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Preparation of a fish embryo for micromanipulation: staging of development, removal of the chorion and traceability of PGCs in *Prochilodus lineatus*

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ABSTRACT Primordial germ cell (PGC) transplantation represents a valuable tool for gene-banking and reconstitution by means of a germline chimera. The development of this technology requires in depth studies of the developmental stages and traceability of PGCs. The objective of this study was to develop a micromanipulation procedure for the future establishment of cryobanks of PGCs in migratory characins. Incubation temperatures were evaluated at 22°C, 26°C and 30°C in order to synchronize developmental stages. The highest hatching rates and the lowest abnormality rate was found to be 26°C, which was considered to be the optimal incubation temperature. Enzymatic removal of the chorion was determined to be best using 0.05% pronase, in which the embryos presented better survival rates. In order to visualize PGCs *in vivo*, artificial *GFP-nos1 3'UTR* mRNA was injected and the migration route was observed *in vivo* as PGCs were visualized firstly at the segmentation stage (6 to 13 somites). The number of GFP positive cells ranged from 8 to 20 per embryo (mean of 13.8; n = 5). After hatching, GFP-positive cells increased to between 14 and 27 embryos (mean of 19.8; n = 5). Visualization of GFP-positive cells was possible at 10 days post hatching, and at this stage, the cells were positioned in the yolk extension region. This is the first report on PGC visualization *in vivo* in Neotropical fish, providing information about the identification and migration of PGCs. The information presented in this work brings new insights into gene banking in Neotropical species and subsequent reconstitution through a germinal germline chimera.

KEY WORDS: *biotechnology, genebank, germline chimera, primordial germ cell, teleost*

Introduction

Several species of fish are considered endangered due to anthropic action, such as pollution, construction of power hydroelectric plants, mining, fishing, and other factors (Agostinho *et al.,* 2005; Bellard *et al.,* 2016; Castro and Vari, 2003). Only in the Neotropical region, it is estimated that more than 312 freshwater species are listed in the IUCN Red Book of Brazilian Fauna (Machado *et al.*, 2008). Thus, this suggests the establishment of *ex-situ* genebanks for the long term (Comizzoli and Holt, 2014). Recently, surrogate technologies, including germ cell transplantation and production of germline chimera, have been developed in fish (Yamaha *et al.*, 2007). In such a procedure, primordial germ cells (PGCs) from endangered fish species are transplanted into a host, which then produce heterologous gametes (Siqueira-Silva *et al.*, 2018). In addition, PGCs preserve genetic diversity and maternal components, such as germplasm and mtDNA. Cryobanking of PGCs in liquid nitrogen is considered a valuable tool for gene banking because germline chimera derived from post-thaw PGCs may be used

Abbreviations used in this paper: BOD, biochemical oxygen demand, CEPTA, National Center of Research and Conservation of Aquatic Biodiversity; DPBS, Dulbecoo's phosphate-buffered saline; GFP, green fluorescent protein; PF, post-fertilization; PGC, primordial germ cell.

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as a reconstitution procedure for endangered or extinct species (Yasui *et al.*, 2011).

The production of a germline chimera involves micromanipulation in order to achieve transplantation of PGCs. In fish, micromanipulation procedures focused on experimental species, including the *Danio rerio* (Lin *et al.*, 1992), *Oryzias latipes* (Wakamatsu *et al.*, 1993) and the *Misgurnus anguillicaudatus* (Yasui *et al.*, 2011); however, some protocols for aquaculture species also exist, as in the case of carp (Yamaha *et al.*, 2001), salmonids (Yoshizaki *et al.*, 2005), and sturgeon (Saito and Psenicka, 2015). In Neotropical fish species, there is no protocol for the micromanipulation of embryos. Such a protocol is very important for several endangered fish species in the Neotropical region (Machado *et al.*, 2008); therefore, reconstitution and gene-bank procedures are necessary. An initial step for PGC transplantation is to synchronize the developmental stages within the donor and host species since the transplantation is generally performed at the blastula or somite stages. Manipulation of incubation temperature is commonly used to synchronize embryo development (Arashiro *et al.*, 2018), but the temperature of incubation is species-specific.

Micromanipulation in fish presents additional barriers, since the fish embryo have developed in the egg envelope. Another step that hinders micromanipulation is the chorion, a physical barrier that makes both collecting PGCs from the donor species and also transferring the collected cells to the host. The chorion may be removed mechanically, but proteolytic enzymes, such as pronase or trypsin, may be used to digest the chorion (Hallerman *et al.*, 1988; Henn and Braunbeck, 2011). The enzymes and their concentrations are species-specific, and in some species, as in the case of Japanese eel (Kawakami *et al.*, 2012), enzymes digest the chorion, making the micromanipulation more difficult and time consuming. When the chorion is removed, a new problem arises, since the denuded embryos must be cultured in a solution that mimics the medium of the perivitelline space. A combination of ions, sugars, buffers, and antibiotics is then necessary to maintain the denuded embryo, but such a combination is also species specific.

The final step for micromanipulation is to verify if the embryos adapt into the micromanipulation procedure. This includes microinjection, manipulation of denuded embryos, and incubation into Petri dishes and incubators. Most of Neotropical migratory species presents pelagic eggs, in which spawning occurs in open waters. It is unknown if the embryos may be maintained in Petri dishes for micromanipulation.

The streaked prochilod *Prochilodus lineatus* is an interesting model fish to evaluate such manipulation conditions because it is a migratory species with easy reproductive management. This species is commonly used in aquaculture due to its growth, adaptation into aquaculture conditions, and easy juvenile production. Studies involving micromanipulation of this species are interesting since other intra-generic prochilodontiid such as the *Prochilodus vimboides* are considered endangered (Machado *et al.*, 2008), and then, the establishment of procedures for micromanipulation may be applicable for such related species.

Based on the aspects above, the aim of this study is to establish a micromanipulation protocol for future establishment of cryobanks of primordial germ cells in migratory characins.

Experimental Protocols

The experimental procedures were conducted in line with the ethics committee for the use of laboratory animals from the National Center of Research and Conservation of Aquatic Biodiversity (CEUA/CEPTA #02031.000033/2015-11). Sampling in a natural environment was performed with the collection permission from Brazil (Sisbio #47741-5). All the experiments were conducted at CEPTA/ICMBio in Pirassununga-SP, Brazil.

Origin of broodstocks and artificial fertilization

Adult fish were selected for artificial propagation based on external characteristics and behavior. Males were selected based on both seminal release after gently stripping of the papillae area and also by breeding sounds, which is characteristic of this species. Females were selected based on reddish coloration in the papilla area and abdominal volume. The spawning of fish was induced using a single dose of pituitary carp extract (EBHC) at 7 mg kg^{-1}. After injection, the fish were kept in a 7000L tanks with constant water flow, as well asnd temperatures at 26 - 29ºC. After 9-13 hours, spawning behavior, such as males following the females, breeding sounds from the male, or the presence of eggs at the tank outlet, were detected. At this stage, the fish were collected and anesthetized in clove oil (100 mg L^{-1}, Biodinâmica, Brazil) and proceeded gamete sampling by stripping. The eggs oocytes were collected in a 30-cm circular glass bowl. During stripping, the oocytes were distributed in the periphery of the bowl, and oocytes contaminated with feces and urine were removed from the egg mass. Males were stripped in the same bowl used for females, but the sperm was distributed in the central region of the bowl. Such a procedure was used in order to standardize the fertilization time. The gametes were activated by addition of 150 mL of hatchery water to the sperm, and the gametes were rapidly homogenized by hand mixing. The fertilized eggs were, then, immediately used for the following experiments.

Temperatures on early development

After fertilization, each spawning was divided into three batches with temperature set at 22°C, 26°C, and 30°C. For each temperature, an aliquot was removed and fixed in 2.5% of glutaraldehyde in DPBS solution (Dulbecco's Phosphate-Buffered Saline) between regular time intervals to observe the embryonic development. The samples were observed using a stereomicroscope (Nikon SMZ

TABLE 1

EARLY DEVELOPMENT OF *PROCHILODUS LINEATUS* INCUBATED AT 22°C, 26°C AND 30°C

							Larvae	
	Unfertilized	Cleavage	Blastula	Gastrula	Somite	Hatch	Normal	Abnormal
22°C	3.8 ± 4.3%	96.15 ± 4.3%	94.0 ± 3.8%	88.4 ± 4.17%	82.6 ± 5.0%	47.2 ± 25.8%	84.2 ± 9.2%	15.7 ± 9.2%
26°C	6.8 ± 7.14%	93.1 ± 7.1%	91.4 ± 6.9%	78.1 ± 7.3%	75.0 ±10.5%	64.2 ± 7.9%	77.1 ± 12.7%	22.8 ± 12.7%
30°C	4.2 ± 2.1%	95.7 ± 2.1%	91.8 ± 3.6%	58.7 ± 28.0%	23.7 ± 19.5%	10.5 ± 12.5%	60.7 ± 15.1%	39.2 ± 15.1%

Newly fertilized eggs were placed in incubators at different temperatures, and the developmental stages were evaluated until the hatching stage.

1500, Nikon, Tokyo, Japan) with a CCD camera (Nikon DS-Fi, Nikon, Japan). Digital images were captured using software NIS - Ar Elements (Nikon, Tokyo, Japan). Samples were collected each 5 minutes until 2 h and 30 min post-fertilization (hours past fertilization (hpf); minutes past fertilization (mpf) and each 10 minutes until 5 hpf, each 15 min until 7 hpf; each 20 min until 11 hpf; and each 30 min until hatching (Arashiro *et al*., 2018; Pereira-Santos *et al*., 2016). Early developments of *Prochilodus lineatus* were divided into zygote, cleavage, blastula, gastrula, segmentation, and hatching, and each period was divided into stages based on studies of Fujimoto *et al.,* (2006) and Kimmel *et al.,* (1995).

Chorion removal and culture of denuded embryos

Some protease enzymes were evaluated in order to digest the egg chorion of *P. lineatus*. In the first series of dechorionation experiments, many fertilized eggs were placed in Petri dishes (90 X 15 mm) containing 20 ml of the five enzymatic media: 1% bromelain, 3% papain, 4% pepsin, 0.6% trypsin, and 0.032% pronase, all of them dissolved in characin medium (12 mM NaCl, 1 mM KCl, 1.5 mM $CaCl_2$ and 1.5 mM $MgCl_2$) and kept at 26°C in a B.O.D. incubator. After the immersion, the digestion of the chorion was evaluated in each solution based on the appearance of the chorion after mechanical stimuli (touch) using a glass pipette. The enzymatic activity was measured according to the following criteria: without alteration to the chorion, eggs may be easily dislocated using the pipette and the chorion remains swollen; eggs may be dislocated using the pipette, but the chorion is weakly softened; eggs may be dislocated using the pipette, but the chorion is strongly softened; digestion on the chorion surface is visible, eggs may not be dislocated using the pipette due to excessively softened chorion; complete digestion (absence) of the chorion.

In the second set of dechorionation assays, the most effective enzymes from the above results were used. The concentration of enzymes was changed in order to maximize dechorionation and provide a safe concentration for the embryos. For this, the eggs were transferred to Petri dishes (90 X 15 mm) coated with agar (100 ml) containing 20 ml of protease solution. Pronase 0.05% (Sigma # SLMQ2345V, St. Louis, USA) or porcine trypsin 0.15% (Sigma #110M7362V, St. Louis, USA) were dissolved in characin medium. The chorion digestion was evaluated using a stereomicroscope (SMZ1500, Nikon®, Tokyo, Japan), and the digestion time was recorded. The survival rate in the stages of cleavage, blastula, gastrula, somite, and hatching, as well as percentage of normal and abnormal larvae were analyzed. As controls, an aliquot of intact eggs was kept in a Petri dish with water.

GFP-nanos1 3'-UTR mRNA synthesis

The GFP-*nos1* 3'UTR mRNA was produced using as a template the vector pCS2-GFP-na*nos1* 3'UTR, kindly provided by Prof. Dr. Takafumi Fujimoto (Hokkaido University, Japan), which contains the 3 'UTR region of the *Danio rerio nanos1* (Koprunner *et al*., 2001) in fusion with the green fluorescent protein (GFP). The vector was cleaved with Not I restriction enzyme (IVGN0014, Anza™, Invitrogen™), and the mRNA was synthetized *in vitro* using a commercial kit mMESSAGE MACHINE Kit® (AM 1340, Ambion®, Life Technologies™). The mRNA produced was precipitated by LiCl and re-suspended in 200 mM KCl at final concentration of 100 ng. μL^{-1}. The mRNA concentration was measured in the NanoDrop spectrophotometer (Thermo Fisher Scientific).

Microinjection and PGC traceability

The GFP-*nos1* 3'UTR mRNA solution was microinjected in the blastodisc of dechorionated embryos of *Prochilodus lineatus* at the 1 cell stage for identification of primordial germ cells (PGCs) and their migration route. For this, a boron silicate micropipette of 10 μm diameter (Drummond, U.S.A) was used with a microinjector (CellTram vario, Eppendorf, Hamburg, Germany) that was connected to the micromanipulator (M-152, Narishige, Tokyo, Japan) under a stereomicroscope (SMZ18, Nikon®, Tokyo, Japan). The microinjected fertilized eggs were kept in petri dishes (90 X 15 mm) with 200 mL of Characin solution at 26°C in a B.O.D. incubator. The embryonic and larval development were analyzed under fluorescence stereomicroscope (Nikon SMZ18, Tokyo, Japan), connected to a CCD camera (DS-Ri2, Nikon®, Japan). Digital images were taken using the Nis-Ar Elements (Nikon, Tokyo, Japan). The control groups, uninjected and uninjected dechorionated, were kept under the same conditions. After hatching, the larvae were observed daily until the fluorescence of the GFP-positive PGCs disappear.

Statistics

Data are shown as mean ± standard error of mean. All data were transformed using the arc sin transformation in order to fit normality and submitted to Kruskal-Wallis' test. The means were compared using non-parametric Tukey's multiple range test.

Results

Embryo and larval development were influenced by incubation at different temperatures. The hatching rate varied among the temperatures, being 47.2 ± 25.8%, 64.2 ± 7.9%, and 10.5 ± 12.5% at 22°C, 26°C, and 30°C, respectively. The highest mortality rates were observed during the gastrula stage, being a critical point in the effect of the temperature on the survival

TABLE 2

DEVELOPMENTAL STGES OF *PROCHILODUS LINEATUS* INCUBATED AT 22°C, 26°C AND 30°C

Stage	22ºC	26ºC	30ºC	Fig.No.
Cleavage				
2 – Cell	1 h	40 min	30 min	1B
4 - Cell	1 h 20 min	50 min	40 min	1C
8 - Cell	1 h 30 min	1 h 00 min	50 min	1D
16 - Cell	1 h 55 min	1 h 15 min	1 h 00 min	1E
32 - Cell	2 h 12 min	1 h 30 min	1 h 10 min	1F
64 - Cell	2 h 37 min	1 h 50 min	1 h 20 min	1G
Blastula				
128 - Cell	3 h 00 min	2 h 07 min	1 h 38 min	1H
256 – Cell	3 h 20 min	2 h 22 min	1 h 53 min	1I
512 - Cell	3 h 40 min	2 h 35 min	2 h 05 min	1J
1000 – Cell	3 h 50 min	2 h 50 min	2 h 20 min	1K
Elongation	4 h 00 min	3 h 20 min	2 h 41 min	1L
Sphere	5 h 00 min	3 h 18 min	2 h 50 min	1M
Dome	6 h 05 min	4 h 00 min	3 h 30 min	1N
Gastrula				
25% epiboly	6 h 35 min	4 h 15 min	4 h 00 min	1O
50% epiboly	7 h 15 min	4 h 50 min	4 h 45 min	1P
Germ ring	7 h 15 min	4 h 55 min	4 h 50 min	1Q
75% epiboly	8 h 45 min	5 h 30 min	5 h 30 min	1R
90% epiboly	11 h 00 min	6 h 40 min	6 h 10 min	1S
Segmentation				
Neurula	11 h 55 min	7 h 25 min	6 h 30 min	2A
Optic vesicle	15 h 00 min	8 h 55 min	7 h 45 min	3A
Otic vesicle	17 h 00 min	10 h 20 min	8h 30 min	3B
Kupffer vesicle	21 h 00 min	11 h 02 min	9 h 30 min	3C
Kupffer vesicle desappearance	22 h 00 min	13 h 14 min	10 h 30 min	3D
Hatching	27 h 00 min	14 h 45 min	11 h 30 min	4H

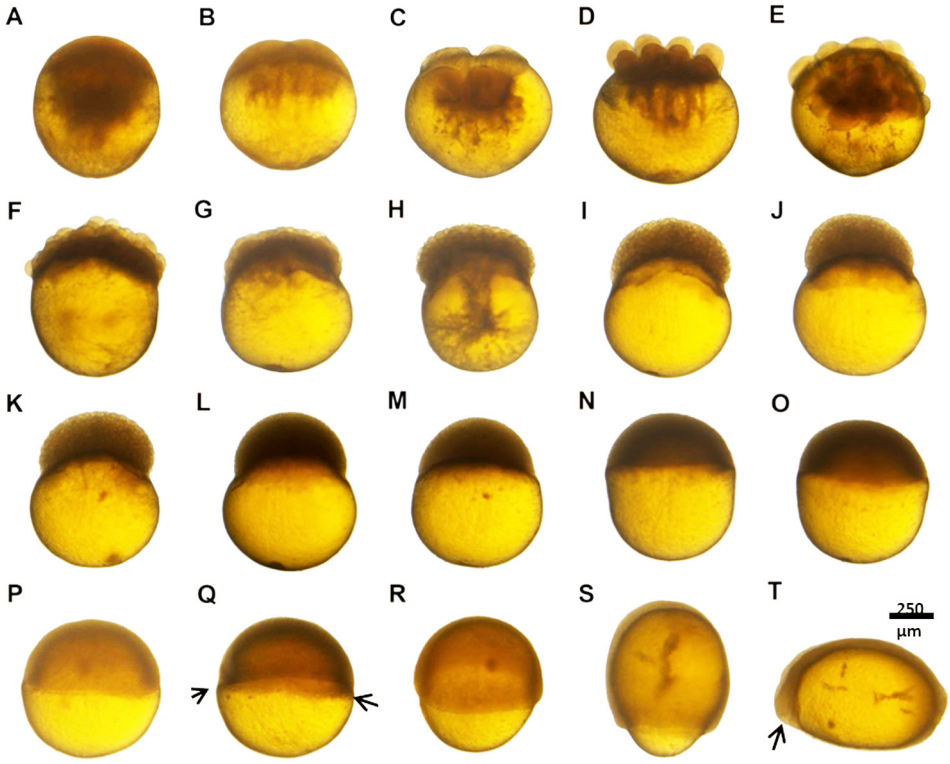

Fig.1. Embryonic development of *Prochilodus lineatus,* after fertilization, at stages of cleavage, blatula, gastrula, and initial segmentation. (A) *formation of blastodisc;* **(B)** *2 cells stage;* **(C)** *4 cells stage;* **(D)** *8 cells stage;* **(E)** *16 cells stage;* **(F)** *32 cells stage;* **(G)** *64 cells stage;* **(H)** *blastula initial stage with 128-cell;* **(I)** *stage of 256-cell;* **(J)** *512-cell stage;* **(K)** *stage with more than 1000-cell;* **(L)** *elongation stage;* **(M)** *sphere stage;* **(N)** *dome stage;* **(O)** *initial gastrula with 25% of epiboly;* **(P)** *stage with 50% of epiboly;* **(Q)** *germ ring stage (arrow indicates germ ring);* **(R)** *70% epiboly stage;* **(S)** *90% epiboly stage;* **(T)** *initial segmentation stage, neurula stage with head and tail differentiation (arrow points to head). Scale bar indicates 250 micrometers.*

of the embryos, especially in extreme temperatures. However, embryos incubated at 30°C had lower survival rates and the highest percentage of abnormal larvae (39.2%) when compared with other temperatures (Table 1). The characteristics of each stage of initial development are presented in detail in Table 2, which includes the embryogenesis intervals of the three incubation temperatures.

Cleavage period

After fertilization and hydration, the chorion begins to expand, giving rise to the perivitelline space. The cytoplasm begins to migrate to the animal pole, initiating the formation of the blastodisc and covering the yolk at the animal pole (Fig. 1A). The blastodisc formed within 1h when incubated at 22°C, 40 minutes when incubated at 26°C, and 30 minutes when incubated at 30°C (Fig. 1B, table 2). As in other teleost species, the cleavage occurred in a partial meroblastic pattern exclusively at the animal pole. Additionally, the cell diameter decreased substantially in each cell cleavage. The embryos reached the stage of 2 cells, called blastomeres. The cell divisions proceeded synchronously. At the 32-cell stage, the cluster of cells begins to overlap irregularly. The cleavage period ends when there are 64 cells, and this occurred over 02 h and 37 min when

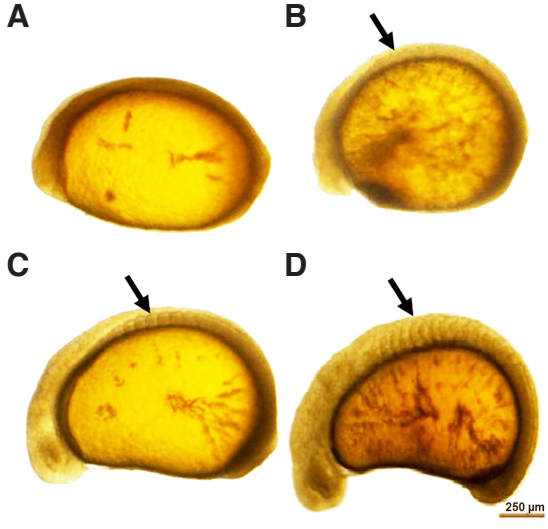

Fig.2 (left). Embryos of *Prochilodus lineatus* at segmentation stage. (A) *initial neurula stage;* **(B)** *3-somites stage;* **(C)** *8-somites stage;* **(D)** *14-somites stage (arrow indicate the somite). Scale bar indicates 250 µm.*

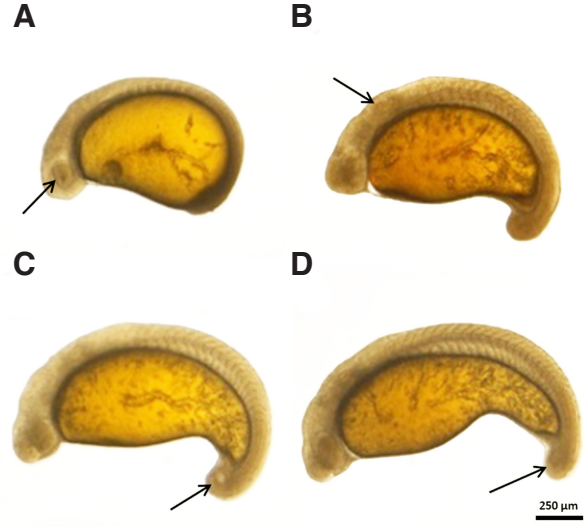

Fig. 3 (right). Embryos of *Prochilodus lineatus* at segmentation stage. (A) *arrow indicates optic vesicle;* **(B)** *arrow indicates otic vesicle;* **(C)** *arrow indicates Kupffer vesicle;* **(D)** *arrow indicates disappearance of Kupffer vesicle. Scale bar indicates 250 µm.*

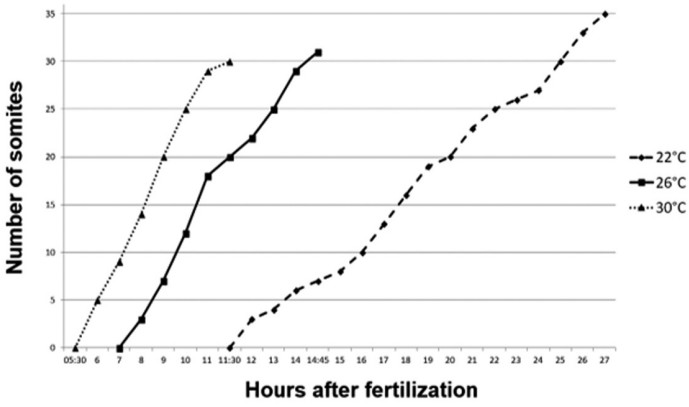

Fig. 4. Increasing the number of somites along the embryonic development of *Prochilodus lineatus* *incubated at 22°C, 26°C, and 30°C.*

incubated at 22°C, 1 h and 50 min at 26°C, and 1 h and 20 min when incubated at 30°C (Fig. 1B-G; Table 2).

Blastula period

The blastula stage continues cell cleavage and formation of the yolk syncytial layer (YSL) or periblast. The blastula period was divided into stages at 128, 256, 512, and more than 1000 cells, including the elongation, sphere, and dome phases. The cells were arranged in an irregular manner and overlapping each other over the yolk (Fig. 1H-I). At the 512 cells stage, the blastocyst is already much higher when compared to any stage of cleavage (Fig. 1J). During the elongation stage, the bulkier blastocyst cel cluster extends over the yolk in an elliptical form (Fig. 1L). The sphere represented the cluster of cells already organized in the yolk, and the surface of the blastomeres presented a flattened appearance, giving the embryos a spherical shape (Fig. 1M). During the dome stage, the cluster of cells began to cover all of the yolk in a movement called epiboly (Fig. 1N). The cluster of cells begins to cover all of the yolk in a movement called epiboly (Fig. 1N). This phase begins at 06 h and 05 min when incubated at 22°C, 4 h at 26°C and 03 h, and 30 min at 30°C (Fig. 1H-N Table 2).

Gastrula period

In this period, the embryos were subject to morphogenetic movement, characterized by the epiboly, involution, convergence, and extension. The progressive epiboly movements gave rise to an embryonic axis head–tail. The steps of this period are divided according to the percentage of yolk that is covered by the blastoderm. Morphogenetic movements of convergence and cel migration began at the border of the blastoderm, presenting about 50% of the epiboly movement when half of the yolk is covered by the blastoderm. In this stage, it was observed that the germ ring (Fig. 1Q) was composed of a superficial layer, the epiblast, and an inner layer, the hypoblast. The gastrula period concluded when the surface of the yolk was covered by the blastoderm, occurring after 11 h when incubated at 22°C, 06 h and 40 min at 26°C, and 06 h and 10 min at 30°C (Fig. 1O-T; Table 2).

Segmentation period

This stage is characterized by the formation of somites, which had a progressive increase (Fig. 2 A-D, 3 A-D and Fig. 4). This period is defined by structures visualized and the count of somites. It begins at the neurula stage and ends at the hatching. During this stage, the embryo begins to develop the rudimentary structures and organ, such as the optic and optic vesicle and the appearance of the kupffer vesicle (Fig. 3).

The first somites appeared at 13 h and 20 min when incubated at 22°C, 08 h and 05 min at 26°C, and 06 h and 10 min at 30°C (Fig. 2 and 3; Table 2).

Hatching period

The incubation temperatures influenced mainly the incubation period until they reached the hatching. For embryos incubated at 22°C, this event occurred at 27 hpf; at 26°C, this event occurred at 14 h and 45 hpf; and at 30°C, this event occurred at 11 h and 30 min (Fig 5, Tab 2). Incubation temperature influenced survival percentages 47.2%, 64.2%, and 10.5% at 22°C, 26°C, and 30°C, respectively. Hatchlings showing free swimming and no malformations were classified as normal (Fig. 5). Rates of normal and abnormal larvae are shown in Table 1. The increased temperatures accelerated the development of somites, but at the lower temperature, a higher number of somites was observed. Embryos incubated at 22°C hatched with 35 somites, while at 26 and 30°C, the embryos hatched with 32 and 30 somites, respectively (Fig. 4).

Enzymatic removal of the chorion

In the first stage of the chorion removal experiment, the dechorionation level was evaluated at 5, 10, and 15 min, based on the

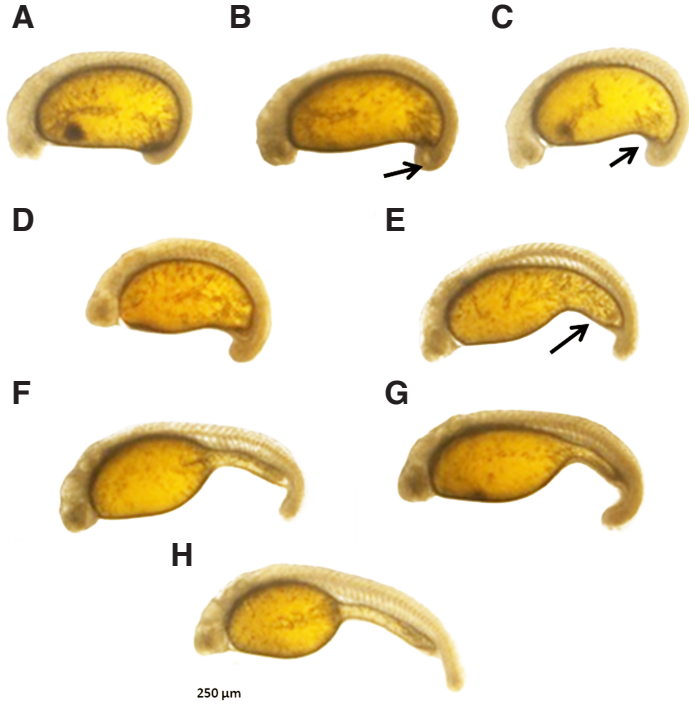

Fig. 5. Embryos of *Prochilodus lineatus* at segmentation stage until hatching. (A) *17-somites stage;* **(B)** *19-somites stage (Arrow indicates the Kupffer vesicle);* **(C)** *22-somites stage (Arrow indicates the tail detachment from yolk);* **(D,** *25-somites stage;* **(E)** *27-somites stage (Arrow point to the yolk elongation);* **(F)** *30-somites stage;* **(G)** *34-somites stage;* **(H)** *hatched larvae. Scale bar indicates 250 µm.*

TABLE 3

ENZYMATIC TREATMENT OF JUST-FERTILIZED EGGS

	Prochilodus lineatus		
Enzyme	**5 min**	**10 min**	**15 min**
Trypsin 0.6%	*****02:00	*****	*****
Pepsin 3%	**	***	***
Bromelain 1%	**	***	***
Papain 4%	***	***	***
Pronase 0.03%	*****02:30	*****	*****

After fertilization, the eggs were hydrated and, then, immersed in five enzymatic solutions. The level of dechorionation was evaluated at 5, 10, or 15 min based on visual inspection (chorion appearance and contact with a fine glass pipette (see score below).

* no alteration on chorion, eggs were easily dislocated using the pipette and the chorion remained swollen.

** eggs may be dislocated using the pipette, but the chorion was slightly softened.

*** eggs may be dislocated using the pipette, but the chorion was strongly softened.

**** digestion of the chorion surface was visible, eggs may not have been dislocated using the pipette due to excessively softened chorion.

***** complete digestion (absence) of the chorion.

visual inspection. In the enzymes pepsin, bromelain, and papain, the appearance of the chorion varied from eggs that may have been dislodged using the pipette, loosely softened chorion, and heavily softened chorion, according to increased exposure to enzymes. In the enzymes trypsin and pronase, the digestion of the chorion was completed after 2 and 02:30 minutes, respectively (Table 3).

In light of the results, the enzymes trypsin and pronase were more effective and, then, chosen to continue the evaluations.

The time of removal of the chorion varied for the enzymes evaluated. In the treatment containing the enzyme trypsin, the eggs were decorated, on average, 34 seconds after immersion in enzymatic solution. While in the treatment with pronase, the digestion of the chorion was later, on average 1 minute and 21 seconds after the exhibition (Table 4).

In both enzymes tested, the fertilization rate values were higher than 93%. From the blastula stage, the embryos exposed to the trypsin enzyme had a lower survival rate than the control group. The hatching rate percentages were similar for that verified in the control and embryos exposed to the enzyme pronase. Although the embryos exposed to the enzymes had a lower normal lava rate than the control, the percentage of normal and abnormal larvae were better for the pronase treatment (Table 4).

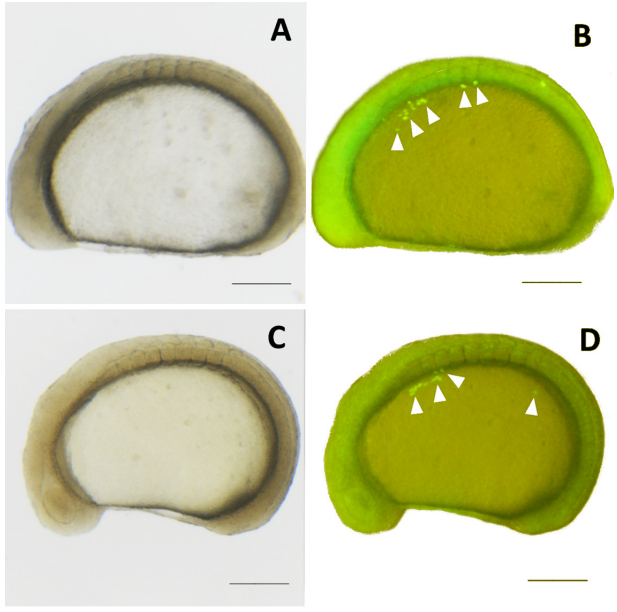

Fig. 6. Visualization of primordial germ cells (PGCs) in an embryo of *Prochilodus lineatus* injected with GFP-*nos1* 3'UTR mRNA. (A,B) *Segmentation embryo with 6 somites (PGCs, arrow tip).* **(C,D)** *Segmentation embryo with 11 somites (PGCs, arrow tip). (B and D are images captured under fluorescence of A and C, respectively).*

Taking into account the values verified for survival rate during embryonic development, the enzyme pronase was the most efficient for percentage of hatching and of normal larvae and, then, chosen to be used for the removal of chorion in *P. lineatus* eggs.

Migration route of GFP-positive primordial germ cells

Primordial germ cells (PGCs) as well as their migratory route were observed with the injection of GFP-*nos1* 3'UTR mRNA into dechorionated eggs of *Prochilodus lineatus*. In total, 142 eggs were injected, where 106 PGCs were GFP-positive *in vivo* when observed under fluorescence with the stereomicroscope. In relation

TABLE 4

SURVIVAL RATE OF DENUDED *PROCHILODUS LINEATUS* EMBRYOS AFTER EXPOSURE TO PROTEASE SOLUTION

								Larvae	
Groups	**Total eggs**	**Digestion time**	**Unfertilized**	**Blastula**	**Gastrula**	**Somite**	**Hatch**	**Normal**	**Abnormal**
Control	99		4.87 ± 0.01%	95.13 ± 0.01%	93.32 ± 0.00%^{a}	92.26 ± 0.01%^{a}	86.02 ± 0.03%^{a}	69.57 ± 0.02%^{a}	30.43 ± 0.02%^{a}
Trypsin	80	00:34	6.62 ± 0.00%	93.38 ± 0.00%	73.76 ± 0.06%^{b}	63.53 ± 0.02%^{b}	62.65 ± 0.02%^{b}	43.61 ± 0.04%^{b}	56.39 ± 0.04%^{b}
Pronase	57	01:21	6.30 ± 0.03%	93.70 ± 0.03%	86.83 ± 0.04%^{ab}	81.77 ± 0.04%^{ab}	82.27 ± 0.07%^{ab}	47.73 ± 0.07%^{ab}	52.27 ± 0.07%^{ab}

Trypsin 0.15% and pronase 0.05%, dissolved in characin medium.

$^{b.\ ab}$ Values with different superscripts differ significantly.

TABLE 5

							Larvae	
Groups	**Unfertilized**	**Cleavage**	**Blastula**	**Gastrula**	**Somite**	**Hatch**	**Normal**	**Abnormal**
Intact control	0.7 ± 0.0%	99.3 ± 0.6%	86.0 ± 5.9%	79.3% ± 8.1%	68.7 ± 12.1%	67.3 ± 12.6%	94.8 ± 2.9 %	5.2 ± 2.9%
Dechorionated control	6.1 ± 0.0%	93.9 ± 4.2%	82.0 ± 1.9%	78.0 ± 3.4%	58.7 ± 2.2%	58.8 ± 2.5%	93.1 ± 0.3%	6.9 ± 0.3%
Injected	4.6 ± 0.0%	95.4 ± 0.9%	83.7 ± 2.3%	76.7 ± 3.0%	57.4 ± 4.1%	56.2 ± 4.9%	91.7 ± 1.4%	8.3 ± 1.4%

Survival of embryos of *Prochilodus lineatus* that were intact (control with chorion), dechorionated (control without chorion), and injected with GFP-*nos1* 3'UTR mRNA during embryo development, in addition to percentage of normal and abnormal larvae.

There were no significant differences among control batches and experimental batches in each developmental stage. ($P > 0.05$).

to survival at different stages of development and percentage of normal larvae, there was no significant difference among embryos injected with GFP-*nos1* 3'UTR mRNA, dechorionated embryos (control without chorion), and intact embryos (control with chorion) (Table 5).

The first PGCs were visualized in the segmentation stage when the embryos had between 6 and 13 somites (Fig. 6 A-D). At this stage, the number of GFP-positive cells ranged from 8 to 20 per embryo (mean 13.8; n = 5). PGCs were located between the first and sixth somites on both sides of the dorsal axis. In the previous stages of development, it was not possible to identify PGCs.

After hatching, the GFP-positive cells migrated to the posterior region of the yolk sac (Fig. 7 A-C), ranging from 14 to 27 per embryo (mean 19.8; n = 5). Screening of PGCs occurred until the tenth day after hatching (Fig. 7 D-F) when they reached the upper part of the intestine where the gonadal ridge is located. During this period, no changes in position were observed, and the number of GFP-positive cells gradually decreased (Fig. 8). On the first post-hatch day, 9 to 19 (mean 12.8, n = 5) PGCs were found by larvae; on the fifth day, it ranged from 6 to 16 (mean 10.6. n = 5); while on the tenth day, there were between 4 and 5 (mean 4.5. n = 5) per larvae.

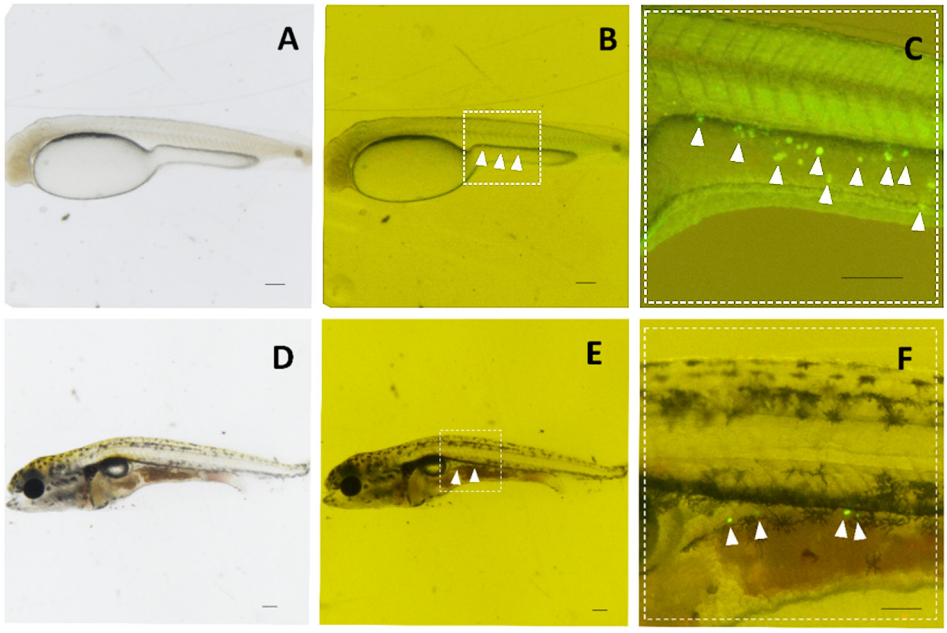

Fig. 7. Visualization of primordial germ cells (PGCs) in larvae of *Prochilodus lineatus* injected with GFP-*nos1* 3'UTR mRNA. (A,B) *Newly hatched larvae (PGCs, arrowhead).* **(C)** *Detail of the region highlighted in B.* **(D,E)** *Larvae 10 days after hatching (CGPs, arrowhead).* **(F)** *Detail of the region highlighted in E. (B and E are images captured under fluorescence of A and D, respectively).*

Discussion

The incubation temperature ranges described have been shown to influence the initial development, hatching time, and rate of abnormal embryo. The embryos of *Prochilodus lineatus* showed tolerance at temperatures of 22°C, 26°C, and 30°C. Higher temperatures at 30°C may be detrimental to incubation.

In a study of Neotropical species, such as *Astyanax altiparanae* (Pereira-Santos *et al.*, 2016), *Brycon amazonicus* (Silva *et al.*, 2017), *Rhandia quelen* (Rodrigues-Galdino *et al.*, 2009), *Pimelodus maculatus* and *Pseudopimelodus mangurusas* (Arashiro *et al.*, 2018), the species exhibited tolerance to the exposed temperatures, and the incubation time for the species were inversely proportional to temperature. Comabella *et al.,* (2014) and Lahnsteiner *et al.,* (2012) also report this inverse relationship between incubation time and temperature for *Atractosteus tristoechus* and *Lota lota* species, respectively.

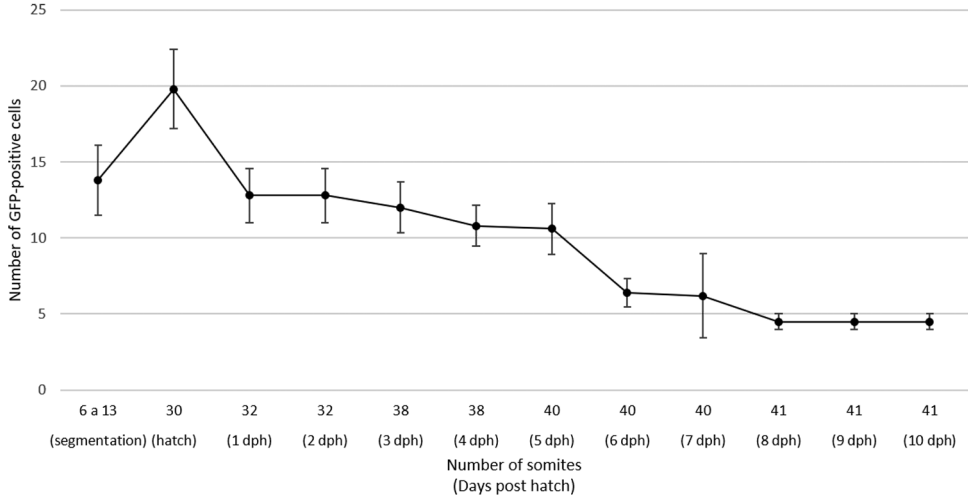

Fig. 8. Mean number of GFP-positive primordial germ cells (PGCs) in *Prochilodus lineatus* *traced during segmentation stage at the 10th day after hatching, incubated at 26°C.*

In fish, the incubation time may be affected by several factors, such as environmental and genetic conditions, that will determine the development period. Falk-Petersen (2005) reports that the duration of each stage of development in teleost is influenced by the size of the egg and the yolk sac and especially the temperature.

The manipulation of water temperature and the detailed description of each stage of embryonic development incubated at different temperatures can be used to manipulate the cell transplantation period. Studies involving cell transplantation require a specific stage and a synchronized embryonic development to collect the donor cells and transplant to the host embryos.

From this study, it is possible to support research in the area of cell transplantation in fish species, providing basis for the continuity of the following steps to prepare an embryo for germ cell transplantation.

Micromanipulation procedures of fish embryos were used in the identification, isolation, screening, transplantation, and cryopreservation of primordial germ cells (PGCs) (Fernández *et al.*, 2015; Fujimoto *et al.*, 2006; Linhartova *et al.*, 2014; Nagasawa *et al.*, 2013; Okutsu *et al.*, 2006; Saito *et al.*, 2006; Yasui *et al.*, 2011), which contributes to the establishment of genetic banks for endangered species.

To develop protocols for micromanipulation, transplantation, and cryopreservation of PGCs, it is necessary to establish methodologies for the management of eggs and embryos, requiring the removal of the chorion, which facilitates embryo manipulation (Hallerman *et al.*, 1988). This removal can be performed mechanically or chemically, the latter being more efficient in the procedure (Henn and Braunbeck, 2011). The chemical removal of chorion has been reported for some species of teleost fish using different protocols and enzymatic concentrations (Henn and Braunbeck, 2011; Morrison *et al.*, 2003; Yamaha *et al.*, 2001).

In this study, the chorion was successfully removed from newly fertilized eggs of *P. lineatus* without affecting its subsequent viability during embryogenesis, and normal larvae arose after hatching. The treatment using with pronase were more efficient, maintaining the integrity of the eggs. The treatment with trypsin was not satisfactory for this species in relation to the survival rate during the development stages and abnormality rate among the hatched larvae.

After removal of the chorion, the marking of the PGCs and the definition of their migration route in *P. lineatus* was made possible by the injection of nanos1 3' UTR of *Danio rerio* mRNA in fusion with GFP, a specific marker for germinal lineage.

The visualization of the first PGCs in *P. lineatus* occurred during the somite stage. In the previous stages of development, the expression of GFP in embryonic cells was still remarkable, making it impossible to identify the PGCs. During the development, the level of GFP expression in the embryonic cells decreased, becoming less evident. According to Mishima *et al.,* (2006), the portion of 3'UTR *nos*1 is degraded in somatic cells throughout the embryonic development but remains stable in germ cells. Saito *et al.,* (2006) suggests that there is a difference between somatic cells and germ cells in the stability of injected mRNA, or in GFP, or in both. In a study carried out with *Clupea pallasii*, *Danio rerio*, *Danio albolineatus*, *Carassius auratus*, *Misgurnus anguillicaudatus*, *Oryzias latepis*, *Leucopsarion petersii* (Saito *et al.*, 2006), *Tinca tinca* (Linhartova *et al.*, 2014), and *Seriola lalandi* (Fernández *et al.*, 2015), the GFP-positive PGCs have become evident in the final stages of the gastrulation stage, suggesting that the function of *nos*1 3'UTR is highly conserved in teleost.

The migration pattern of PGCs in *P. lineatus* showed some differences in the migration route at the final location of these cells when compared with the species studied by Linhartova *et al.,* (2014) and Saito *et al.,* (2006), although these cells are limited the medial region of the embryo and the yolk extension region.

The establishment of reproductive methodologies, including micromanipulation, differentiation, and migration route of PGCs, as well the knowledge of the embryonic development at different temperature, provide important information. It constitutes a base for the implementation of advanced reproductive biotechniques, such as chimerism. The data obtained in the present study provides essential information for production of this important species, as well to the development of techniques and future works applied in the conservation and formation of genetic banks of fish species.

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