

Research Article

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Spawning induction and embryonic development of *Salminus hilarii* (Characiformes: Characidae)

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Summary

The present study aimed to evaluate two different methods of artificial reproduction induction, and characterize the embryonic development of *Salminus hilarii*. Different than observed for other tropical fish species, artificial reproduction induction followed by hand-stripping of gametes was considered unfeasible for *S. hilarii*, as no gamete fertilization was observed. However, females that were induced and spawned naturally presented a fertilization rate of $65.64 \pm 0.54\%$. With a mean temperature of $26.20 \pm 0.90^\circ\text{C}$ it was possible to clearly distinguish a large perivitelline space at 14 min after fertilization (AF) and at 49 min AF more than 50% of the embryos presented two blastomeres, and these cleavages occurred until 1 h 54 min AF. The gastrula phase was characterized at 3 h 56 min AF, and blastopore closure was observed at 8 h 31 min AF. At 9 h 1 min, organogenesis started, with a clear distinction of the yolk mass, embryonic axis, cephalic and caudal regions; at 11 h 51 min AF the embryos already had advanced segmentation and a free tail. Total hatching occurred at 21 h 17 min and after opening the mouth, which occurred at 33 h 9 min, the larvae of *S. hilarii* presented a strong and characteristic cannibalism. This information can be considered fundamental to improving *S. hilarii* production in captivity and for collaboration with a conservation programme in the upper Tietê river basin.

Introduction

Salminus hilarii (Valenciennes, 1850), commonly known as *tabarana* or *dourado branco*, is a rheophilic teleost fish, carnivorous, medium-sized (first maturation in captivity: 29.40 ± 2.40 cm for females and 23.80 ± 0.70 cm for males), which can be considered of great importance in the food chain in the upper Tietê river basin (São Paulo/SP, Brazil). This species can also be found in rivers of the upper Paraná, São Francisco and Tocantins, upper Amazonas and upper Orinoco basins. Factors such as: (i) pollution, mainly from the residential and industrial effluents, (ii) population growth, (iii) eutrophication, (iv) fishing, and (v) dam construction has significantly reduced the *S. hilarii* natural stocks in the upper Tietê basin (Honji *et al.*, 2009, 2011, 2013; Araújo *et al.*, 2012).

As described by Agostinho *et al.* (2003), *S. hilarii* has preference for small water bodies and this, therefore, makes the species more susceptible to local extinctions from pollutions and impoundments. Similarly, dam construction for energy production or water storage directly interferes in the migratory course of rheophilic fish species, preventing reproduction and consequently reducing their natural stocks (Honji *et al.*, 2011; Araújo *et al.*, 2012). *S. hilarii* was classified in the 2008 and 2010 Red List of São Paulo state (Assembleia Legislativa do Estado de São Paulo, 2008; Bressan *et al.*, 2009) as ‘almost threatened’, however *S. hilarii* captures are almost rare, suggesting ineffective survey and data collection of fauna by the competent agency. Production in captivity can be considered a viable strategy to reduce the anthropic pressure and collaborate with the conservation efforts for native rheophilic species (Silva *et al.* 2006). However, basic information about biology, genetics, reproductive physiology and larval and embryonic development are essential to improve the productive performance in captivity.

Previous studies have been carried out with *S. hilarii* especially focused on reproduction (Honji *et al.*, 2009, 2013), larval rearing (Araújo *et al.*, 2012), and population genetics (Silva *et al.*, 2015) and providing essential information about this species production. Embryonic

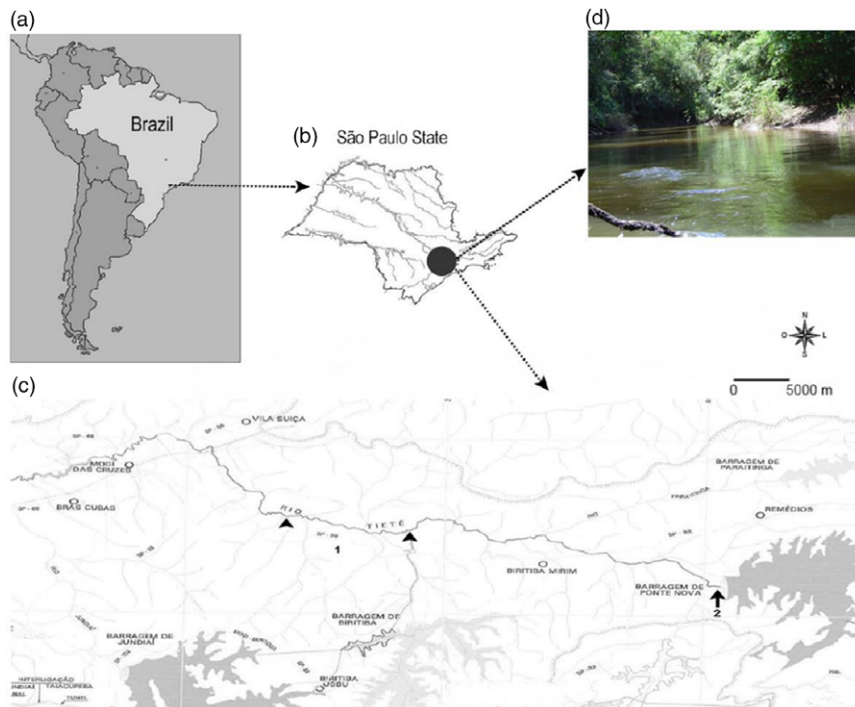


Figure 1. Map of the upper Tietê river basin and type locality of *Salminus hilarii* sampling sites. (a, b) Map of the Upper Tietê River Basin, São Paulo State (Brazil). (c) Upper Tietê River Basin with the collection site (1) (between the arrowhead) and Ponte Nova fish farm (2) (arrow). (d) Type locality of *S. hilarii*, Upper Tietê River in areas in which the water is clean, shallow and running. Source: Modified from DAEE (Departamento de Águas e Energia Elétrica), Brazil, and Gomes *et al.* (2016).

and larval studies are essential to understand fish development, mainly those performed with commercial interests or endangered species, not only to generate knowledge about their development characteristics, but also to have a comparison model to know when normal development is altered by external factors (Coelho *et al.*, 2019). According to Morrison *et al.* (2001) knowledge about embryonic development in fish is essential to define adequate strategies to avoid high mortality rates influenced mainly by embryo deformities and bad formations. Due to this importance, several studies have been conducted in Brazil to describe the initial developmental phase of fish species with commercial interest or strong environmental appeal, such as *Pseudoplatystoma corruscans* (Cardoso *et al.*, 1995), *Brycon cephalus* (Romagosa *et al.*, 2001), *Brycon insignis* (Andrade-Talmelli *et al.*, 2001), *Steindachneridion parahybae* (Honji *et al.*, 2012), among others.

Against this background, we investigated *S. hilarii* reproduction, embryonic and larval phase development to understand the reproductive biology and ontogenetic development to guide reproductive techniques and captivity production of this species. Our goals were: (i) to assess the performance of two different spawning techniques (dry stripping of gametes and natural spawning); and (ii) to describe the initial developmental stages, from activation of the oocyte to the initial larval period.

Materials and methods

Fish stocks, rearing conditions, and fish collection

Sexually adult wild *S. hilarii* females and males were collected from the Tietê River in the São Paulo States, Brazil (at intervals 23°32'45.3''S, 46°08'03.2''W and 23°34'36.5''S, 45°54'23.9''W) using artisanal fisheries (Fig. 1). These animals were transported

individually to the Ponte Nova hatchery (23°35'33.8''S, 45°58'09.1''W), located in Salesópolis city (São Paulo, Brazil). *S. hilarii* broodstock were maintained in two ponds (300 m² each) during the experimental period under a natural photoperiod and fed with commercial extruded diet for carnivorous fish (40% crude protein) with 2% of the biomass per day.

Broodstock selection, hormonal induction and spawning

Salminus hilarii reproductive period normally occurs from October to February, with a peak in December and January (Honji *et al.*, 2009, 2011). In December, artificial reproductions were performed using three females and three males (in each different groups explained below) that were selected by morphological characteristics of sexual ripeness, according to Honji *et al.* (2011). Males were chosen according to colour (white) and running sperm were observed after a gently abdominal massage. Females were selected by the presence of a large, soft abdomen, and also by gonadal characteristics (biopsy procedure). Oocytes were collected to examine size, appearance, and diameter under a stereomicroscope, these are important criteria used to establish the spawning readiness of a total spawning *S. hilarii* broodstock (Honji *et al.*, 2009, 2011).

After broodstock selection, animals were transferred to laboratory tanks (3.6 m²), and artificial reproduction induction was performed on *S. hilarii* using a routine protocol (Honji *et al.*, 2011). Concisely, the females were induced using two intraperitoneal injections of carp pituitary extract (cPE; Fish Braz). Doses were 0.5 mg cPE/kg (first injection) and 5.0 mg of cPE/kg (second injection), diluted in 0.9% sodium chloride solution (considering 1.0 ml in each dose). The second injection (females) was administered 10 h after the first. Additionally, a single dose of cPE injection (3 mg cPE/kg also diluted in 0.9%

Table 1. Data on water temperature and dissolved oxygen during artificial reproduction (first and second doses; laboratory ponds), embryonic development (conical incubators), and the annual mean (broodstock ponds) at the Ponte Nova hatchery (Departamento de Águas e Energia Elétrica, São Paulo, Brazil) during embryonic and larval development study. Data are presented as the mean \pm standard error of the mean

Period of induced reproduction	Water temperature (°C)	Dissolved oxygen (mg/l)
First injection	25.25 \pm 1.25	6.60 \pm 1.10
Second injection	26.65 \pm 0.35	7.05 \pm 0.05
Spawning	26.20 \pm 0.90	7.20 \pm 0.30
Annual average	19.97 \pm 1.53	7.07 \pm 0.50

sodium chloride solution) was applied to the males at the same time as the second cPE application in females.

After first hormone induction until second dose, the females were kept in separate tanks to the males, however under the same conditions. After final hormonal induction, males and females were kept in the same tank to allow natural spawning (Group 1 – G1) and consequently natural fertilization. Subsequently, water flow in the tanks was increased and the water temperature was monitored. The time for natural gamete releasing, counted from the second hormone administration until spawning, was calculated as accumulated thermal units (ATU), or degree-hours following Weingartner and Zaniboni-Filho (2005). The second group (G2) was established to compare the eggs and larval development using a standard protocol applied to other teleost species, in which males and females were stripped (from the results obtained by the ATU). The dry stripped oocytes from three females were mixed with sperm from three males. Subsequently, gametes from males and females were stripped in plastic containers and dry extrusion was carried out. Oocytes were weighed, sperm were added and then the mixture was gently homogenized. Water was added for hydration to allow fertilization (in accordance with the protocol described by Caneppele *et al.*, 2009). In all groups, the estimated number of oocytes in *S. hilarii* was calculated indirectly by weighing the eggs and counting subsamples in triplicate (Caneppele *et al.*, 2009; Honji *et al.*, 2011). After spawning (natural or stripped), females were transferred back into their original ponds (100% survival). Additionally, data on broodstock weight were: G1 (871.33 \pm 99.30 for females and 541.33 \pm 24.30 for males) and G2 (674.67 \pm 39.30 for females and 393.33 \pm 45.00 for males).

Eggs were transferred to four 60-litre fibreglass conical incubators, in which they were kept for 3 days until hatching and yolk-sac absorption. After this period, the larvae were transferred to horizontal fibreglass incubators (0.5 m²), in which they were fed with *Artemia* sp. *nauplii*. At 6 h after fertilization (AF), when the gastrula stage was well established, the fertility rate was calculated using the equation: F = (number of fertilized eggs \times 100)/number of total eggs (Vazzoler, 1981, 1996), for both experimental groups.

Characteristics of the freshly spawned egg, terminology used for embryonic and larval development analysis and morphometric analyses

The main morphological features of each stage of embryonic and larval development of *S. hilarii* were described meticulously. Oocytes, eggs and larvae at each sampling were measured using

a Leica EZ4 stereomicroscope (Leica Microsystems, Wetzlar, Germany) with coupled camera and using a computer image capture (Leica Application Suite v2.1.0). Every 5 min, fresh eggs samples were collected for analysis and a photographic record was taken until hatching. After hatching, the larvae were sampled every hour and a photograph was also recorded. The developmental stage was established when more than 50% of the specimens were at the same stage. This parameter was measured using five subsamples of the same group with 20 eggs and/or larvae samples. Additionally, at the end of each observation, characterization, and photographic record, the original volume of water and eggs/larvae was re-housed in the fibreglass conical incubators or horizontal fibreglass incubators. Embryonic and early larval development stages were identified according to descriptions established for others Characidae (Andrade-Talmelli *et al.*, 2001; Reynalte-Tataje *et al.*, 2004; Nakaghi *et al.*, 2006; Alexandre *et al.*, 2009; Faustino *et al.*, 2010a; Nakauth *et al.*, 2016) and also by Nakatani *et al.* (2001) and Richards (2006), who described the ontogenetic development of several fish species.

For morphometrical analysis of embryonic and larval development in *S. hilarii* during artificial reproduction in captivity, the following parameters were considered: arrangement of the development at the egg phase, total length of the larvae (L_t in millimetres), yolk sac length (YL) and yolk sac height (YH) (for both, in millimetres). YL and YH were measured shortly after hatching in this way: volumes were approximated by the formula for a prolate spheroid $\{V(\text{mm}^3) = [\pi \times l \times (h^2)]/6\}$, in which l is YL and h is YH (Bagarinao, 1986). For analysis of L_t and yolk volume, measurements were analyzed using Leica Application Suite Professional LAS v3.6 software.

Water quality analysis and additional information

Water quality, temperature (°C) and dissolved oxygen (mg/l), during artificial reproduction (first and second doses; laboratory ponds), embryonic development (fibreglass conical incubators), and the annual mean (broodstock ponds) were monitored daily using an oximeter (YSI model 55) and are shown in Table 1. Additionally, all procedures used in the sampling of the broodstocks, eggs and larvae were in agreement with the Institutional Animal Care Protocols (Comissão de Ética em Manipulação e Experimentação Animal, CEMEA) of UMC University (protocol number and approval 0.28.04, 21 May 2004), and with the System of Authorization and Information on Biodiversity of the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) (protocol number and approval 125, 29 December 2004).

Results

Broodstock selection, hormonal induction and spawning

All broodstock from G1 induced to the reproductive-specific protocol responded positively to artificial induction and naturally spawned in the tank (100% survival). Additionally, *S. hilarii* underwent external fertilization and produced spherical, transparent, pelagic eggs. Spawning occurred at 148.5 \pm 13.5 ATU (under 26°C) with 127.10 g of spawned eggs and a fertilization rate of 65.64 \pm 0.54%. By monitoring ATU for G1, it was possible to know the exact moment for extrusion of G2. In this way, G2 also responded positively (also 100% survival) but only 7.23 g of spawned eggs were obtained, and no fertilization was observed in this group.

Table 2. Time at the main morphological observations and brief description during the embryonic and larval development of *Salminus hilarii*

Time after fertilization	Stage	Brief description
0	Zygote	Just after fertilization to form blastodisc, a prominent layer of cytoplasm, it moves towards the animal pole; perivitelline space was clearly defined
14 min	Zygote	Characterized by a large perivitelline space (that persisted until the embryo development in the animal pole)
49 min	Cleavage	Up to 50% with two blastomeres (in a 1 × 2 arrangement of cells)
57 min	Cleavage	Up to 50% with four blastomeres (in a 2 × 2 arrangement of cells)
1 h 10 min	Cleavage	Up to 50% with eight blastomeres (in a 2 × 4 arrangement of cells)
1 h 35 min	Cleavage	Up to 50% with 16 blastomeres (in a 4 × 4 arrangement of cells)
1 h 42 min	Cleavage	Up to 50% with 32 blastomeres (in a 4 × 8 arrangement of cells)
1 h 54 min	Cleavage/morula/ blastula	Up to 50% with 64 blastomeres (in a 4 × 8 × 2 arrangement of cells); morula (more than 64 blastomeres); blastula (characterized when the periblast and blastoderm region or embryonic cells became clear until gastrula stage)
3 h 56 min	Gastrula	Beginning of the cell movement (with identification of embryonic cells boundaries); up to 50% with 25% epiboly (observation of the morphogenetic movement), which resulted in rearrangement of the blastoderm relative to the yolk mass and establishes of the embryonic axis
4 h 26 min	Gastrula	Up to 50% with 50% epiboly
6 h 16 min	Gastrula	Up to 50% with 90% epiboly
8 h 31 min	Gastrula	Final gastrula stage and blastopore closure (when yolk mass was completely surrounded by the blastoderm forming the yolk sac)
9 h 1 min	Organogenesis	Early segmentation: establishment of the embryonic axis and clear distinction of the yolk sac
9 h 31 min	Organogenesis	Early segmentation: clear distinction between cephalic and caudal region (the tail still attached from the yolk sac); evident somites

Embryonic and larval developmental stages

Main embryonic events, such as zygote, cleavage, morula, blastula, gastrula, organogenesis, hatching and other phases, and some behavioural information such as cannibalism were observed and registered (Table 2). All stages were characterized in detail with the specific time of occurrence and a brief description (Table 2).

As fertilization occurred in tanks, eggs were observed after hydration (Fig. 2a, b) and were characterized by the appearance of a cell region that was formed at the blastodisc division; it was possible to visualize a prominent cytoplasm layer. Activated eggs were composed of the chorion, perivitelline space and animal pole (Fig. 2c) that persisted until embryo development at the animal pole (more detail in Fig. 2d), in which the perivitelline space was large, clear and translucent.

First cleavage was observed for fertilization at 49 min AF, and was characterized by the appearance of a cellular region above the yolk sac and division of the blastodisc into two blastomeres of the same size (the first cleavage, 49 min AF; Fig. 2e). Second and third cleavages occurred, generating four blastomeres (second cleavage, 57 min AF; Fig. 2f) and eight blastomeres (third cleavage, 1 h and 10 min AF; Fig. 2g), respectively. Fourth and fifth cleavages produced 16 blastomeres (fourth cleavage, 1 h and 35 min AF; Fig. 2 h) and 32 blastomeres (fifth cleavage, 1 h and 42 min AF; Fig. 3a). Following development, the sixth cleavage give rise to 64 blastomeres (sixth cleavage, 1 h and 54 min AF; Fig. 3b), at which time the number of blastomeres was still evident. After this stage cell cycles lost synchrony and it became difficult to observe the number and the pattern of cleavage.

Morula stage was identified as the successive cell division of more than 64 blastomeres and was arranged in a half-berry shape;

the periblast and blastoderm became visible. Blastula stage was characterized by the presence of a cup-like shape with spaces between blastomeres at the animal pole; the beginnings of cell movement started at the gastrula stage. At this stage it was possible to observe morphogenetic movement (epiboly), which produced rearrangement of blastoderm to the yolk mass and originated the germinative follicles and embryonic axis. Epiboly was detectable at three different times, 25% epiboly (3 h 56 min AF; Fig. 3c), 50% epiboly (4 h 26 min AF; Fig. 3d) and 90% epiboly (6 h 16 min AF; Fig. 3e) and finally, the final gastrula stage and blastopore closure (8 h 31 min AF; Fig. 3f).

Next, organogenesis started and the process was divided into early segmentation phase and late segmentation phase. At the early segmentation phase it was possible to observe the cephalic and caudal regions and yolk sac (9 h 01 min AF; Fig. 3g), in which the tail was very short and attached to the yolk mass, and it was clearly possible to distinguish the optic vesicle and the somites that posteriorly originated the muscle tissue (9 h 31 min AF; Figs 3h and 4a, b). This subdivision ended when the tail detached and the embryo start growing with the tail well extended and completely free from the yolk sac (11 h 51 min AF; Fig. 4c). After this stage, the embryo started conspicuous muscular contractions (Fig. 4d) with a heterogenic egg wall (Fig. 4e), followed by hatching (Fig. 4f). At 12 h after hatching (HAH), an initial cannibalistic behaviour was observed (Fig. 4g, h) and at 33 HAH this behaviour clearly became more intense.

Additionally, other morphological aspects of *S. hilarii* larvae were measured, such as larval length and yolk sac volume at hatching, and were compared with aspects of other teleost fish larvae (Table 3). *S. hilarii* larvae hatched with a 4.68 ± 0.13 mm length and yolk sac volume was 0.97 ± 0.04 mm³.

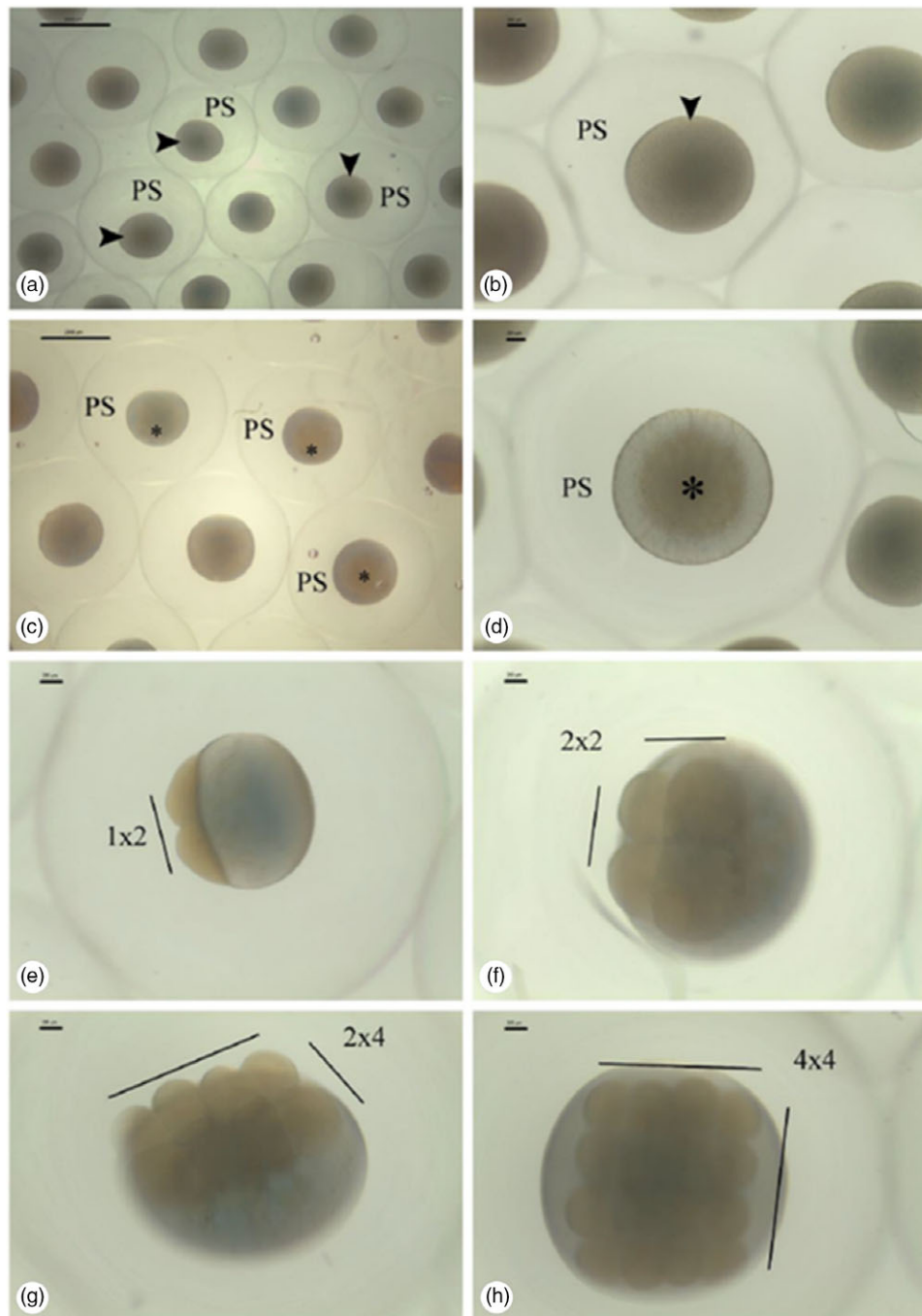


Figure 2. Macroscopic aspects of embryonic development of *Salminus hilarii*. (a) Group 1 (G1 – natural spawning) egg hydrated soon after fertilization (AF) (time zero); egg cell (arrowhead). (b) Group 2 (G2 – stripped spawning) egg hydrated AF (time zero) egg cell (arrowhead). (c) Activated egg (G1) with animal pole (asterisk) (14 min AF). (d) More detail of activated egg (G1) with animal pole (asterisk) (14 min AF). (e) G1: two blastomeres (49 min AF). (f) G1: four blastomeres (57 min AF). (g) G1: eight blastomeres (1 h 10 min AF). (h) G1: 16 blastomeres (1 h 35 min AF). PS: perivitelline space. Bars: 2000 μ m (a, c); 200 μ m (b, d–h).

Discussion

In general, *S. hilarii*-induced reproduction using the cPE technique is recommended followed by natural spawning in the tank. This species presented similar embryogenesis and larval development compared with a congeneric species (*S. brasiliensis*), with larvae containing a large yolk sac and strong pigmentation in the body and eyes, and with a very strong cannibalistic behaviour that generally can be minimized by selection by size during development.

Water quality parameters observed in this study were similar to those observed in previous trials carried out with *S. brasiliensis*. In the same way, reproductive protocols used for this congeneric species were successfully applied for *S. hilarii* (Weingartner and Zaniboni-Filho, 2005; Zanandrea *et al.*, 2016). In contrast, animals from the G2 group released a low amount of eggs and we failed to obtain fertilized eggs by manual stripping. This failure is probably related to either water volume at the moment of fertilization, oocyte quality, or synchronization of the micropyle opening time

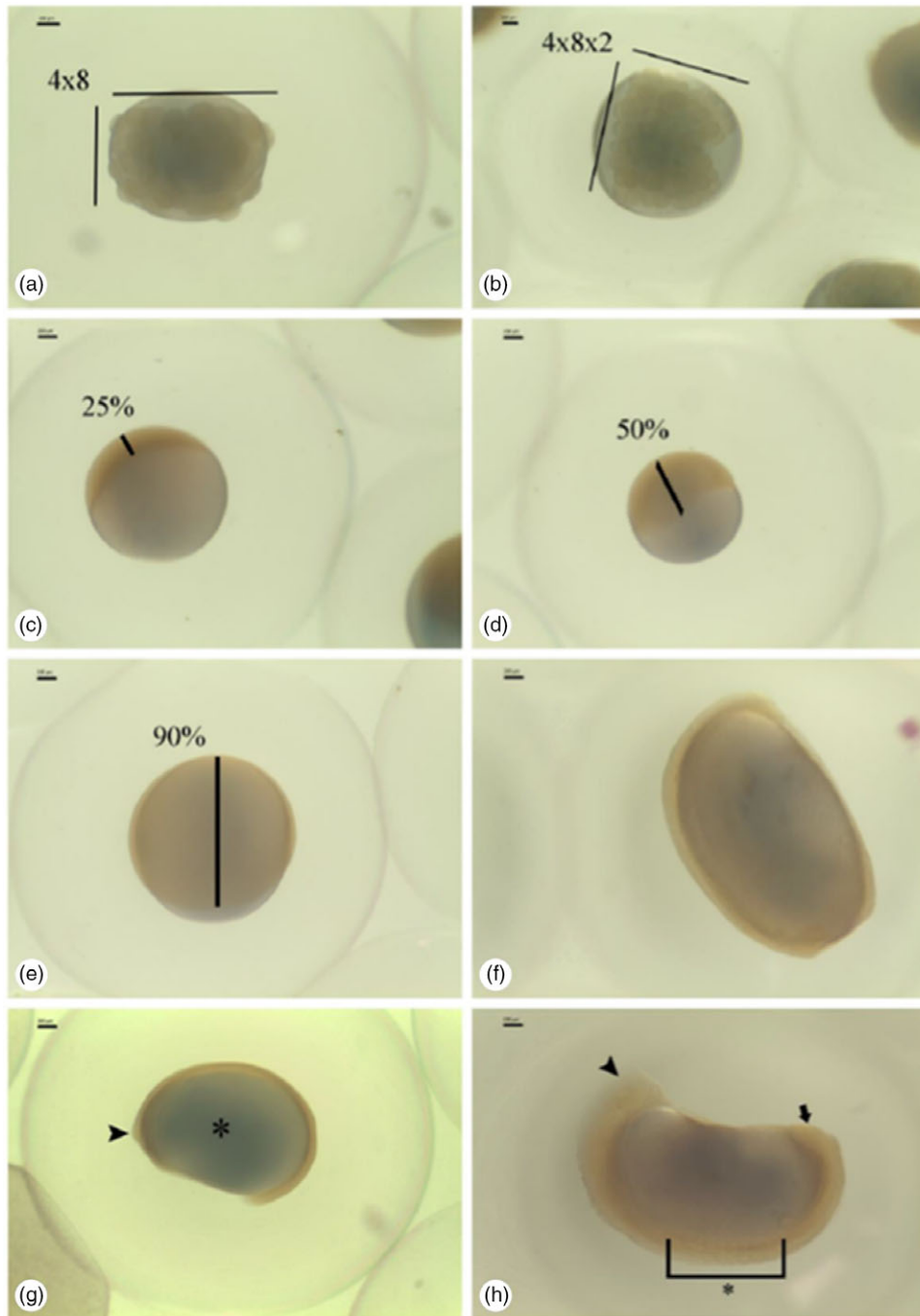


Figure 3. Macroscopic aspects of embryonic development of *Salminus hilarii*. (a) G1 (Group 1 – natural spawning): 32 blastomeres (1 h 42 min AF). (b) G1: 64 blastomeres (1 h 54 min AF). (c) G1: gastrula stage: epiboly of 25% (3 h 56 min AF). (d) G1: gastrula stage: epiboly of 50% (4 h 26 min AF). (e) G1: gastrula stage: epiboly of 90% (6 h 16 min AF). (f) G1: final gastrula stage and blastopore closure (8 h 31 min AF). (g) G1: differentiation of embryonic layers, head (arrowhead), tail (arrow), and yolk sac (asterisk) (9 h 1 min AF). (h) G1: distinguish between the cephalic (arrowhead) and caudal (arrow) regions, somites (asterisk), and optic vesicle (arrowhead) (9 h 31 min AF). Bars: 200 μ m (a–h).

(Mylonas *et al.*, 2010). Compared with G2, fertilized eggs were observed in animals from G1, nevertheless compared with the other Characidae species, fertilization rates were considered lower (65%) (Luz *et al.*, 2001; Andrade-Talmelli *et al.*, 2002; Narahara *et al.*, 2002; Weingartner and Zaniboni-Filho, 2005; Honji *et al.*, 2012). Additionally, *S. hilarii* ATU in both groups were close to those of previous studies performed with Characidae, with only a few slight variations observed according to species. Therefore,

natural spawning after hormonal induction is highly recommended for *S. hilarii*.

In general, embryogenesis is characterized mainly by fertilization to zygote formation, when it is possible clearly distinguish the blastodisc from the vegetative pole (Richards, 2006; Nakatani *et al.*, 2001; Buzollo *et al.*, 2011). In *S. hilarii* this stage was characterized from the moment of fertilization up to 14 min AF, while for others Characidae species this process occurred later, as observed for

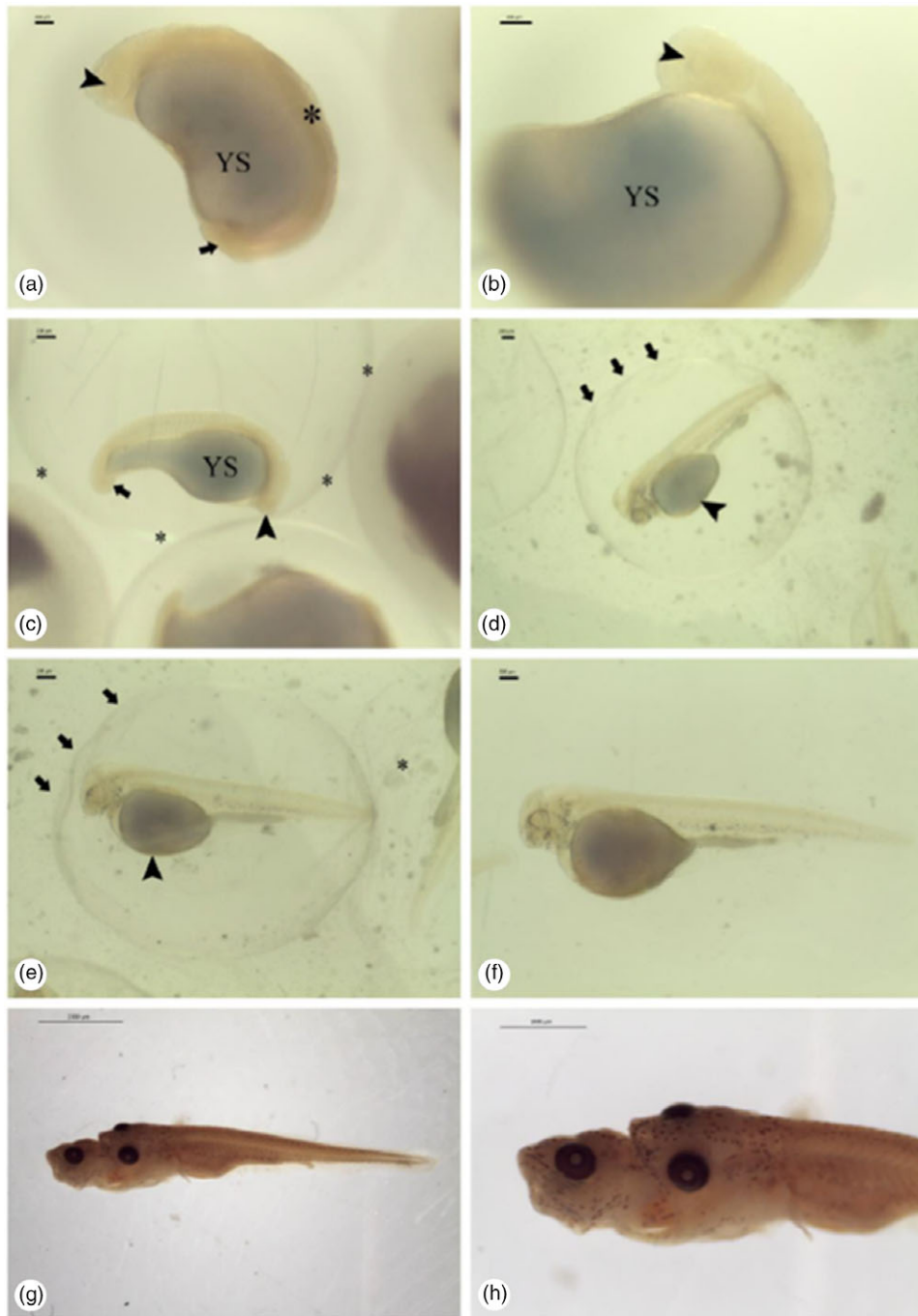


Figure 4. Macroscopic aspects of embryonic development of *Salminus hilarii*. (a) G1 (Group 1 – natural spawning): distinguish between the cephalic with optic vesicle (arrowhead) and caudal (arrow) regions, somites details (asterisk), and yolk sac (YS) (10 h 1 min AF). (b) G1: detail of cephalic region with optic vesicle detail (arrowhead) and yolk sac (YS) (10 h 1 min AF). (c) G1: complete development of larvae (arrowhead), the tail release (arrow), and homogenous egg wall (asterisk) (11 h 51 min AF). (d, e) G1: complete development of larvae (arrowhead) with movement, beginning (d), advanced (e) of heterogenic of egg wall (arrows), and next (e) to hatching (asterisk) (20 h 46 min AF). (f) Hatching (time zero after hatching – AH). (g, h) G1: cannibalism (g) and detail (h) among the *S. hilarii* larvae (12 h 23 min AH). Bars: 200 μ m (a–f); 2000 μ m (g); 1000 μ m (h).

B. cephalus (25 min at 26.8°C; Alexandre *et al.*, 2009), *Brycon gouldingi* (45 min at 26.4°C; Faustino *et al.*, 2010a), *Prochilodus lineatus* (75 min at 24°C; and 34 min at 28°C, Ninhaus-Silveira *et al.*, 2006). Previous studies performed with *B. cephalus* (Alexandre *et al.*, 2009), and *S. brasiliensis* (Nakagui *et al.*, 2006) showed a large perivitelline space in the egg similar to that observed for *S. hilarii* ($937.89 \pm 107.19 \mu$ m). According to

Nakatani *et al.* (2001) the perivitelline space is described as ‘large’ when it reaches an area between 20 and 29.9% of total egg volume; this characteristic is considered an advantage as could prevent possible damage in the eggs, and consequently improves the survival rate (Andrade-Talmelli *et al.*, 2001).

Cleavage stage is characterized by successive cell divisions that result in a blastomere volume increase, consequently decreasing

Table 3. Overview of larval length, yolk volume and/or yolk diameter measurements at hatching in several teleost species. Data are presented as the mean \pm standard error of the mean

Species	Larval length at hatching (mm)	Yolk volume (unit)	Source
Tabarana (<i>Salminus hilarii</i>)	4.68 \pm 0.13	0.9700 \pm 0.04 (mm ³)	This study
Summer flounder (<i>Paralichthys dentatus</i>)	2.85 \pm 0.18	0.3480 (mm ³)	Johns and Howell (1980)
Milkfish (<i>Chanos chanos</i>)	3.46	0.4744 (μ l)	Bagarinao (1986)
Seabass (<i>Lates calcarifer</i>)	1.72	0.0859 (μ l)	Bagarinao (1986)
Rabbitfish (<i>Siganus guttatus</i>)	1.50	0.0251 (μ l)	Bagarinao (1986)
Lemon sole (<i>Microstomus kitt</i>)	4.01 \pm 0.21	1.26 \pm 0.08 (μ l)	Rønnestad et al. (1992)
Trout (<i>Salmo trutta</i>)	–	63.58 \pm 22.72 (mm ³)	Bonislawska et al. (2001)
Rainbow trout (<i>Oncorhynchus mykiss</i>)	–	55.24 \pm 15.75 (mm ³)	Bonislawska et al. (2001)
Pike (<i>Esox lucius</i>)	–	6.50 \pm 0.87 (mm ³)	Bonislawska et al. (2001)
Lavaret (<i>Coregonus lavaretus</i>)	–	4.25 \pm 1.05 (mm ³)	Bonislawska et al. (2001)
Whitefish (<i>Coregonus albula</i>)	–	1.29 \pm 0.35 (mm ³)	Bonislawska et al. (2001)
Bream (<i>Abramis brama</i>)	–	0.51 \pm 0.06 (mm ³)	Bonislawska et al. (2001)
Three-spined (<i>Gasterosteus aculeatus</i>)	–	1.24 \pm 0.15 (mm ³)	Bonislawska et al. (2001)
Bleak (<i>Alburnus alburnus</i>)	–	0.46 \pm 0.06 (mm ³)	Bonislawska et al. (2001)
Rudd (<i>Scardinius erythrophthalmus</i>)	–	0.38 \pm 0.14 (mm ³)	Bonislawska et al. (2001)
Spring baltic (<i>Clupea harengus</i>)	–	0.25 \pm 0.02 (mm ³)	Bonislawska et al. (2001)
Perch (<i>Perca fluviatilis</i>)	–	0.52 \pm 0.08 (mm ³)	Bonislawska et al. (2001)
Goldfish (<i>Carassius auratus</i>)	–	0.46 \pm 0.10 (mm ³)	Bonislawska et al. (2001)
Sun bass (<i>Leucaspius delineatus</i>)	–	0.34 \pm 0.05 (mm ³)	Bonislawska et al. (2001)
Plaice (<i>Pleuronectes platessa</i>) *Irish Sea	6.97	0.89 (mm ³)	Kennedy et al. (2007)
Plaice (<i>Pleuronectes platessa</i>) *Norwegian coastal waters	6.62	1.42 (mm ³)	Kennedy et al. (2007)
Sharpsnout seabream (<i>Diplodus puntazzo</i>)	2.20 \pm 2.15	0.359 \pm 0.017 (mm ³)	Klimogianni et al. (2011)
Angelfish (<i>Pterophyllum scalare</i>)	2.60	0.64 (mm ³)	Korzelecka-Orkisz et al. (2012)
Common snook (<i>Centropomus undecimalis</i>) at 25°C	1.57	0.811 (mm ³)	Barón-Aguilar et al. (2013)
Common snook (<i>Centropomus undecimalis</i>) at 28°C	1.68	0.749 (mm ³)	Barón-Aguilar et al. (2013)
Common snook (<i>Centropomus undecimalis</i>) at 31°C	1.65	0.764 (mm ³)	Barón-Aguilar et al. (2013)
African catfish (<i>Clarias gariepinus</i>)	4.24 \pm 0.15	0.99 \pm 0.31 (mm ³)	Ing and Chew (2015)
Silver perch (<i>Bidyanus bidyanus</i>)	2.85 \pm 0.06	0.58 \pm 0.06 (mm ³)	Sulaeman and Fotedar (2017)
Patagonian sprat (<i>Sprattus fuegensis</i>)	3.56 \pm 0.34	0.18 \pm 0.03 (mm ³)	Leal et al. (2017)
Paraitinga (<i>Brycon nattereri</i>)	6.32 \pm 0.69	3.64 \pm 0.39 (mm ³)	Maria et al. (2017)

the cytoplasm volume (Gilbert, 2003; Marques et al., 2008). In *S. hilarii*, the 2–64-blastomeres stage was observed between 49 min AF and 1 h 42 min AF; this phase is considered critical mainly due to a high deformity incidence and consequently higher mortality (Morrison et al., 2001; Ninhaus-Silveira et al., 2006). Morula and blastula stages are mainly distinguished by differentiation in the blastoderm (embryonic cells) and periblast (yolk syncytial layer) (Marques et al., 2008). The blastoderm stage presented more than 64 blastomeres arranged in two or more layers, and separated from the blastocoele periblast (large space between the yolk and the blastomeres). Additionally, the periblast plays an essential role during the period of endogenous feeding, as it is directly related to nutrient absorption processes. In *S. hilarii* these stages particularly occurred from 1 h 54 min AF, whereas for

B. cephalus it occurred from 1 h 30 min (26.8°C; Alexandre et al., 2009), in *L. macrocephalus* and *P. corruscans* from 2 h 0 min AF (29°C; Buzollo et al., 2011; 27°C; Marques et al., 2008 respectively) and in *P. lineatus* from 3 h 0 min AF (24°C; Ninhaus-Silveira, et al., 2006). These differences showed a distinct pattern according to species specificity and temperature.

Gastrula phase is initially characterized by the first cellular movements in an event called epiboly, and is finished by closure of the blastopore, resulting in blastoderm rearrangement in relation to yolk mass (Honji et al., 2012; Buzollo et al., 2011; Nakagui et al., 2006; Mello et al., 2018). Gastrula stage in *S. hilarii* was initiated at 3 h 56 min to 9 h 1 min, this time was considered late and no longer compared with other species such as *P. maculatus* (2 h 15 min to 5 h 0 min at 29°C; Buzollo et al.,

2011), *B. cephalus* (1 h 45 min to 7 h 45 min at 26.8°C; Alexandre *et al.*, 2009), and *S. parahybae* (2 h 40 min to 8 h 40 min at 24°C; Honji *et al.*, 2012).

Initially, organogenesis is marked by notochord development, which will set the caudal cephalic corporeal axis, allowing distinction between the embryonic axis and the yolk sac (Nakatani *et al.*, 2001). The main features during this phase were optic vesicle maturation, a marked increase in somites, and pigmentation in distinct body regions, which occurred at 9 h 1 min to 20 h 45 min AF. At the end of organogenesis, muscular segmentation was observed, and the tail was completely released from the yolk sac (Andrade-Talmelli *et al.*, 2001; Faustino *et al.*, 2010a,b; Chalde *et al.*, 2011; Honji *et al.*, 2012). At approximately 20 h 46 min AF, an intense pigmentation in *S. hilarii* was observed in the cephalic and ventral posterior regions, this verified an intense muscular activity that consequently resulted in chorion rupture, initiating the hatching process.

After 21 h 17 min AF at 26°C, 100% of *S. hilarii* larvae were hatched, while in *S. brasiliensis* total hatching was observed after 18 h 0 min AF at 23.4°C (Nakaghi *et al.*, 2006; Vega-Orellana *et al.*, 2006), and in *B. orbignyanus* after 14 h 0 min AF at 27.9°C (Nakatani *et al.*, 2011). Based on these events, we can suggest that *S. hilarii* presented a late development. After hatching, *S. hilarii* larvae presented a large yolk sac ($0.9700 \pm 0.04 \text{ mm}^3$) this characteristic is considered an important advantage, as fish species with this profile have more energy-containing substrates and consequently more time to complete their development and find food. Additionally, in this shift phase, high mortality rates are normally observed, mainly influenced by the transition event from endogenous to exogenous feeding (Bagarinao, 1986; Jaworski and Kamler, 2002; Yufera and Darias, 2007; Jaroszewska and Dabrowski, 2011; Barón-Aguilar *et al.*, 2013; Sulaeman and Fotedar, 2017).

After hatching, *S. hilarii* larvae presented a large yolk sac, subtly pigmented eyes, and an adhesive gland present in the frontal cephalic region that have the main function to keep the larvae adhered to different substrates. *S. hilarii* larvae showed an intense cannibalistic behaviour, which was observed from 33 h AF and continued throughout development; this was similarly observed for the congeneric species *S. brasiliensis* (Nakaghi *et al.*, 2006; Vega-Orellana *et al.*, 2006). Therefore, to avoid high mortality rates by cannibalism, *S. hilarii* larvae should be fed intensively (especially using *Artemia* sp. *nauplii*) associated with size grading. This technique is highly effective in decreasing cannibalism and consequently the mortality rate (Buzollo *et al.*, 2011).

In conclusion, the most effective protocol to artificially reproduce *S. hilarii* is hormonal induction using cPE, followed by spontaneous spawning in the tank, and then oocyte collection. The results clearly showed that manual extrusion followed by dry fertilization of gametes could not be applied for this species, or needs to be adapted, as no fertilized eggs were obtained. Ontogeny and embryonic development were very similar to that of other teleost species, especially compared with *S. brasiliensis*. However, the time at which the events occurred were largely influenced by water temperature. The results obtained in this study could be considered fundamental to improving reproduction and consequently enhancing the survival of *S. hilarii* in captivity, allowing their massive production and directly contributing to an effective conservation programme in the upper Tietê river basin. However other studies mainly related to their basic biology,

reproductive physiology and first stage nutrition are still essential to this species production.

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Conflicts of Interest. None.

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