
	Maintenance	6,70 ^b (6,61-6,74)	3,92 ^b (3,71-4,07)	49,06 ^b (45,70-50,10)	0,52 ^{bB} (0,51-0,54)	0,17 ^b (0,14-0,18)	1,16 ^{bAB} (1,08-1,32)	38,20 ^{bAB} (36,00-41,00)
Postflexion	Renewal	7,71 ^a (7,37-7,83)	6,49 ^a (6,02-7,05)	83,95 ^a (71,70-102,80)	0,02 ^{aX} (0,01-0,03)	0,01 ^a (0,01-0,02)	0,42 ^a (0,28-3,05)	53,00 ^{aX} (41,80-59,40)
	Maintenance	6,77 ^b (5,76-7,05)	3,83 ^b (3,15-4,43)	47,00 ^b (43,80-56,60)	0,51 ^{bB} (0,44-0,73)	0,13 ^b (0,12-0,18)	1,63 ^{bB} (1,29-1,88)	40,60 ^{bB} (29,00-48,60)

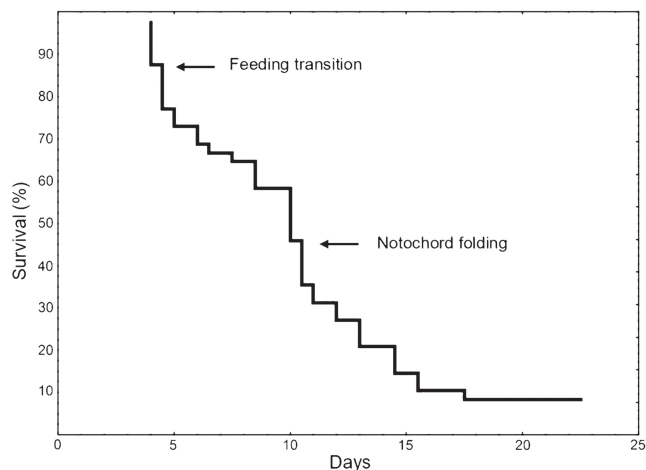


Fig. 5. Survival curve, from hatching to the end of the postflexion stage, of *Astyanax lacustris*. Arrows indicate the most critical periods for larval survival.

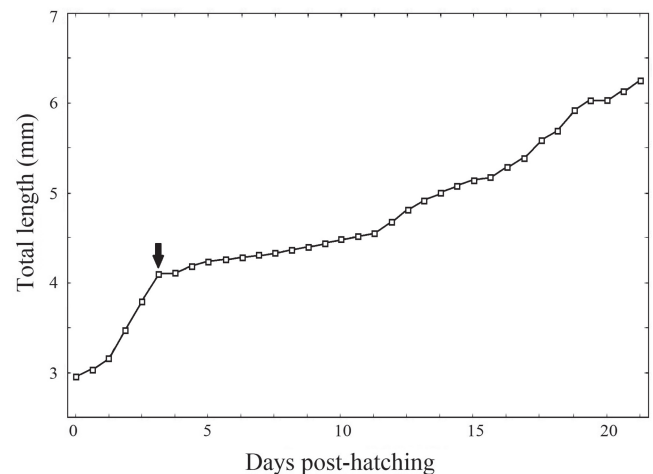


Fig. 6. Total length (mm) of *Astyanax lacustris* during its larval development. Arrows indicate the total consumption time of yolk reserves.

Discussion

According to Nakatani *et al.* (2001), most fish species present a relatively similar morphological post-hatching pattern. Some of the morphological characteristics mentioned in their study are also observed in our study, in the larvae of *A. lacustris*, such as the presence of small pigments formed by dendritic chromatophores, spread through the outer edges of the yolk-sac and near the eyes. In addition, the post-hatching morphometric parameters analysed in this study agree with those described by Nakatani *et al.* (2001) also for *A. lacustris* larvae, although in their study, specimens were collected from the natural environment.

An important characteristic of the *A. lacustris* larvae is the presence of the adhesive gland. According to Godinho *et al.* (2003), in the natural environment, this gland allows the larvae to remain in the hatching place when dragged by water currents, increasing their dispersion capacity. The gland was not recorded in animals after 98 hph.

During the first hours following hatching, there was greater pigmentation by dendritic chromatophores, spread throughout the region above the notochord, and by punctate chromatophores, scattered around the cranial region. According to Meijide, Guerrero (2000), studies concerning fishes body pigmentation are essential for the identification of larval stages in various species. Larvae of

the species *Atherinella brasiliensis* (Franca *et al.*, 2007) and *Rhinelepis aspera* (Perini *et al.*, 2010) have only one type of chromatophore. Other species have both types of chromatophores, such as *Pimelodus maculatus* (Buzollo *et al.*, 2011) and *Rhamdia quelen* (Amorim *et al.*, 2009). At the yolk-sac larval stage, we also observed the development of the digestive tract structures, in particular of those associated with the opening and functionality of the buccal cavity, together with the development of the digestive tube and anal opening.

With the appearance of the swim bladder and the development of the pectoral and caudal fins, the preflexion stage started. According to Trotter *et al.* (2003), as soon as the swim bladder becomes functional, the larvae need to capture atmospheric air. The development of these structures provides better swimming balance and control, increasing the potential for food capture and, consequently, the survival of larvae.

Regarding fin development, the pattern observed in this study was similar to that reported by Bialecki *et al.* (2001) for *Auchenipterus osteomystax*, which start with the metamorphosis of the embryonic caudal membrane. The same process is also observed in the larvae of *Danio rerio* (Parichy *et al.*, 2009), which is followed by appearance of the button of the pectoral fins (yolk-sac larval period), disappearance of the embryonic membrane, emergence of the caudal fin (preflexion stage), and development of the anal and dorsal fins in the postflexion stage.

The period length until the absorption of yolk reserves observed in this study differed from the one reported by Almeida (2007) for specimens of the same species. According to the author, full absorption of yolk reserves and the beginning of exogenous feeding occurred at 18 hph (yolk-sac larval stage). In the present study, these processes were observed only at 74 hph, close to the developmental time reported for larvae of *Brycon orbignyanus* (Maciel *et al.*, 2010; Nogueira *et al.*, 2014).

In Nakatani *et al.* (2001), total absorption of the yolk-sac occurred when the specimens of *A. lacustris* reached a TL of 4.50 mm (preflexion). However, in our work, the larvae had a TL of 3.90 mm, which can indicate the presence of genetic variations within the same species, or that the ontogenetic development in the laboratory differs from the patterns observed in nature. In natural environments, animals are often exposed to different water temperatures during early development, in contrast to laboratory conditions.

The transition period from endogenous to exogenous the nutrition is characterized by a high mortality rate during the first days of life. Between 62 and 86 hph, the presence of food in the digestive tract of larvae was observed, which indicates that the transition occurred when the specimens still had traces of yolk reserves. According to Guerrero Alvarado (2003), larvae do not immediately recognize inert artificial diets as food, which may explain the great range of time observed (about 24 h) between the record of food in the digestive tract in the first and last analysed

specimens of *A. lacustris*. Pittman *et al.* (2013) reported that some factors, such as TL and mouth size, are essential during the early stages to increase the chances of larvae survival, while they are related to a greater ability to capture food. The same authors also report that, in the absence of predators and with high food availability, this advantage no longer exists, since there are no literature reports showing that bigger larvae necessarily become bigger juveniles and adults. In addition, in laboratory studies, one of the main factors associated with the success of the fish feeding process is the adaptation of the early life stages to external foods, especially when using inert feeds. This process can be affected both by the type of food and by the structure where the animals are maintained. In this study, since it was not possible to promote continuous resuspension of food in the tissue-culture plates, the feed particles were sedimenting over time, hindering or even preventing the fish access to them.

When larvae reached an average TL of 4.93 mm (11-13 hph), folding of the notochord caudal tip started. To our knowledge, there are no other records of the time of notochord folding for this species. In larvae of *Brycon hilarii*, total folding of the notochord occurs when individuals reach a TL of 9.00 mm (Oliveira *et al.*, 2012). In larvae of *Pyrrhulina australis*, notochord folding occurs when the specimens reach a TL of about 5.33 mm (Taguti *et al.*, 2009).

During this stage of development, the number of pre- and post-anal myomeres ranged from 13 to 17 and 13 to 16, respectively. In Nakatani *et al.* (2001), the total number of myomeres in *A. lacustris* ranged from 32 to 37 during the postflexion stage. In our work, the maximum number of myomeres observed during the folding stage of the notochord was 33. There were no visible myomeres during the postflexion larval stage. The number of myomeres differentiates *A. lacustris* from other species of the Characidae family, such as *Pyrrhulina australis* (Taguti *et al.*, 2009), *Brycon orbignyanus* (Faustino *et al.*, 2011), and *Brycon hilarii* (Oliveira *et al.*, 2012).

According to Nakatani *et al.* (2001), individuals of the species *A. lacustris* reach the postflexion stage with a TL of 8.10 mm, and scales are visible when the specimens reach a TL of 13.08 mm. In our observations, the larvae reached the notochord postflexion stage with a TL of 4.98 mm and showed scales with a TL of 5.97 mm (17 hph). Again, these differences may be related to genetic variations or due to the laboratory conditions.

Although there are great individual variations in the number of fin rays, no studies are available regarding this morphological feature during the early development stages of *A. lacustris*. The rays of the pectoral, anal, caudal, and dorsal fins allowed differentiation of the *A. lacustris* specimens observed by Nakatani *et al.* (2001). This supports that, even after completion of the larval period, some morphological alterations may still occur in this species.

In order to use larvae in laboratory experiments, one of the challenges to overcome is water quality deterioration

in the experimental setting, which is caused by increased production of metabolic waste and decomposition of unconsumed diet. Such processes could be mainly noted by the increase in the concentrations of ammonia and CO₂ that occurred during the development of the individuals. In a study performed by Dal Pont (2012) on ammonia toxicity in adult *A. lacustris*, the CL₅₀ at 24 h was more than 100 mg L⁻¹. However, there is no detailed information on the toxicity of ammonia or CO₂ or about the critical levels of the other water quality parameters to the larvae of *A. lacustris*. Therefore, the hypothesis that the alterations in the baseline of these compounds in the water may have affected the growth rate or even the survival of animals cannot be excluded a priori.

Despite variations in water quality, larvae kept at 25 °C were able to survive (even in relatively low proportion), adapting to the intense and unavoidable manipulations to which they were subjected throughout the experiment. In addition, it was possible to observe that these animals effectively fed and showed an adequate development of their morphological and anatomical structures. The results corroborate the potential use of tetra, *Astyanax lacustris*, as model animals in laboratory trials.

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