

## **Embryonic development and larval stages of *Steindachneridion parahybae* (Siluriformes: Pimelodidae) - implications for the conservation and rearing of this endangered Neotropical species**

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*Steindachneridion parahybae* is a freshwater catfish endemic to the Paraíba do Sul River and is classified as an endangered Neotropical species. An increasing number of conservation biologists are incorporating morphological and physiological research data to help conservation managers in rescue these endangered species. This study investigated the embryonic and larval development of *S. parahybae* in captivity, with emphasis in major events during the ontogeny of *S. parahybae*. Broodstocks were artificially induced to reproduce, and the extrusion occurred 200-255 degree-hours after hormonal induction at 24°C. Larval ontogeny was evaluated every 10 minutes under microscopic/stereomicroscopic using fresh eggs samples. The main embryogenic development stages were identified: zygote, cleavage, including the morula, blastula, gastrula phase, organogenesis, and hatching. The extruded oocytes showed an average diameter of  $1.10 \pm 0.10$  mm, and after fertilization and hydration of eggs, the average diameter of eggs increased to about  $1.90 \pm 0.60$  mm, characterized by a large perivitelline space that persisted up to embryo development, the double chorion, and the poles (animal and vegetative). Cell division started about 2 minutes after fertilization (AF), resulting in 2, 4, 8 (4 x 2 arrangement of cells), 16 (4 x 4), 32 (4 x 8) and 64 (2 x 4 x 8) cells. Furthermore, the blastula and gastrula stages followed after these cells divisions. The closed blastopore occurred at 11 h 20 min AF; following the development, the organogenetic stages were identified and subdivided respectively in: early segmentation phase and late segmentation phase. In the early segmentation phase, there was the establishment of the embryonic axis, and it was possible to distinguish between the cephalic and caudal regions; somites, and the optic vesicles developed about 20 h AF. Total hatching occurred at 54 h AF, and the larvae average length was  $4.30 \pm 0.70$  mm. Gradual yolk sac reduction was observed during the first two days of larval development. The first feeding occurred at the end of the second day. During the larval phase, cannibalism, heterogeneous larval growth and photophobia were also observed. This information will be important in improving the artificial reproduction protocols of *S. parahybae* in controlled breeding programs.

*Steindachneridion parahybae* é um bagre de água doce, endêmico do rio Paraíba do Sul e é classificado como espécie neotropical ameaçada. Um número crescente de biólogos conservacionistas estão incorporando dados de pesquisas morfológicas e fisiológicas para ajudar os gestores de conservação no resgate destas espécies ameaçadas de extinção. Este estudo investigou o desenvolvimento embrionário e larval de *S. parahybae* em cativeiro, com ênfase nos principais eventos durante a ontogenia de *S. parahybae*. Reprodutores foram artificialmente induzidos à reprodução e a extrusão ocorreu com 200-255 horas-graus após a indução hormonal a 24°C. A ontogenia larval foi avaliada a cada 10 minutos sob microscópio/estereomicroscópio, utilizando-se amostras de ovos recém coletados. Os principais estágios de desenvolvimento embrionário foram identificados: zigoto, clivagem, incluindo as fases, mórula, blástula e gástrula, organogênese e eclosão. Os ovócitos extrusados apresentaram uma média de diâmetro de  $1,10 \pm 0,10$  mm e depois da fertilização e hidratação dos ovos, a média de diâmetro dos ovos aumentou para  $1,90 \pm 0,60$  mm, caracterizado pelo grande espaço vitelínico que persistiu até o desenvolvimento do embrião, duplo córion e os polos (animal e vegetal). A divisão celular iniciou-se aproximadamente dois minutos após a fertilização (AF), resultando em 2, 4, 8 (4 x 2 arranjo das células), 16 (4 x 4), 32 (4 x 8) e 64 (2 x 4 x 8) células. Adicionalmente, seguiram as fases de blástula e gástrula depois das divisões celulares. O fechamento do blastóporo ocorreu às 11 h 20 min AF; seguindo o desenvolvimento, os estágios de organogênese foram identificados e subdivididos,

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respectivamente em: fase de divisão inicial e fase de divisão avançada. Na fase de divisão inicial, depois do estabelecimento do eixo do embrião, foi possível distinguir as regiões cefálica e caudal; os somitos e as vesículas ópticas se desenvolveram com aproximadamente 20 h AF. A eclosão total ocorreu com cerca de 54 h AF e o comprimento médio foi de  $4,30 \pm 0,70$  mm. A redução gradual do saco vitelínico foi observada durante os primeiros dois dias de desenvolvimento larval. A primeira alimentação ocorreu no final do segundo dia. Durante a fase larval, canibalismo, crescimento larval heterogêneo e fotofobia também foram observados. Estas informações serão importantes para aperfeiçoar o protocolo de reprodução artificial em *S. parahybae* em programas de reprodução controlada.

**Key words:** Artificial reproduction, Early development, Eggs, Larvae, Surubim do Paraíba.

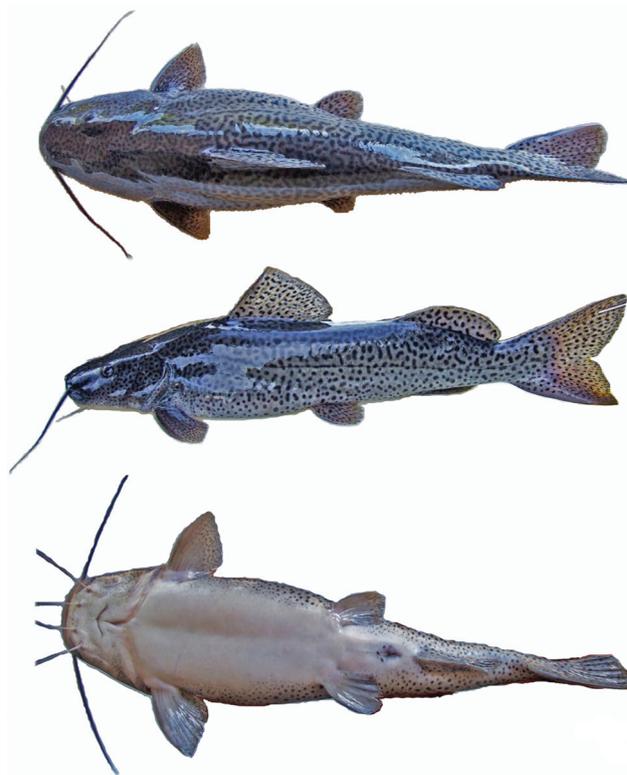
### Introduction

The teleost order Siluriformes, which includes the largest number of living teleost fish species, contains 3093 species popularly known as “catfish” (Ferraris, 2007). In this group the family Pimelodidae is a large and diversified family of freshwater catfishes (Burgess, 1989). The Neotropical catfish genus *Steindachneridion* within the order Siluriformes, family Pimelodidae (Garavello, 2005), has six species described: *S. parahybae* (Steindachner, 1877), *S. amblyurum* (Eigenmann & Eigenmann, 1888), *S. doceanum* (Eigenmann & Eigenmann, 1889), *S. scriptum* (Miranda Ribeiro, 1918), *S. punctatum* (Miranda Ribeiro, 1918), *S. melanodermatum* Garavello, 2005. Little is understood about the reproductive biology of this genus. This lack of knowledge is likely because the species within this genus have a naturally restricted environment and are relatively hard to collect, resulting in a limited number of *Steindachneridion* species available for biological studies (Garavello, 2005). Additionally, many of these species are at risk for extinction because of environmental disturbances that result in the reduction of their natural habitat (Honji *et al.*, 2009).

The surubim do Paraíba, *Steindachneridion parahybae* (Fig. 1), is a medium-sized Neotropical catfish freshwater species endemic to the Paraíba do Sul River basin, which crosses three Brazilian states (Rio de Janeiro, Minas Gerais, and São Paulo States), and is critically threatened (Caneppele *et al.*, 2008; Honji *et al.*, 2009; Polaz *et al.*, 2011). The main threats to *S. parahybae* are the construction of dams, riparian habitat destruction, water pollution and fishing (Honji *et al.*, 2009). Since 2003, CESP has supported researches on the biology of *S. parahybae*, including the maintenance of broodstocks and successful larval rearing, in order to ensure the conservation of this species. There have been no recent reports of this species in the southern rivers of the Paraíba do Sul River basin (São Paulo, 2008). However, *S. parahybae* was commonly captured in the commercial fisheries (around 1,000 kg/year) along the Paraíba do Sul River during the 1950s (Machado & Abreu, 1952). Nowadays, this species is only captured in Paraíba do Sul River in Rio de Janeiro State; and Pomba and Paraíba Rivers in Minas Gerais State, as observed in professional fishing data (Polaz *et al.*, 2011).

There are few studies on the reproductive biology of Brazilian Pimelodidae, although, it has been observed a number of descriptions of the spawning strategies and larvae development of some Pimelodidae species as:

*Pseudoplatystoma fasciatum* (Leonardo *et al.*, 2004), *Pseudoplatystoma corruscans* (Campos, 2005), *Rhamdia quelen* (Baldiasseroto & Neto, 2005), *Pimelodus maculatus* (Buzollo *et al.*, 2011), *Pauliceia luetkeni*, and *Iheringichthys labrosus* (Nakatani *et al.*, 2001). Furthermore, reproductive status of broodstock when reared under different rearing systems and hormonal treatment (hormone stimulation techniques to induce ovulation and spermiation) of Pimelodidae species were also described (Batlouni *et al.*, 2006; Andrade *et al.*, 2010; Romagosa, 2010). On the other hand, considering the number of species and their threatened situation, similar to the genus *Steindachneridion*, there is little descriptive information on reproduction and early development stages of this genus, such as: *S. melanodermatum* (Ludwig *et al.*, 2005; Feiden *et al.*, 2006); *S. scripta* (Adamante *et al.*, 2007; Zaniboni-Filho *et al.*, 2008), and no information is available to *S. parahybae*. According



**Fig. 1.** Dorsal, lateral and ventral views of *Steindachneridion parahybae*. Broodstock female, 520 mm total length, Paraíba do Sul River basin, Paraíba, São Paulo State, Brazil.

to Feiden *et al.* (2006), it is very important to study embryos and larvae development, within two main goals, conservation and commercial fish farming.

Studies on the early development of fish not only yield information on spawning locations and the development features of the different species, but also allow comparisons between normal and altered development patterns (Meijide & Guerrero, 2000), and/or environmental influences (*i.e.*, temperature and photoperiod), on the mechanisms of development during early-life stages (Feiden *et al.*, 2006; Schütz *et al.*, 2008; Zaniboni-Filho *et al.*, 2008). These data will be also of great use in determining the biological changes when the wild broodstocks are transferred to captivity; therefore, they have a practical application in fish farming and fisheries biology (Meijide & Guerrero, 2000; Wikelski & Cooke, 2006). Furthermore, these studies are even essential in the Paraíba do Sul River basin, considering that the rivers of this basin receive a large discharge of domestic and industrial pollution (Polaz *et al.*, 2011).

Many teleost species have reproductive dysfunction in captivity (Mylonas *et al.*, 2010), including *S. parahybae*, which results in problems with the production of fingerlings of native species in Brazil. Therefore, fish conservationists must investigate embryonic and larval development to find ideal conditions for the production of fingerlings and to obtain higher rates of survival in captivity (Honji *et al.*, 2011). Furthermore, the information garnered from field investigation is essential to ensure the success of conservation programs (Wikelski & Cooke, 2006). Similarly, conservation efforts in the field could be more successful if corroborated by captivity studies. Therefore, study of the development of various fish species in captivity is one of the first steps in helping conservation biologists rescue endangered species, such as, many Pimelodidae: *Steindachneridion parahybae* (Honji, 2011), *S. melanodermatum* (Feiden *et al.*, 2006), *S. scripta* (Adamante *et al.*, 2007), *S. scriptum* (Schütz *et al.*, 2008), *Rhamdia quelen* (Soso *et al.*, 2007), *Pseudoplatystoma corruscans*, and *Lophosilurus alexandri* (Santos & Luz, 2009).

Captivity studies of fish are also applicable to aquaculture. The elucidation of feeding behavior, prey preferences, the ecology of the larvae of cultured fishes, and the marking of developmental time-points like yolk sack absorption and mouth opening (which varies among species) will determine the requirements for exogenous food during larval growth (Appelbaum & McGeer, 1998). For example, the high rate of cannibalism observed in cultured fishes is frequently due to inadequate larval food and an improper feeding regime, demonstrating the importance of studying larval development (Appelbaum & McGeer, 1998).

The first step in the study of any species is to obtain knowledge of the embryonic and larval stages. This knowledge allows for morphological and physiological understanding of the species and helps to control the period of the larvae vulnerability. With information about appearance of organs, their functionality and their exact time of emergence, it is

possible to establish management protocols and specific food recommendations for this critical phase. This information is even more important in this study, because is the first characterize *S. parahybae* development, considering the status of conservation, *i.e.*, listed in the Brazilian red list (Caneppele *et al.*, 2009), and regionally extinct in the São Paulo State (São Paulo, 2008; Honji *et al.*, 2009).

The aim of this study was to describe the morphological events related to embryogenesis and larval ontogenesis of *S. parahybae*. We induced spawning in captive *S. parahybae* and followed progeny development from oocyte activation until the beginning of the juvenile period. This study is important in understanding the early development of genus *Steindachneridion* in order to contribute to the conservation of this species.

## Material and Methods

This study was performed at the Hydrobiology and Aquaculture Unit of the Energetic Company of São Paulo State (CESP) located in Paraíba city, São Paulo State, Brazil (23°41'39"S 45°60'42"W). This study is also associated with the conservation program of endangered species of the Paraíba do Sul Basin, undertaken by CESP (Caneppele *et al.*, 2009). During 2003, fifteen *S. parahybae* specimens were caught in the Paraíba do Sul River, near the town of Rio das Flores in the State of Rio de Janeiro (22°13'54"S 43°25'15"W), and these individuals were brought to the CESP hatchery to induce spawning. Descriptions of the fishing gear, catch operations and induced spawning of this wild broodstock were followed in accordance with Caneppele *et al.* (2009).

### Broodstock selection, hormonal induction and spawning

The reproductive period of *S. parahybae* at CESP Fish Farm was from late spring (November) until March, with a reproductive peak in December and January. Therefore, in December 2007 and January 2008, two artificial reproductions were performed using two females and two males in each experiment, that were selected on the basis of the typical morphological characteristics of sexual ripeness, according to the principles previously established for *S. parahybae* (Caneppele *et al.*, 2009). Briefly, the females were selected by external characteristics of the hyperemic genital pore and swollen abdomen. Furthermore, a biopsy procedure was conducted, cannulating the gonoduct with a fine polyethylene tubing (5 mm diameter) attached to a plastic syringe through the gonad papilla. This procedure allowed us to collect several oocytes to better observe their size, appearance, and diameter homogeneity, which are important criteria used to assess the status of female broodstock for the hormonal induction (Harvey & Carolsfeld, 1993). The features of oocytes' diameters and appearance were analyzed under a stereomicroscope (Leica S6D stereomicroscope), and some oocytes were fixed in Bouin's solution for 24 hours for routine histological techniques (described below). Males were chosen according to the color and running sperm when the abdominal

region was gently massaged. The protocol for broodstock selection for artificial reproduction was previously described for the genus *Steindachneridion* (Ludwig *et al.*, 2005; Adamante *et al.*, 2007; Caneppele *et al.*, 2009).

Animals were caught in ponds, transferred to the laboratory at the same moment, kept in 1,000 L tanks, and shortly after, induced to reproduce by combining carp pituitary extract (CPE; Fish Braz) and human Chorionic Gonadotropin (hCG; Pregnyl – Organon) in the same application. For the hormonal induction, the CPE protocol was performed according to methods previously described for *S. parahybae* (Caneppele *et al.*, 2009). Two CPE doses (0.6 mg and 5.4 mg of CPE per kg of body weight, diluted in 0.9% sodium chloride solution) were given to females with a 12 hour interval between doses. Males were given a single dose at the same time as the females' second dose; this dose contained 3 mg of CPE per kg and was also diluted in 0.9% sodium chloride solution. A single dose of hCG application was given to both females and males at the same time as the second CPE application. The hCG concentration used was 2 IU per kg<sup>-1</sup> for females and 1 IU per kg<sup>-1</sup> for males. The dosages used in this study and the broodstock's weight and length are displayed in Table 1.

After hormone induction, each couple was placed in a glass tank (1,000 L) to facilitate the observation of their sexual behavior during spawning. The time necessary for gamete elimination, counted from the second hormone administration until spawning, was calculated as Unit Thermal Accumulated (UTA), or degree-hours. Therefore, the UTA is the sum of the water temperature per hour, from the second hormonal application until spawning (Weingartner & Zaniboni-Filho, 2005). Subsequently, the gametes from males and females were stripped in plastic containers and the dry extrusion was carried out. The oocytes were weighed, kept in a bowl, the sperm were added and then the mixture was gently homogenized. Water was then added for hydration to allow fertilization (according to the protocol described by Caneppele *et al.*, 2009). Furthermore, following the gametes extrusion, the broodstocks were transferred back into their original ponds (100% survival).

Six hours after the eggs were placed in the conical incubator, when the gastrula stage was well established, the fertility rate was calculated using the equation  $F = (\text{number of fertilized eggs} \times 100) / \text{number of total eggs}$  (Vazzoler, 1981, 1996). This protocol is a routine adopted at CESP, where the

fertilized eggs were distinguished from the unfertilized eggs according to the general aspect and color, considering that the unfertilized ones are opaque. We also measured the temperature and dissolved oxygen every hour (Horiba - ModU10), and all recorded values were expressed as the mean  $\pm$  standard error of the mean (M  $\pm$  SEM).

### Water quality

The water quality during the artificial reproduction and embryonic development is shown in Table 2. The average water temperature and dissolved oxygen concentration during the artificial hormonal reproduction (first and second doses), spawning season, and annual mean, respectively, were as follows:  $23.75 \pm 0.75^\circ\text{C}$  and  $7.94 \pm 0.20 \text{ mg/l}$ ;  $23.70 \pm 1.10^\circ\text{C}$  and  $7.67 \pm 1.03 \text{ mg/l}$ ;  $24.13 \pm 0.47^\circ\text{C}$  and  $7.70 \pm 0.79 \text{ mg/l}$ ;  $21.10 \pm 0.14^\circ\text{C}$  and  $7.58 \pm 0.36 \text{ mg/l}$ . Furthermore, during the ten first days of the embryonic/larval development the average water temperature and dissolved oxygen was:  $22.40 \pm 0.27^\circ\text{C}$  and  $7.30 \pm 0.33 \text{ mg/l}$ .

### Characteristics of the freshly spawned egg and larval analysis

The main morphological features of each developmental stage were described. The oocytes, eggs and larvae at each sampling, were measured using a Leica DM1000 microscope or Leica S6D stereomicroscope connected Leica DFC295 camera and using a computer image capture Leica Application Suite professional. Every ten minutes, fresh eggs samples were collected for analysis and a photographic record was taken until hatching. After hatching, larvae were sampled at every twenty four hours during the first ten days (at the same hour of day).

After hatching and the macroscopic observation of absorption of yolk sac, *Astyanax altiparanae* larvae (*ad libitum*) were offered during the first three days, followed by *Prochilodus lineatus* larvae until the 10<sup>th</sup> day. At the same time when *P. lineatus* larvae were offered, inert feeding was also initiated, with a prepared food (commercial fish food - 45% of crude protein; and milled fish in a 4:1 ratio). At the 10<sup>th</sup> day of age the larvae were eating only this prepared food, without any other live prey. The developmental stage was established when more than 50% of the specimens were in the corresponding stage. This parameter was measured using five sub-samples of the same group.

**Table 1.** *Steindachneridion parahybae* reproduction at CESP Fish Farm in December/2007 and in January/2008. Data are presented as the mean  $\pm$  standard error.

Animals		N	Mean $\pm$ SD	hCG (IU/kg)	First CPE dose (mg/kg)	Second CPE dose (mg/kg)	Eggs spawned weight (g)
Females	Total weight (g)	4	2025.00 $\pm$ 319.83	2	0.60	5.40	92.20 $\pm$ 29.99
	Total length (cm)		55.75 $\pm$ 2.32				
Males	Total weight (g)	4	1675.00 $\pm$ 209.66	1	-	3.00	
	Total length (cm)		53.00 $\pm$ 2.12				
Interval between doses					12 hours		
Accumulated thermal unit (ATU)					200-255 (231 $\pm$ 16.25)		
Fertilization rate					69.50 $\pm$ 10.20 %		

**Table 2.** Water quality during the period of artificial reproduction at CESP Fish Farm. Data are presented as the mean  $\pm$  standard error.

Period of artificial reproduction	Water temperature (°C)	Dissolved oxygen (mg/L)
First injection (0.6 mg CPE/kg)	23.75 $\pm$ 0.75	7.94 $\pm$ 0.20
Second injection (5.40 mg CPE/kg)	23.70 $\pm$ 1.10	7.67 $\pm$ 1.03
Spawning	24.13 $\pm$ 0.47	7.70 $\pm$ 0.79
Ten first days	22.40 $\pm$ 0.27	7.30 $\pm$ 0.33
Annual mean	21.10 $\pm$ 0.14	7.58 $\pm$ 0.36

We also collected samples at each major macroscopic ontogenetic change, and fixed some eggs and larvae in Bouin's solution for 20-24 hours (depending on the samples size) for routine histological techniques. Briefly, samples were dehydrated through an ascending series of increasing concentrations of ethanol, cleared in dimethylbenzene (xylene), and embedded in Paraplast (Erv-Plast; Erviegas Instrumental Cirúrgico, São Paulo, Brazil), and 5  $\mu$ m thick sections were stained with hematoxylin-eosin (Behmer *et al.*, 1976). The slides were examined, and imaged using the Leica microscope and camera.

All eggs and larvae were fixed according to the institutional animal care protocols and approval (Protocol: 072/2008). Histological slices are preserved in the collection of the Laboratório de Metabolismo e Reprodução de Organismos Aquáticos (LAMEROA), Instituto de Biociências da Universidade de São Paulo.

### Terminology for embryonic development

The nomenclature describing embryonic development and the early larval stages can sometimes be confusing because of the variety of terms used by different authors. In this study, we identified the embryonic and larval development stages according to the descriptions established for other Siluriformes studied so far (Cardoso *et al.*, 1995; Buzollo *et al.*, 2011) and also by Nakatani *et al.* (2001) and Richards (2006).

## Results

### Broodstock selection, hormonal induction and spawning

In the present study, all broodstocks (100% survival) induced to spawning with this specific protocol responded positively to the artificial reproduction, and have external fertilization and produce pelagic eggs.

The spawning (by dry method) occurred within 200-255 (231  $\pm$  16.25) ATU (about 24°C), with 92.20  $\pm$  29.99g of eggs spawned (weight), and the fertilization rate was about 69.50  $\pm$  10.20% (Table 1). Additionally, we will describe the stages of embryogenesis when the eggs were incubated at a temperature of 23.75  $\pm$  0.75°C.

### Characteristics of the freshly spawned egg and larval analysis

The following embryonic and larval development stages were established:

**Egg stage:** the period from fertilization until hatching. In this stage, an egg consists of a chorion, perivitellinic space and the embryo with a yolk sac observed in the center.

**Larval stage with yolk sac:** This stage includes the time from hatching until the start of the exogenous feeding. The duration of this stage varies widely among fish species, which depends on the water temperature.

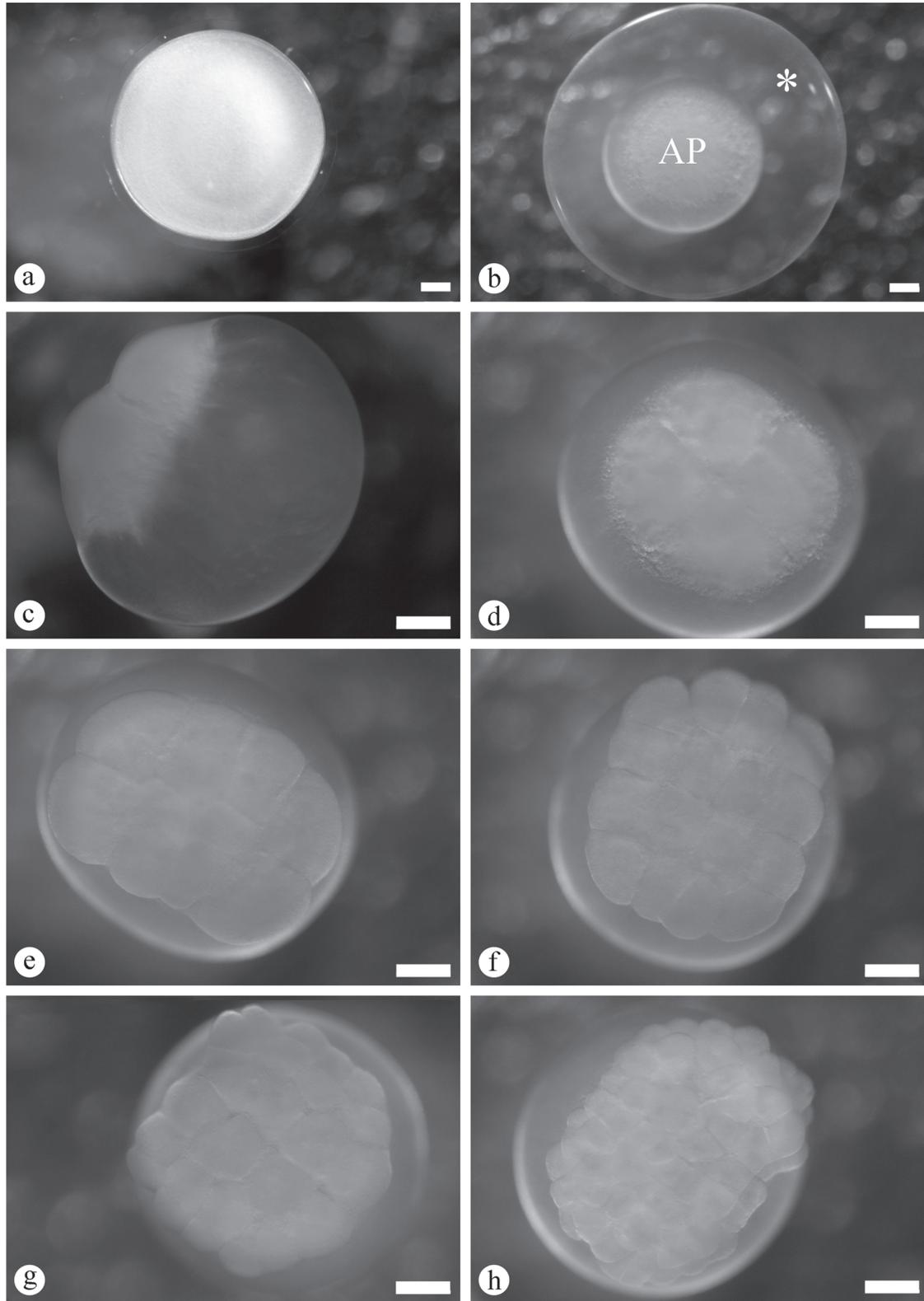
**Larval stage - exogenous feeding:** This stage includes the start of exogenous feeding and can last for a few days to months (depending on the temperature and species). In this study, the duration of this stage was established as ten days after the start of exogenous feeding.

### Egg stage

Following spawning and fertilization until hatching period, we observed different stages of development, as follows: zygote, cleavage, including the morula phase, blastula, gastrula, organogenesis, and hatching, with particular features for *S. parahybae* embryos and larvae. Additionally, heterogeneity was observed during the embryo development, *i.e.*, embryos at different embryonic stages were observed at the same moment, and due to this heterogeneity of development, the different stages of development were characterized when 50% of the eggs/embryos/larvae were in this specific stage. The time after fertilization (AF) is reported in parentheses.

**Zygote stage.** Initially, before hydration, it was possible to visualize that the eggs were yellow, nonadhesive, and ovoid in shape with an average diameter of 1.10  $\pm$  0.10 mm (Fig. 2a). After fertilization and hydration of eggs, easily evident (within 10-30 min AF), fertilization activates cytoplasmatic movements (cytoplasm streams towards the animal pole, where the nucleus is positioned, to form a prominent layer of cytoplasm known as blastodisc), and the average diameter of eggs increased to about 1.90  $\pm$  0.60 mm (Fig. 2b), characterized by a large perivitelline space (Fig. 2b) that persisted up to embryo development, the double chorion, and the poles (animal and vegetative). The yolk was concentrated in the vegetative pole, while the cytoplasm, nucleus and organelles were distributed over the animal pole (Fig. 2b).

**Cleavage stage.** The cleavage was typically characterized by creating a cellular region above the yolk sac, which was firstly divided in the blastodisc (the first cleavage, 1 h 20 min AF), dividing this blastodisc into two blastomeres of equal size (embryonic cells, Fig. 2c). Second and third cleavages occurred, giving rise to four blastomeres (in a 2 x 2 arrangement of cells, 1 h 40 min AF, Fig. 2d), and eight blastomeres (in a 2 x 4 arrangement of cells, 1 h 50 min AF, Fig. 2e). Following the cleavage (fourth and fifth cleavages), respectively, sixteen (in a 4 x 4 arrangement of cells, 2 h AF, Fig. 2f) and thirty-two blastomeres (in a 8 x 4 arrangement of cells, 2 h 10 min AF, Fig. 2g) were lying in only one layer, and possibly curved on the yolk sac surface. The sixth cleavage or sixty-four blastomeres (in a 2 x 4 x 8 arrangement of cells, 2 h 40 min AF, Fig. 2h) was still easily evident for observation;



**Fig. 2.** Macroscopic aspects of embryonic development of *Steindachneridion parahybae*. **a)** oocyte extruded (just after spawning, and before fertilization and hydration) -  $1.10 \pm 0.10$  mm; **b)** egg hydrated soon after fertilization (time zero AF) -  $1.90 \pm 0.60$  mm; **c)** two blastomeres (1 h 20 min AF); **d)** four blastomeres (1 h 40 min AF); **e)** eight blastomeres (1 h 50 min AF); **f)** sixteen blastomeres (2 h AF); **g)** thirty-two blastomeres (2 h 10 min AF); **h)** sixty-four blastomeres (2 h 40 min AF). (\*): perivitellinic space; (AP): animal pole. Bars: (a-b) 0.2 mm; (c-h) 0.3 mm.

however some blastomeres completely covered others. Beyond the sixth cleavage, the number of small cells became increasingly higher and it was more difficult to discern any stereotypical arrangement of the blastomeres, besides; the cell cycles begin to lose synchrony and any patterns of cleavage plan.

The morula stage was characterized by successive cells divisions (more than sixty-four blastomeres) and arranged in a “half-berry”-like shape; the blastula stage was identified when the periblast and blastoderm region or embryonic cells became clear, and the animal pole presented a cup-like shape (without identification of embryonic cells boundaries) up to the beginning of the cell movement; when it began to characterize the gastrula stage, *i.e.*, in this stage it was observed the occurrence of morphogenetic movement, named epiboly, which resulted in a rearrangement of the blastoderm relative to the yolk mass, and ultimately forms the germinative follicles and establishes the embryonic axis. The morphogenetic movement was divided in three steps: epiboly of 25% (Fig. 3a), epiboly of 50% (Fig. 3b), and epiboly of 90% (Fig. 3c); and subsequently, the final gastrula stage, and blastopore closure (Fig. 3d). The morula stage occurred between 2 h 50 min and 11 h 20 min AF.

Following the development, the next stage identified was the organogenesis stage (Figs. 3e, f, g, h; 4a), which was subdivided respectively in: early segmentation phase and late segmentation phase (between: 14 h 25 min and 25 h AF). In the early segmentation phase, subsequently of the establishment the embryonic axis, it was possible to distinguish between the cephalic and caudal regions (14 h 25 min AF, Fig. 3f), where the tail was short and attached to the yolk mass, and along this axis, non-pigmented regions were observed; the optic vesicle was present (Fig. 3g); and the somites, that have a mesoderm origin and will give rise to the muscle, was also identified (Figs. 3g, h); this subdivision ended, when the tail detached and embryo started growing (Fig. 4a). Therefore, the late segmentation phase was characterized by the presence of the tail well extended and free from the yolk sac (25 h AF, Fig. 4a), many somites, optic vesicle well developed, and embryo growth. Additionally, during the late segmentation phase and before hatching, the embryo showed conspicuous muscular contraction.

**Hatching stage (Fig. 4b).** Vigorous muscular twitches of the enclosed embryo were important to break the chorion, which was softened and slimed now, and finally this movement resulted in the tearing of the chorion, and fry release (hatching start). Total hatching occurred at 54 h AF, and the average length was  $4.30 \pm 0.70$  mm; thus the larval stage began.

#### Larval stage with yolk sac and larval stage - exogenous feeding

**Day 1 (average length  $4.50 \pm 0.80$  mm).** After hatching (AH), the larvae were still translucent, and the body was non-

pigmented (Fig. 4c). Following the growth (23 h AH), we observed a slight reduction in the yolk sac (Fig. 4c), the early formation of barbells, and the mouth was not completely formed yet (not functional).

**Day 2 (average length  $4.60 \pm 1.20$  mm).** A reduction in the yolk sac was observed, and the barbells were more visible (38 h 30 min) (Fig. 4d). Gill development was observed, and in some larvae, but not all, the mouth and jaws were formed and mobile (40 h AH) (Fig. 4d). Approximately 50 h AH, exogenous food composed of ‘lambari’ (*A. altiparanae*) larvae was offered (Fig. 4e), and it was observed predation of ‘lambari’ by *S. parahybae* larvae. At that point, most *S. parahybae* larvae had open mouths, eyes with pigmented retinas, and still a smaller yolk sac.

**Day 3 (average length  $5.40 \pm 2.10$  mm).** Exogenous feeding started by this time, therefore, there was a period of combined endogenous and exogenous food supply. Following the growth, about 60-70 h AH, we observed that the *S. parahybae* larvae were completely full of exogenous food (Fig. 4f). We could no longer detect the yolk sac by histological analysis, and the barbells were completely formed and well developed (Fig. 4e), as well as the caudal and anal fins, while dorsal and pectoral fins were still in the first steps of development. At this time (about 60-70 h AH), we also observed some cannibalism among the *S. parahybae* larvae (Figs. 5a, b).

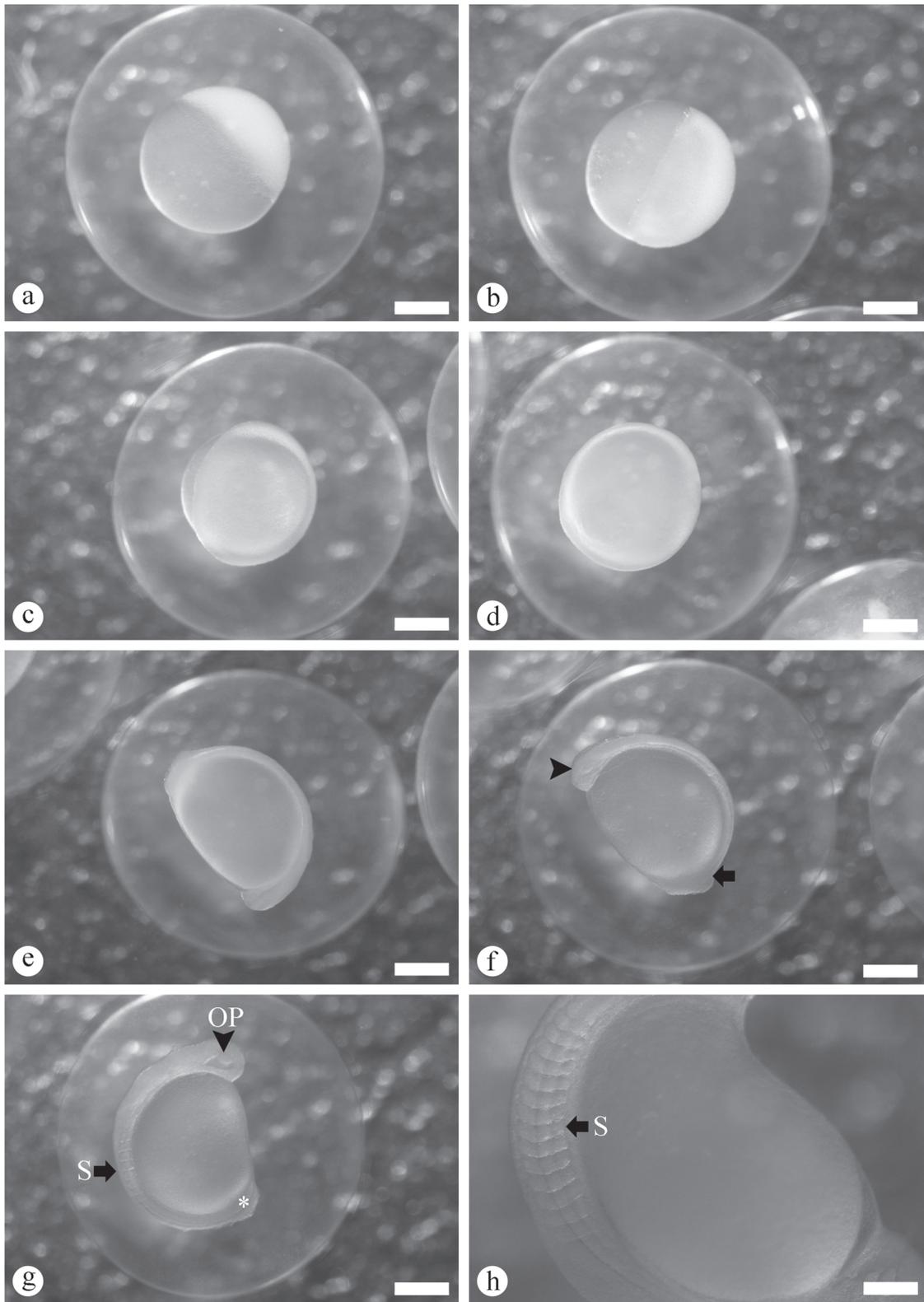
**Days 4 to 10.** At approximately 80 h AH, ‘curimbatá’ (*P. lineatus*) larvae were offered, and we observed intense cannibalism. After 100 h 57 min AH, exogenous feeding was initiated with a prepared food (Fig. 5c, d). Even when ‘curimbatá’ larvae were offered with prepared food (mixture of commercial fish food and milled fish) (Fig. 5d), we observed cannibalism among *S. parahybae* larvae. However, some larvae fed on prepared food (Fig. 5c, d) while others fed on ‘curimbatá’ larvae (Fig. 5d), or both (Fig. 5d). We also observed that the *S. parahybae* larvae displayed photophobia.

After three months, *S. parahybae* juvenile were an average length of  $11.00 \pm 2.20$  cm. The time scales of the major events in the early development of *S. parahybae* are shown in figure 6.

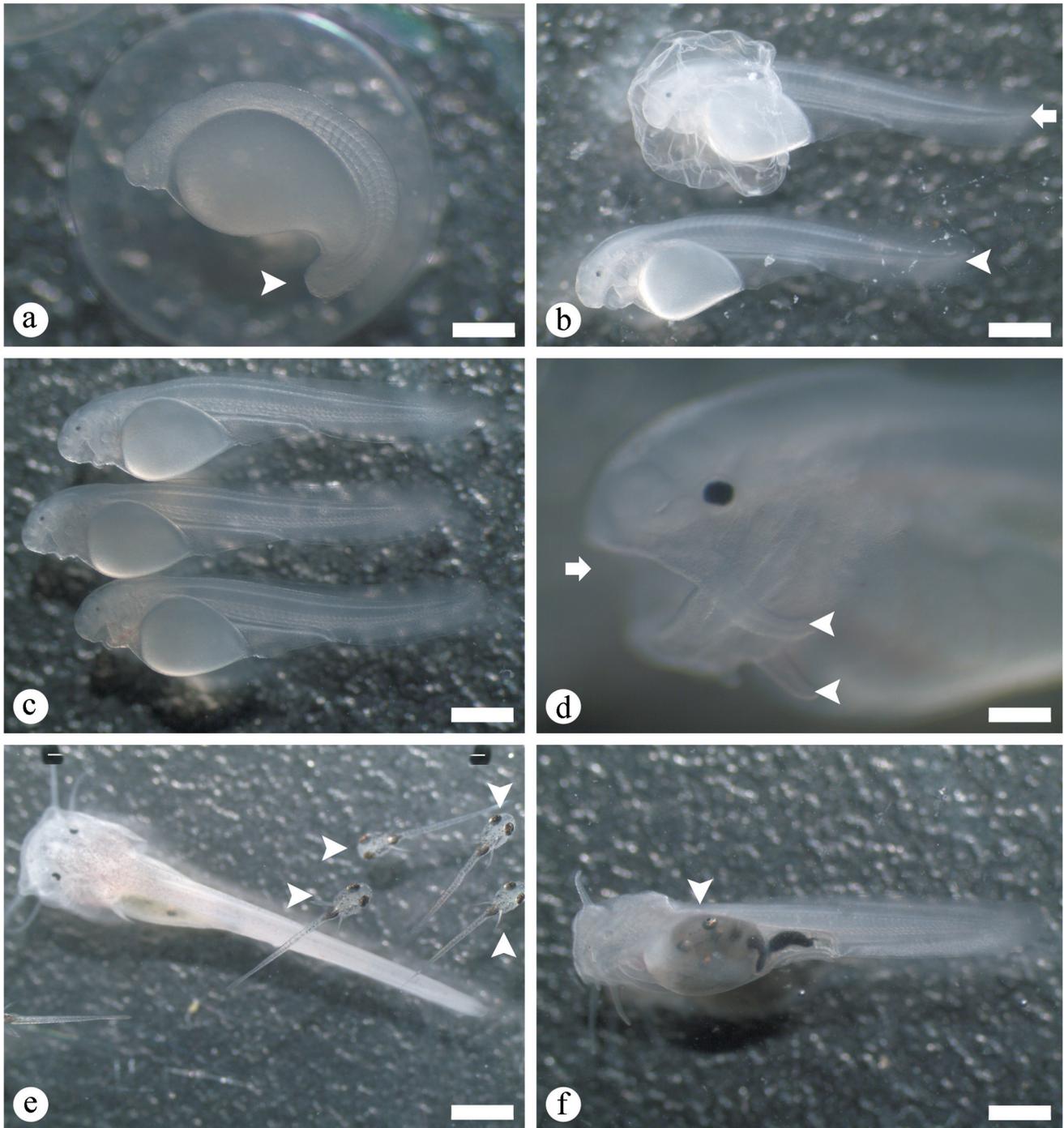
#### Histological analysis

In the present study, the females presented vitellogenic oocytes with central or slightly eccentric nuclei (Fig. 7a) with small nucleoli. Furthermore, the follicular layers were developed and could be easily seen even at this level of analysis (Fig. 7a). After fertilization and egg activation (0 m AF), it was possible to identify the perivitellinic space and the yolk granules (Fig. 7b). The final gastrula and blastopore closure (11 h 20 min AF) and the differentiation between the cranial and caudal regions was observed microscopically (Fig. 7c).

**Day 1 (after hatching).** In the cranial region, we could identify the head, attached to the yolk sac, and the neurocranium. In the caudal region, we could identify the embryonic tail (Fig. 7d). At 34 h AH, the main structures of the larvae head were identified:



**Fig. 3.** Macroscopic aspects of embryonic and larval development of *Steindachneridion parahybae*. **a-c**) gastrula stage: **a**) epiboly of 25%; **b**) epiboly of 50%; **c**) epiboly of 90%; **d**) final gastrula stage and blastopore closure (11 h 20 min AF); **e-h**) organogenesis stage: **e**) organogenesis and embryo growth (beginning of differentiation of embryonic layer); **f**) differentiation of embryonic layers, head (arrowhead) and tail (arrow) (14 h 25 min AF); **g**) distinguish between the cephalic (arrowhead) and caudal (asterisk) regions, somites (S), and optic vesicle (OP); **h**) Detail of somites development (S). Bars: (a-g) 0.3 mm; (h) 0.2 mm.



**Fig. 4.** Macroscopic aspects of larval development of *Steindachneridion parahybae*. **a)** complete development and the tail release (arrowhead) (25 h AF); **b)** hatching (time zero AH) -  $4.30 \pm 0.70$  mm (54 h). Hatched (arrowhead), and hatching (tearing of the chorion, and fry release; arrow); **c)** after hatching, the larvae were still translucent with a non-pigmented body; **d)** early formation of barbells (arrowhead) (38 h 30 min AH), and larvae with opened mouth (arrow) were observed although exogenous feeding have not been observed yet (50 h AH); **e)** larvae fully developed (all structures) with barbells (60 h AH), and exogenous feeding of *S. parahybae* with *A. altiparanae* larvae (arrowhead) (100 h 57 min AH); **f)** Detail of *S. parahybae* feeding at *A. altiparanae* larvae (arrowhead). Bars: (a) 0.3 mm; (b-c) 0.9 mm; (d) 0.2 mm (e-f) 0.8 mm.

eye, operculum, gill arches, and neurocranium (Fig. 7e). By the end of the second day, we could also identify the main features of the fish: head, tail, stomach, and others structures (Fig. 7f).

Early on the third day, we did not detect food in the larvae's stomach, despite the mouth being open (Fig. 7f). However, at 60 h AH, we could identify all features of the digestive system as shown in Figures 7g, h, i (mouth, esophagus, stomach, gut, and other), and some food in the digestive system ('lambari' or 'curimbatá' larvae).

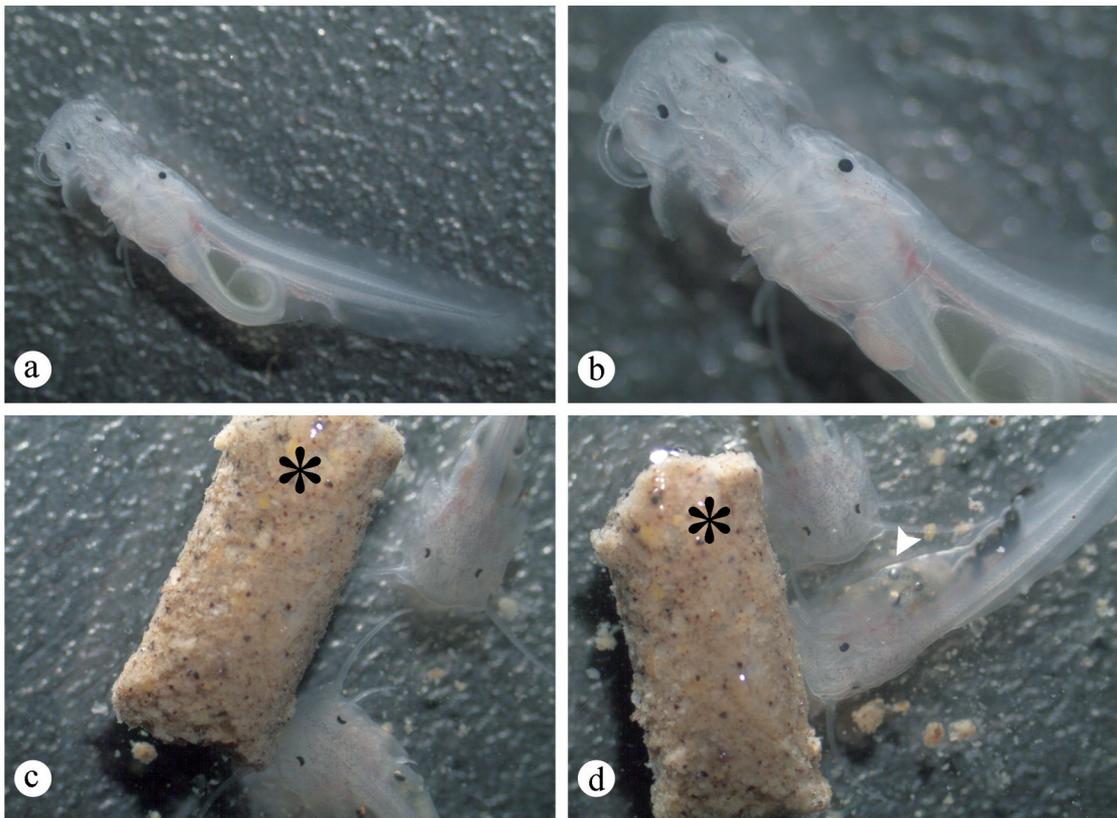
### Discussion

The water quality parameters analyzed in the present study were close to other research's which observed embryonic and larval development in other Neotropical species, mainly for the Siluriformes order, *i.e.*, *P. maculatus*, *R. quelen*, *P. fasciatum* (Barcellos *et al.*, 2004; Leonardo *et al.*, 2004; Weingartner & Zaniboni-Filho, 2004), and fish of the same genus of *S. parahybae*, as *S. scripta* (Adamante *et al.*, 2007), *S. scriptum* (Schütz *et al.*, 2008), and *S. melanodermatum* (Feiden *et al.*, 2006).

The ATU values observed in this study were similar to those of other catfish species in the Pimelodidae family

(Weingartner & Zaniboni-Filho, 2004; Campos, 2005), including other wild broodstocks of *S. parahybae* in captivity (Caneppele *et al.*, 2009). Furthermore, the results of spawning induction experiments in *S. parahybae* from the wild broodstocks indicated that artificial reproduction is viable although the 'surubim do Paraíba' females release only a small amounts of eggs (~53g per females) (Caneppele *et al.*, 2009). Using hormonal induction, we observed a slight increase in the amount of eggs released (~92g per females). Despite this slight increase, the amount of eggs that was released is below that observed in other Neotropical catfish, such as: *R. quelen* (Baldisserotto & Neto, 2005), *P. corruscans* (Campos, 2005), *S. melanodermatum* (Ludwig *et al.*, 2005), *P. fasciatum* (Leonardo *et al.*, 2004).

The small quantity of eggs released during the artificial induction of *S. parahybae* can reflect the gonadosomatic index values, which increased during the final gonadal maturation and were similar after the hormonal induction of domesticated broodstock (Honji, 2011). Macroscopic and microscopic analysis of the gonads of *S. parahybae* may help explain the small quantity of eggs released. The pattern of oocyte development seems to be multiple-batch group-synchronous (Rocha & Rocha, 2006) during the reproductive



**Fig. 5.** Macroscopic aspects of larval development of *Steindachneridion parahybae*. **a-b)** cannibalism among the *S. parahybae* larvae; **a)** start of cannibalism among the *S. parahybae* larvae (60 h AH); **b)** detail of cannibalism; **c-d)** exogenous feeding with prepared food; **c)** exogenous feeding was initiated with a prepared food (100 h 57 min AH); **d)** exogenous feeding of *S. parahybae* with *Prochilodus lineatus* larvae (arrowhead). (\*) commercial fish food. Bars: (a, c, d) 0.8 mm; (b) 0.4 mm.

season (Honji, 2011), a reproductive strategy also suggested for another Pimelodidae, *P. corruscans* (Godinho *et al.*, 2007), a species commonly known as single spawners for most authors so far (Bazzoli, 2003; Godinho & Godinho, 2003; Sato & Godinho 2003; Campos, 2005).

Hand-stripping, followed by dry gamete fertilization is a good technique for *S. parahybae* (Caneppele *et al.*, 2009), and the embryonic and larval development is similar to other Siluriformes species. The animal pole forms in *S. parahybae* at about 30 min, which was similar to *P. maculatus* at 23.1°C (Luz *et al.*, 2001). In *P. maculatus* the beginning of successive divisions in the animal pole was observed at 40 min AF; 32 blastomere was observed at 1 h 40 min AF; and the blastopore closed was identified after 5 h 50 min AF (Luz *et al.*, 2001). These results show that *P. maculatus* developed faster than *S. parahybae*. In *P. corruscans* the appearance of the animal pole and successive divisions began around 1 h and high blastula stage occurred within 2 h 50 min AF (at 23.5-25.0°C) (Cardoso *et al.*, 1995). The blastopore in *S. parahybae* closed at 11 h 20 min AF, slightly later than that of *P. corruscans*, which occurred after 6 h AF under similar temperatures. Furthermore, the *P. corruscans* larvae at 25-26°C displayed blastopore closure at 6 h 30 min AF (Cardoso *et al.*, 1995). Other Siluriformes species like *Heterobranchus longifilis* and *Clarias gariepinus* have an early embryonic development similar to *S. parahybae*, indicating that the development of surubim do Paraíba eggs generally follows that of other fish species, with small differences in time of development (Legendre & Tcugcls, 1991).

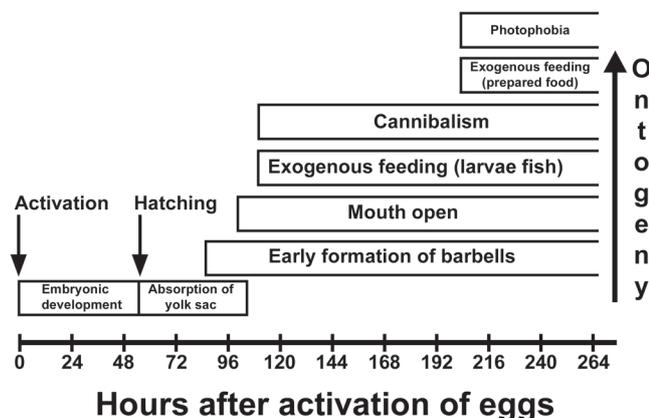
At 11 h 20 min AF, it was possible to distinguish the cranial and caudal region in *S. parahybae*. However, in *P. corruscans* (Cardoso *et al.*, 1995), *P. maculatus* (Luz *et al.*, 2001), and *Rhamdia hilarii* (Godinho *et al.*, 1978), this distinction occurred at 7 h 50 min, 10 h 50 min, and 7 h 30 min AF, respectively, under similar temperature conditions. Furthermore, the somites and optical vesicle of *P. corruscans* were visible during this period, but they were not visible in *S. parahybae* until 20 h 10 min AF. This result demonstrates the

great difference in embryonic development between species, even those belonging to the same family.

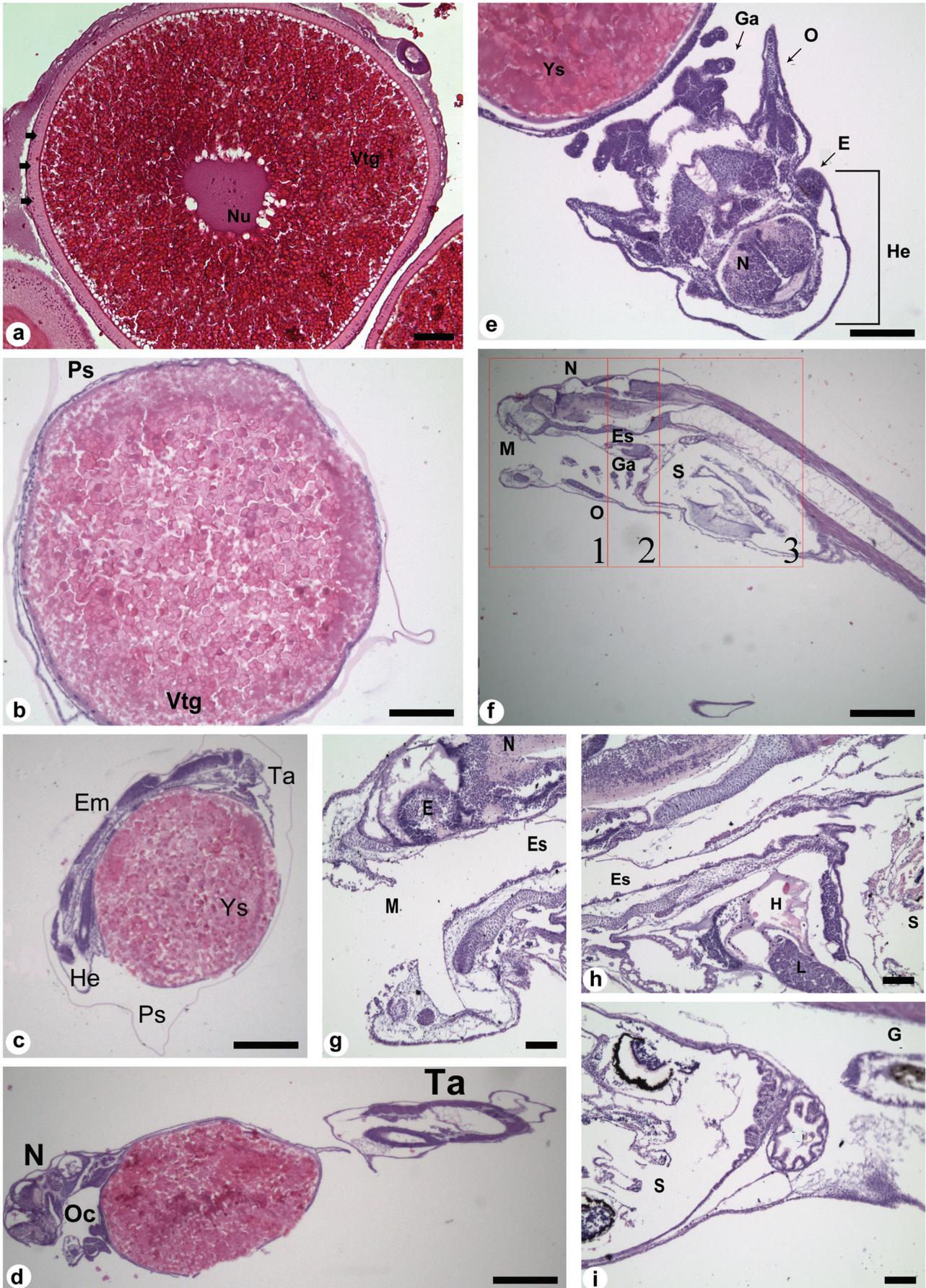
The hatching period of *S. parahybae* (54 h at 23°C) is later than other Siluriformes species, such as *P. corruscans*, which hatched 16 h AF at a temperature of 24.5°C when the larvae were an average size of 2.51 mm (Nakatani *et al.*, 2001). Similar times were observed in *H. longifilis* and *C. gariepinus* (Legendre & Tcugcls, 1991). *P. maculatus* larvae hatched at 14 h 30 min AF at 25.5 ± 0.3°C with the larvae measuring 2.56 ± 0.13 mm, or at 12 h AF when reared at 27.6 ± 1.4°C (Luz *et al.*, 2001). Most fish species which migrate to reproduce have a rapid embryonic development, *i.e.*, 22 h at 24°C for *Prochilodus scrofa* (= *P. lineatus*) (Cavicchioli & Leonhardt, 1993), 18 h at 25-26°C for *Colossoma macropomum*, and 22 h at 25-26°C for *Piaractus mesopotamicus* (Ribeiro *et al.*, 1994). Interestingly, even though *S. parahybae* migrate to mate, their progeny do not develop quickly. The embryonic development time showed by *S. parahybae* is similar to or even faster than non-migratory fish species, such as *Hoplias malabaricus* and *Parauchenipterus galeatus*, which hatched 44 h at 24-26.5°C and 64 h at 27-28°C AF, respectively (Sanches *et al.*, 1999, 2001). However it must be considered that the experimental temperature for *S. parahybae* in the present study was lower (23°C) than most of the species above described, another reason for the slower development speed, since teleosts are ectothermic vertebrates.

During the larval development, adequate levels and intensity of light are required to optimize growth of many fish species (Boeuf & Bail, 1999). The rapid growth of larvae during this period depends upon vision in order to find food, select prey, and avoid predators (Appelbaum & Mcgeer, 1998; Boeuf & Bail, 1999). In contrast, other species, including Siluriformes, such as, *C. gariepinus* (Appelbaum & Mcgeer, 1998), *P. maculatus* (Luz *et al.*, 2001), *R. quelen* (Boeuf & Bail, 1999), *Steindachneridion* sp. (Feiden *et al.*, 2005, 2006), *S. scriptum* (Schütz *et al.*, 2008), the larvae can feed independent of the vision, because they show photophobia, having a better development when they are stored in dark or low intensity light (Appelbaum & Mcgeer, 1998; Schütz *et al.*, 2008). Furthermore, studies with Siluriformes species indicate that dark environments also reduce the stress and consequently improve larval development (Appelbaum & Mcgeer, 1998; Feiden *et al.*, 2005, 2006). Previous studies of *S. parahybae* larvae (Caneppele *et al.*, 2009) and this study observe photophobia in *S. parahybae* larvae. Therefore, further studies of the influence of light on *S. parahybae* larval growth should be performed.

The main problem with the larviculture of Neotropical species in Brazilian Fish Farms (including *S. parahybae*) is the lack of knowledge about feeding during the larval development, mainly due to a lack of ecological, behavioral, and ontogenetic studies during this period. Much larvae mortality is directly related to inadequate feeding practices, which, in most cases, do not meet the real nutritional needs of larvae (Appelbaum & Mcgeer, 1998). Although some techniques that use inert feed (starters) are well known and efficient for some species, many others species do not become accustomed to this management



**Fig. 6.** *Steindachneridion parahybae*: major events during ontogeny of the surubim do Paraíba larvae.



(Kamler, 1992). Thus, the employment of live food, as performed in the present study is recommended. We offered live food to *S. parahybae* larvae at first and transitioned to inert food on the eighth day. In our study, the 'surubim do Paraíba' larvae accepted this transition period, although we did observe some cannibalism during this period. Additionally, carnivorous fish larvae do not have a fully developed gastrointestinal tract and enzymatic activity when exogenous feeding begins (Dabrowski, 1984). Consequently, these animals are not able to digest inert food. This fact furthermore suggests that these larvae use the enzymes present in live food for digestion, in a process known as autolysis (Dabrowski, 1984).

Cannibalistic behavior, present when feeding practices are not adequate, is the main source of larvae mortality and production decrease in aquaculture. During rearing, which typically occurs in small ponds in larviculture, the chance of cannibalism is higher because of a higher density of larvae and a decreased chance of escape from predation (Smith & Reay, 1991). Furthermore, heterogeneous growth in fish larvae is always observed in many teleost, such as seen in *C. gariepinus* (Hecht & Pienaar, 1993) and in *S. parahybae* larvae (Caneppele *et al.*, 2009) as also evidenced by the present study.

Cannibalism behavior was observed in 36 of 410 teleost families, with several species showing this behavior approximately 50 h AF dependent upon temperature (Smith & Reay, 1991; Nelson, 2006). To decrease the intensity of cannibalism, the following techniques can be applied: increase food availability, maintain shorter intervals of feeding, and

lower the stocking density (Howell *et al.*, 1998). *S. parahybae* larvae showed a greater growth homogeneity and survival, with reduced cannibalism, when they were maintained in dark environment, at low stocking density, and with plenty of food available, ideally live food (fish prey) followed by inert food (Caneppele *et al.*, 2009). These techniques, applied in the present study, were also observed in the genus *Steindachneridion*, such as *S. melanodermatum* (Ludwig *et al.*, 2005; Feiden *et al.*, 2006), *S. scriptum* (Schütz *et al.*, 2008), and *S. scripta* (Adamante *et al.*, 2007) when larvae were started with live food followed by inert food.

Many recent studies have shown the importance of conservation biology in the rescue of endangered wildlife (Wikelski & Cooke, 2006). There are implications for the conservation of *S. parahybae* from the present study. The events in embryonic development and feeding behavior of fish larvae observed here allow for the development of strategies to obtain success in larval rearing. For example, a live food diet (forage larvae) in the early days of larval development and a gradual transition to an artificial diet will increase the survival of larvae. Since *S. parahybae* is endangered, this information is critical in contributing to the success of artificial reproduction programs (Honji *et al.*, 2009). It is our recommendation that immediate action be taken in order to avoid the extinction of this species in the Paraíba do Sul River basin.

In conclusion, this study investigated the ontogeny and organogenesis of early development of *S. parahybae*. These data provide information that can be used to improve feeding protocols and to establish specific conservation management for this endangered catfish. Our data, together with the findings previously described by Caneppele *et al.* (2009), contribute to the recovery of this threatened species by giving biological tools to conservation biologists and aquatic physiologists to predict and solve future problems.

### Acknowledgements

The authors would like to thank the local fishermen who helped to collect the wild females, Ms. Eduardo Sanches for providing the cannibalism pictures, CESP (Companhia Energética de São Paulo) for providing the Fish Farm facilities and FAPESP (2007/55949-7 and 2008/57687-0) for financial support.

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**Fig. 7. (left page)** Main features of oocytes and the phases of embryonic and larval development of *Steindachneridion parahybae*. **a)** Micrograph of oocyte in advanced maturation stage, showing the main characteristics of oocyte: follicular layers (arrow), yolk granules (VtG), and nucleus (Nu). These oocytes were collected after cannulating the gonoduct with a fine polyethylene tubing attached to a plastic syringe; **b)** Egg hydrated, immediately after spawning and fertilization (time zero AF): showing the yolk granules (VtG) and perivitelline space (Ps); **c)** Final gastrula stage and blastopore closure - followed by embryo (Em) differentiation of head (He), tail (Ta) and yolk sac (Ys) inside the perivitelline space (Ps); **d)** Hatching (time zero AH): showing the neurocranium (N), tail (Ta), and oral cavity (Oc); **e)** Detail of the head (He) - showing the main structures of He: neurocranium (N), eyes (E), operculum (O), gill arches (Ga), and yolk sac (Ys); **f)** Neurocranium (N), opened mouth (M), and the main structures of digestive and respiratory system: gill arches (Ga), stomach (S), esophagus (Es), and operculum (O). The number indicate the levels of histological section shown in detail in: **g, h, and i; g, h, i)** Details of the digestive, sensorial, and respiratory system: eyes (E); esophagus (Es); gut (G); heart (H); liver (L); mouth (M); neurocranium (N); stomach (S). Hematoxylin-eosin staining. Bars: (a, b, e, g, h, i) 100 µm; (c, d, f) 400 µm.

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Submitted July 18, 2011

Accepted May 3, 2012

Published June 29, 2012