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, Dulbecco’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 micro-injection, manipulation of denuded embryos, and incubation into Petri dishes and incubators. Most of Neotropical migratory species present 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 Pirassunungua-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 papilla 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 - 29°C. 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**

| Temperature | Unfertilized | Cleavage | Blastula | Gastrula | Somite | Hatch | Larvae |
|-------------|--------------|----------|----------|----------|--------|-------|--------|
|             | %            | %        | %        | %        | %      | %     | 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%  | 56.7 ± 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.
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. Thus, all of them were dissolved in characin medium (12 mM NaCl, 1 mM KCl, 1.5 mM CaCl2, and 1.5 mM MgCl2) 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 # SLMO2345V, St. Louis, USA) or porcine trypsin 0.15% (Sigma #110MT7362V, 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 solution was microinjected in the blastodisc of dechorinated 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®). Digital images were taken using the Nis-Elements software (Nikon, Tokyo, Japan). Digital images were captured using software NIS - AR (Nikon, Tokyo, Japan). Samples were collected each 5 minutes until 2 h and 30 min post-fertilization (hours past fertilization (hpf)); minutes past fertilization (min) 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).

**Microinjection and PGC traceability**

The GFP-nos1 3’UTR mRNA solution was microinjected in the blastodisc of dechorinated 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®). Digital images were taken using the Nis-Elements software (Nikon, Tokyo, Japan). The control groups, un.injected and uninjected dechoronated, 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 STAGES OF *PROCHILODUS LINEATUS* INCUBATED AT 22 °C, 26 °C AND 30 °C

| Stage       | 22°C | 26°C | 30°C | Fig. No. |
|-------------|------|------|------|----------|
| Cleavage    | 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 25 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        | 5 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 | 1D     |
| 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     |
| 15 h 00 min | 8 h 55 min | 7 h 45 min | 2 A      |
| Optic vesicle| 17 h 00 min | 10 h 20 min | 8 h 30 min | 3B      |
| 21 h 00 min | 11 h 02 min | 9 h 30 min | 3C      |
| 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      |
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 1 h 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. 1. Embryonic development of *Prochilodus lineatus*, after fertilization, at stages of cleavage, blastula, 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.

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 280 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.
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The 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...
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 was better for the pronase treatment (Table 4).

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 *Prochilodus 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-nos 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 to the embryos exposed to the enzymes, a lower normal lava rate was observed *in vivo*.

**Table 3**

| Enzyme    | 5 min | 10 min | 15 min |
|-----------|-------|--------|--------|
| Trypsin   | 6.6 ± 0.3\% | 6.3 ± 0.3\% | 6.1 ± 0.3\% |
| Pepsin    | 6.9 ± 0.3\% | 6.3 ± 0.3\% | 6.1 ± 0.3\% |
| Bromelain | 6.9 ± 0.3\% | 6.3 ± 0.3\% | 6.1 ± 0.3\% |
| Papain    | 6.9 ± 0.3\% | 6.3 ± 0.3\% | 6.1 ± 0.3\% |

**Table 4**

| Groups               | Total eggs | Digestion time | Unfertilized | Cleavage | Blastula | Gastrula | Somite | Hatch          |
|----------------------|------------|----------------|--------------|----------|----------|----------|--------|----------------|
| Control              | 99         | 4.87 ± 0.01\% | 95.13 ± 0.01\% | 93.32 ± 0.00\% | 92.28 ± 0.01\% | 86.02 ± 0.03\% | 69.57 ± 0.02\% | 30.43 ± 0.02\% |
| Trypsin              | 80         | 6.62 ± 0.00\% | 93.38 ± 0.00\% | 73.76 ± 0.06\% | 63.53 ± 0.02\% | 62.65 ± 0.02\% | 43.61 ± 0.04\% | 56.39 ± 0.04\% |
| Pronase              | 57         | 6.30 ± 0.03\% | 93.70 ± 0.03\% | 86.83 ± 0.04\% | 81.77 ± 0.04\% | 82.27 ± 0.07\% | 47.73 ± 0.07\% | 52.27 ± 0.07\% |

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

**Table 5**

| Groups                  | Unfertilized | Cleavage | Blastula | Gastrula | Somite | Hatch          |
|-------------------------|--------------|----------|----------|----------|--------|----------------|
| 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-nos 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.

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.](image-url)
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 nano1 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 was made possible by the injection of nano1 3' UTR of Danio rerio mRNA in fusion with GFP, a specific marker for germinal lineage.

of nos1 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 chimera. 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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