Primordial germ cells identification and traceability during the initial development of the Siluriformes fish Pseudopimelodus mangurus.

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Research Article

Keywords: micromanipulation, whole-mount in situ hybridization, in vitro RNA synthesis, RNA microinjection, nanos3 3'UTR, bucky ball 3'UTR, germline chimera fish

Posted Date: March 15th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1427882/v1

Abstract

Primordial germ cells (PGCs) are responsible for the generation of all germ cells, and therefore, they are essential targets to be used as a tool for the production of germline chimera. The labeling and route of PGCs were evaluated during the initial embryonic development of *Pseudopimelodus mangurus*, using whole-mount *in situ* hybridization (WISH) and mRNA microinjection in zygotes. The WISH method was synthesized a specific RNA antisense probe using part of the coding region from *P. mangurus nanos3* cDNA. The RNA microinjection was performed using the GFP gene reporter regulated by translation regulatory *P. mangurus bucky ball* and *nanos3* 3'UTR sequences, which are germline-specific markers used to describe *in vivo* migration of PGCs. The *nanos3* and *bucky ball* gene expressions were evaluated in tissues for male and female adults and initial development phases and larvae from first to seventh days post-hatching. The results from the WISH technique appointed to the origin of PGCs in *P. mangurus* from the aggregations of *nanos3* mRNA in the cleavage grooves and the signals obtained of *nanos3* probes corresponded topographically to the migratory patterns of the PGC reported for other fish species. The microinjection of *GFP-nanos3* 3'UTR mRNA in the zygotes of *P. mangurus* was not successful for PGC labeling, but it was possible to identify three embryos injected, presenting 3 to 5 evidence of possible PGC labeled in the hatching phase. The *nanos3* and *bucky ball* gene expression was reported in the female gonads and from fertilized eggs until the blastula phase. These results provide information about the PGC migration of *P. mangurus* and the possible use of PGCs for the future generation of germline chimera to be applied in the conservation efforts of Neotropical Siluriformes species, contributing to the establishment of genetic banks, manipulation of organisms, and assisting in biotechnologies such as the transplantation of germ cells in fish.

Introduction

In teleost fish, the primordial germ cells (PGCs) can be detected in the early ontogenetic stages (Nagai et al., 2001), and maternally delivered germ cells determinants specify these cells (Bontems et al., 2009; Škugor et al., 2016). Genes expressed from the maternal genome during oogenesis start the formation of germ cells by controlling the zygotic genes' expression toward specifying the PGCs (Wylie, 1999). The spermatogenesis and oogenesis processes are initiated during early embryonic development when PGCs are identified into the inner-cell mass of the blastocyst. PGCs present specialized germplasm with a maternally synthesized collection of cytoplasmic organelles containing specific noncoding RNAs, mRNAs, and proteins that confer typical electron-dense structures, known as nuage or germ granules, and a large cell size, which permits to be identified using morphological criteria. The mechanism of PGCs specification is common to fish and other organisms where inheritance of asymmetrical localized cytoplasmic determinants are directed to the cells of germline lineage (Raz 2003). Therefore, PGCs arise from a few cells and segregate from the somatic lineage early in embryonic development. In fish, PGCs are differentiated prior to the formation of the somatic portion of the gonad at different localizations in the embryo and migrate towards the cells comprising the somatic portion of the gonad during the embryogenesis, using a specific migration route (Weidinger et al., 1999, Raz 2003, Fujimoto et al., 2006).

After colonization in the gonads, the primordial germ cells differentiate into spermatogonia and oogonia, giving rise to the germline, with sperm and oocytes as the final product (Nakamura and Seydoux, 2008; Raz, 2003). Studies show that gonadal development, sexual differentiation, and fertility are related to PGC proliferation and migration (Lewis et al., 2008; Saito et al., 2007; Tzung et al., 2015; Xu et al., 2010).

The identification and characterization of germline marker genes are essential for knowing the migration route, gonad formation, and sexual differentiation is considered a prerequisite for establishing reproductive biotechniques such as germ cell transplantation (Ricci et al., 2018). Some germ cell marker genes have already been identified, the *vasa* gene being the most studied and widely used in fish species (Knaut et al., 2000; Li et al., 2009; Raz, 2003; Shinomiya et al., 2000; Yoon et al., 1997; Yoshizaki et al., 2000).

The Nanos protein is involved in segregation, organization, maintaining germ cells, supporting their proliferation and suppressing cell death, protecting migratory PGCs from apoptosis (Köprunner et al., 2001; Suzuki et al., 2010; Tsuda et al., 2003). The maintenance of the undifferentiated state of germ cells by Nanos protein, in a prolonged G1 phase, is due to the repression translational of specific mRNAs, such as *cyclin B1* (Hashimoto et al., 2010). In Drosophila, it was proposed that Nanos (Nos) protein, in combination with Pumilio (Pum) and Brain tumor (Brat) proteins, binds to specific sites in the developmental morphogen (*hunchback*) 3'UTR mRNA, leading to two regulatory mechanisms post-transcriptional, translational repression and mRNA degradation (Arvola et al., 2017). *Nanos* expression can be observed in PGCs until shortly after its establishment in bipotential female and male gonads (Suzuki et al., 2010; Tsuda et al., 2003). The Bucky ball protein plays a role in the organization and aggregation of germplasm during oogenesis and can be identified in embryos after fertilization (Škugor et al., 2016; Ye et al., 2018). In this study, we have analyzed the migration route of the PGC in the initial development of *P. mangurus* by WISH technique, using antisense RNA probe of *nanos3* cDNA from *P. mangurus*. Alternately, we have used the microinjection of *in vitro* synthesized mRNA to visualize PGC from the constructed vector of the *GFP* gene reporter in fusion with the 3'UTR region from zebrafish (*nanos1*) and *P. mangurus* (*nanos3* and *bucky ball*). The *nanos3* and *bucky ball* gene expressions were evaluated in initial development phases, larvae from hatching until seventh-day post-hatching, and different tissues of male and female adults of *P. mangurus* by conventional PCR. Identifying PGCs and their migratory route, as basic knowledge about molecular control over the development of these type cells during fish ontogenesis, is essential for the generation of germline chimera to be used in biotechnologies applied to the endangered species, as in the *P. mangurus*.

Materials And Methods

Animals stocks and sampling

P. mangurus were collected in natural environment during reproduction period and the gametes were obtained from males and females by hypophysation using crude carp pituitary extract, as Arashiro and collaborators (2018) described, and artificial reproduction was performed. After *in vitro* fertilization,

approximately 150 eggs were stored in Petri dishes contending 20 mL of water and incubated at 26°C in BOD (Biological Oxygen Demand) incubators. Embryonic development was monitored, and samples were collected periodically from cleavage to hatching stage using a stereomicroscope (Nikon SMZ 1500, Japan) to be processed by WISH methodology. For RNA microinjection, an aliquot of eggs fertilized was used in each experiment. In addition, two adult animals (male and female), the pool of embryos in initial stages phases, and six larvae from the first to $^{\text{the seventh}}$ day of development were collected for PCR analysis. Different tissues from adult animals were collected, such as kidney, spleen, brain, hypophysis, muscle, liver, and gonads, and stored at -80°C until RNA extraction. The samples of embryos and larvae were stored in the same conditions.

Whole-mount in situ hybridization (WISH)

The identification of *P. mangurus* PGCs during embryogenesis was determined by detecting *nanos3* mRNA-related gene expression using the WISH method. WISH was performed by immunohistochemistry using an alkaline phosphatase-conjugated antibody against digoxigenin and a chromogenic substrate, as previously described by Thisse and Thisse (2008), with adjustments.

The mRNA of *nanos3* was detected with a digoxigenin-labeled antisense *nanos3* RNA probe contending 576 bp from *P. mangurus nanos3* cDNA. All gene sequence used in this study was obtained from the egg transcriptome of *P. mangurus* (data not published). For the synthesis of antisense RNA probe, labeled with digoxigenin-linked nucleotides, the total RNA was extracted from eggs using TRIzol (Ambion). The cDNA synthesis was performed using the SuperScript III First-Strand kit (Invitrogen). For amplification, the primers drawn for the *nanos3* gene (Table 1) with *EcoRI* and *HindIII* restriction sites were used. The amplified fragment was visualized in 1% agarose gel and recovered using EZN.A Gel Extraction (Omega). The amplified fragment was cloned into pGEM vector (Promega) and subcloned into the pSP64 vector (Promega), using the *EcoRI* and *HindIII* restriction enzymes. The pSP64-*Pm-nanos3* vector constructed was used for the *in vitro* synthesis of antisense RNA using DIG RNA Labeling Mix (Roche) and mMESSAGE mMACHINE SP6 kit (Invitrogen). Antisense RNA probe was used in the WISH technique, according to recommended (Thisse and Thisse, 2008).

Table 1

Primers used for vector construction used in WISH and microinjection procedures, identification of specific tissue in adults, and expression analysis in the initial development and larvae.

Primer	Sequence 5'→3'	Amplicon (bp)
Primer for vector construction to the synthesis of antisense RNA (WISH) and gene expression analysis in the tissues from adults		
Wish-nanos3-F	GAATTCTTTTCTCTGCTGCACTACG	576
Wish-nanos3-R	AAGCTTGGTGTACACGGAGCTGTA	
Wish-bucky ball-F	GAATTCAGGAACCCAATGTGATGTTT	853
Wish-bucky ball-R	AAGCTTATCGGCAGGATCTGTTTC	
Primers for vector construction to the synthesis of in vitro mRNA (microinjection)		
Pm-nanos3 3'UTR-F	TCTAGAACAACACGTACAGCTCCGTGT	867
Pm-bucky ball 3'UTR-F	TCTAGACGATGAGCACTGCTTTAAT	605
AUAP-EcoRI-R	GAATTCGGCCACGCGTCGACTAGTAC	-
EmGFP-F	AAGCTTATGGTGAGCAAGGGCGAG	720
EmGFP-R	TCTAGATTACTTGTACAGCTCGTC	
Primers for gene expression analysis in embryos and larvae		
nanos3-F	CACCAGGAGGTCGGTTTTAC	320
nanos3-R	TAAGGACACACCACCTCTCC	
bucky ball-F	TGGACAATATGGCTCCCAG	201
bucky ball-R	TGCATGCTCATCTGCTGAA	
β-actin-F	CGTGCTGTCTTCCCATCCA	86
β-actin-R	TCACCAACATAGCTGTCCTTCTG	

The embryos collected were dechorionated by pronase, and samples from each stage were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 5.5, for 20 to 30 h at 4°C. The pigmentation of the fixed embryos was removed with a solution of 3% H_2O_2 in 0.5% KOH for 60 min at room temperature. After, the embryos were dehydrated gradually using a sequence of methanol solution (25%, 50%, and 75% methanol in PBS) and stored in 100% methanol at -20°C until use.

The embryos were gradually rehydrated by successive dilutions of methanol in PBS (75%, 50%, 25% methanol in PBS and PBS) and washed four times, for 5 min each, with PBS containing 0.1% Tween 20 (PBT), at room temperature. The embryos were then treated with proteinase K (10 µg/mL in PBT) at room temperature, for 1 min for the blastula to gastrula period, for 2 min for 1-somite to 8-somite stage embryos, for 6 min for 9 to 18-somite stage, and for 20 min for embryos older than the 18-somite stage.

Embryos were postfixed in 4% paraformaldehyde in PBS for 20 minutes and then washed four times in PBT for 5 minutes per wash.

The embryos were prehybridized for 5 hours at 70°C in hybridization mix (HM), composed of 50% deionized formamide, 5× standard sodium citrate (SSC), 0.1% Tween, 50 µg/mL of heparin, 500 µg/mL of tRNA adjusted to pH 6.0 by addition of citric acid. The hybridization was done in an HM solution containing approximately 50 ng of antisense DIG-labeled RNA probes overnight at 70°C. The embryos were transferred to HM solution, without tRNA and heparin, and gradually changed from HM to 2×SSC through four series of 10 min (75%, 50%, 25% HM, and 100% 2×SSC) at 70°C.

The embryos were washed twice with 2×SSC at 70°C for 30 min, and then the 2×SSC was progressively replaced per PBT through four series of 10 min at room temperature in a horizontal shaker (40 rpm). The embryos were incubated in blocking buffer (PBT with 2% goat serum and 2 mg/mL bovine serum albumin) for 4 hours at room temperature and hybridized overnight in blocking buffer contending 1/10,000 anti-digoxigenin-AP Fab fragments (Roche) at 4°C, with gentle agitation on a horizontal shaker (40 rpm). The embryos were then washed six times, 15 min per wash, in PBT and three times, 5 min per wash, in alkaline Tris buffer (100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl and 0.1% Tween 20) at room temperature with gentle agitation.

The embryos were replaced with 0.7 mL staining solution (NBT/BCIP), freshly prepared and kept in the dark to prevent background staining, and periodically monitored until the color develops to the desired extent: 1 hour for the cleavage stage; 2–4 hours for the blastula to gastrula period; 4–8 hours for segmentation. When the desired staining intensity was reached, they were washed three times for 15 min with a stop solution (1×PBS, pH 5.5, 1 mM EDTA, 0.1% Tween 20). They were then incubated in 1×PBS at pH 3.0 and observed with a stereomicroscope (Nikkon SMZ 1500, Japan), being taken digital images through a CCD camera (Nikon DSFI1, Japan) coupled to the microscope. Digital images were obtained by Nis-Ar Elements software (Nikon, Tokyo, Japan).

mRNA microinjection

The sequences of 3’UTR regions of *bucky ball* and *nanos3* of *P. mangurus* were obtained from egg transcriptome (data not published) e completed using 3' RACE System for Rapid Amplification of cDNA Ends (Invitrogen). The amplifications were performed using a GSP of initial 3'UTR regions, the oligo dT primer from the RACE kit, and the cDNA of the egg. The amplification products were recovered from 1% agarose gel, cloned in the pGEM-T, and the insert in the vectors were sequenced. The forward and reverse primers (Table 1) for 3’UTR regions of *bucky ball* (*Pm-bucky ball* 3’UTR-F) and *nanos3* (*Pm-nanos3* 3’UTR-F) were designed, including the restriction sites for *XbaI* in both. The reverse primer (AUAP-*EcoRI*-R) was designed using the sequence of the AUAP primer from the 3' RACE kit, plus the sequence of the restriction site for *EcoRI*. The 3'UTR regions were amplified and cloned in the pGEM-T. To construct the transcription vectors as templates for *in vitro* RNA synthesis, the coding region of GFP was amplified using the pFUGW vector as a template and the EmGFP-F and EmGFP-R primers (Table 1), with restriction sites *HindIII* e *XbaI*, respectively. The coding region of the GFP was cloned into the pGEM vector and

subcloned into transcription vector pSP64, generating the pSP64-GFP vector. The 3'UTR regions were excided from the pGEM vector and cloned into pSP64-GFP, producing the transcription vectors (pSP64-GFP-*Pm-nanos*3 3'UTR and pSP64-GFP-*Pm-bucky ball* 3'UTR). These vectors and the pCS2-GFP-*nos1* 3'UTR (zebrafish), gently provided for Dr. Takafumi Fujimoto, were used as a template for *in vitro* RNA synthesis, using the mMESSAGE-mMACHINE SP6 kit (Invitrogen).

For mRNA injection, freshly fertilized eggs of *P. mangurus* went through the enzymatic removal of the chorion in a Characin solution (NaCl 12 mM, KCl 1 mM, $CaCl_2$ 1.5 mM, and $MgCl_2$ 1.5 mM) containing 0.25% pronase (Sigma). The mRNAs for GFP-*nanos1* 3'UTR from *D. rerio*, GFP-*bucky ball*, and GFP-*nanos3* from *P. mangurus* were microinjected in blastodisc in the stage of one cell to identify primordial germ cells and their migration route. For this, a boron silicate micropipette with 10 µm in diameter (Drummond, USA) was connected to a microinjector (CellTram Vario, Eppendorf, Hamburg, Germany), coupled to the micromanipulator (M-152, Narishige, Tokyo, Japan) under a stereomicroscope (SMZ18, Nikon®, Tokyo, Japan). The microinjected fertilized eggs and the control groups, intact and not injected decorated, were kept in Petri dishes (90 x 15 mm) with 200 mL of Characin solution at 26°C in a BOD incubator. Embryonic and larval development was analyzed under a fluorescence stereomicroscope (Nikon SMZ18, Tokyo, Japan), connected to a CCD camera (DS-Ri2, Nikon®, Japan), observing the moment of evidence of GFP positive as reported by Coelho and collaborators (2019).

Specific tissue gene expression in adults and analysis of the expression in initial stages of development and larvae

The total RNA was extracted using TRIzol (ambion) of *P. mangurus* samples in the initial stages of development (fertilized eggs, two cells, eight cells, blastula, gastrula with 50% epiboly, 10-somite stage and hatching), larvae from the first to the seventh day of development and from female and male adult's tissues (kidney, spleen, brain, hypophysis, muscle, liver and gonad). The quantity and quality of the isolated RNA were evaluated using a QIAxpert spectrophotometer (Qiagen), and 1 µg of RNA, previously treated with DNAse 1, was used for cDNA synthesis, using the SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen). The primers used for gene expression analysis of the *nanos*3, *bucky ball*, and the endogenous *β-actin* in the initial developmental phases, larvae, and tissue-specific expression in adults are shown in Table 1.

According to the manufacturer, amplifications were performed using 1 µL of cDNA as template and Taq DNA Polymerase recombinant (Invitrogen). Parameters cycling started with an initial denaturation step of 5 min at 94°C, followed by 35 cycles, each as follows: (i) denaturation for 45 s at 94°C, (ii) annealing for 30 s at 55°C, and (iii) extension for 30 s at 72°C. The final extension was performed for 10 min at 72°C. PCR products were electrophoresed on agarose gel, detected by staining with SYBER Safe, and were visualized under ultraviolet light (ENDUROTM GDS, Labnet International, Inc).

Sequence analysis

The sequences from *Pm-bucky ball* 3'UTR and *Pm-nanos3* 3'UTR were blasted with deposited sequences and aligned using BLASTN (Altschul et al., 1997) and MultAlin (Corpet 1988) programs.

Results

Localization of nanos3 mRNA during embryonic development by WISH

The vector pSP64-*Pm-nanos3* constructed and used as a template for RNA probe synthesis was evaluated by map restriction, using the restriction enzymes *EcoRI* and *HindIII*, and the sizes of the cleavage products were confirmed in 1% agarose gel (data not shown). To traceability of the primordial germ cells in the early embryonic development of marbled catfish *P. mangurus*, the *nanos3* transcripts signals were observed by WISH using *P. mangurus* antisense *nanos3* RNA probes. At the early cleavage period, 2-cell, 4-cell, and 8-cell stages, *nanos3* mRNA is strongly aggregated at both ends of cleavage furrows (Fig. 1A-B). Signals of *nanos3* transcripts were also observed at one or both ends of the third cleavage furrows at the 8-cell stage (Fig. 1C).

Nanos3 transcripts signals were observed distributed on cell boundaries in the 16-cell stage, though weakly compared with those in the earlier cleavage furrows (Fig. 2A). From the 64-cell stage, the transcripts signals began to differ among embryos and aggregate in clusters on blastomere boundaries (Fig. 2B).

At the blastula stages, *nanos3* transcript signals were observed in small spots distributed on the blastoderm marginal part, some of them in pairs (Fig. 3A, B). The clusters of proliferated *nanos3* positive cells were present in the limit between the blastoderm and the yolk region (Fig. 3C). At the early gastrula stages, *nanos3* positive cells were observed frequently located in the marginal region of the blastoderm.

From the late gastrula stages, some *nanos3* positive cells clusters began to rearrange and rally on the embryonic shield (Fig. 4A, B). At the 90% epiboly stage, were observed *nanos3* positive cells located between the dorsal equatorial and ventral posterior regions (Fig. 4A). At the early segmentation period, *nanos3* positive cells were observed to gather around both sides of the embryonic body. *Nanos3* positive cells formed clusters on the region near the first's somites (Fig. 4C, E). At the 8- to 28- somite stage, many *nanos3* positive cells were clustered on both sides of the gonadal ridge of the embryonic body (Fig. 4D, F).

mRNA microinjection

The GFP-*nos1* 3'UTR (zebrafish), GFP-*Pm-bucky ball* 3'UTR and GFP-*Pm-nanos3* 3'UTR (*P. mangurus*) mRNAs at 100 ng/µL were microinjected in 235, 246 and 213 decorated eggs of *P. mangurus*, respectively. The embryonic development was monitored to record the exact moment of evidence of PGC-positive for GFP. The embryos injected with GFP-*nos1* 3'UTR and GFP-*Pm-bucky ball* 3'UTR mRNAs did not show GFP positive PGCs. The embryos injected with the respective mRNAs remained throughout the

embryonic development and post-hatch with high GFP expression in all cell types (Fig. 5A-D), making it impossible to distinguish germ cells from somatic cells.

The embryos injected with GFP-*Pm-nanos3* 3'UTR mRNA did not show PGCs during embryonic development; all cell types showed expression for GFP (Fig. 5E, F). The first possible PGC-positive for GFP was seen at hatching (Fig. 6A, B) and post-hatching day (Fig. 6C, D). However, only eight embryos showed possible PGC-positive markings for GFP, with only three showing more significant evidence those were PGCs, which varied from 3 to 5 GFP-positive PGCs per embryo (Fig. 6A-F).

Nanos3 and bucky ball expressions in embryonic development, larvae, and tissues of adults

Nanos3 and *bucky ball* expressions were detected in the initial stages of development of *P. mangurus* from fertilized eggs until blastula phases, a decreasing of expressions along with the development (Fig. 7). From gastrula until the seventh-day post-hatching were not observed *nanos3* and *bucky ball* expressions (data not shown).

The *nanos3* and *bucky ball* specific tissue expression analysis was performed, and the expressions were observed only in female gonads (Fig. 8). These results suggest that these mRNAs are stored in oocytes as maternal heritability is involved in embryonic development. *B-actin* expression was detected in all samples evaluated.

Sequences analysis

The poly (A) tails were not used in the identity analysis. For *the Pm-bucky ball* 3'UTR sequence, only three nucleotides were not precisely identified in the sequencing, and it was used 572 nucleotides in the analysis being obtained 85.74% identity with *Ictalurus punctatus* sequence (accession code: XM_017495056.1) and 85.34% identity with *Pangasianodon hypophthalmus* (accession code: XM_026940979.2), with query cover of 100% and 99%, respectively. All species belong to the Siluriformes order, suggesting that *bucky ball* 3'UTR is conserved in these Siluriformes species, besides *P. hypophthalmus* and *I. punctatus* 3'UTR sequences are longer about 430 and 110 nucleotides, respectively, than *P. mangurus*. The alignment of the *bucky ball* 3'UTR sequences is shown in Figure S1. In the *P. mangurus* egg transcriptome, two transcripts for *nanos3* mRNA differ between them only in the 3'UTR sequences. One transcript (transcript 1 with 430 nucleotides) does not present part of the sequence found in the other transcript (transcript 2 with 867 nucleotides), from 101 to 537 after stop codon, and all others nucleotides are identical (Figure S2). *Nanos1* and *nanos2* mRNAs were not found in the *P. mangurus* egg transcriptome. The transcript with a more extended sequence (transcript 2) was used in this work, and it showed 72.14% and 64.59% identity with predicted *nanos3* 3'UTR from *I. punctatus* (accession code: XM_017457116.1) and *Tachysurus fulvidraco* (accession code: XM_027136480.1), with 54% and 72% query cover, respectively. Compared with *bucky ball* 3'UTR, *nanos3* 3'UTR sequences are less conserved in Siluriformes, but some regions are conserved (Figure S3).

Discussion

In this study, we have used two strategies for labeling and traceability in the initial embryonic development of *P. mangurus* PGC, WISH, and mRNA microinjection methodologies, aiming to develop technology for future application in conservationist actions for this endangered species. The WISH technique was performed using a specific *P. mangurus nanos3* RNA antisense probe to obtain essential knowledge about the PGC migration route in *P. mangurus*. However, this methodology cannot be used for PGC transplantation to generate germline chimera. Then, we have mRNA microinjection to describe *in vivo* PGC migration, targeting *P. mangurus* PGC for transplantation to sterile recipients. For this, we have used two specific germline markers, *nanos* and *bucky ball* 3'UTR regulating the translation of the reporter GFP gene. We have used two *nanos* 3'UTR, the *nanos1* 3'UTR from zebrafish, commonly used for PGC labeling of various fish species, and *nanos3* 3'UTR from *P. mangurus*. Only *nanos3* homolog was found in the egg transcriptome of *P. mangurus* (data not published), corroborating with the results obtained using *Larimichthys crocea*, that only *nanos3* transcripts signals were detected during embryogenesis, suggesting *nanos3*, not *nanos1* and *nanos2* is a germ cell marker gene in this species (Han et al., 2018).

RNA microinjection methodology has been used in different fish species to identify and describe the migration route of PGCs (Yoon et al., 1997; Shinomiya et al., 2000; Otani et al., 2002; Saito et al., 2004 and 2006; Fujimoto et al., 2006; Mishima et al., 2006; Nagasawa et al., 2013; Linhartova et al., 2014; Fernández et al., 2015; Wu et al., 2018; Coelho et al., 2019). This technique contributes to establishing genetic banks, manipulating organisms, and assisting in biotechnologies such as the transplantation of germ cells in fish.

To identify PGCs in embryonic development by WISH, we used an RNA probe synthesized from genetic material extracted from the own marbled catfish eggs. However, the use of probe from one species can be used for other species as have been reported in other related studies, such as Loach and Goldfish that was used the *vasa* probe from *Danio rerio* (Otani et al., 2002; Fujimoto et al., 2006), and for ukigori that was used a *Leucopsarion petersii* probe (Saito et al., 2004). These studies point to the conserved sequence of the genes specific for PGC.

The signals obtained from the *P. mangurus nanos3* probes correspond topographically to the migratory patterns of the PGC reported in other fish species (Otani et al., 2002; Saito et al., 2004; Fujimoto et al., 2006; Ricci et al., 2018). Therefore, the results suggest that the *nano3* positive cells correspond to *P. mangurus* PGCs and the description of the migratory route is accurate.

The origin pattern of *P. mangurus* PGCs observed was similar to the *vasa* mRNA aggregations in the cleavage grooves, as in zebrafish (Yoon et al., 1997), goldfish (Otani et al., 2002), ukigori (Saito et al., 2004) e loach (Fujimoto et al., 2006), and unlike the one presented in medaka (Shinomiya et al., 2000) with *vasa* mRNA distribution throughout the blastodisc.

The *nanos1* 3'UTR from *Danio rerio* have been used to *in vivo* visualization of PGCs for different species, with different efficiency in PGC labeling. For *Leucopsarion petersii*, it was obtained 100%, 97.7% *Oryzias*

latepis, 100% *Carassius auratus*, 100% *Danio rerio*, 98.5% *Danio albolineatus*, 100% *Clupea pallasii*, 99.55% *Misgurnus anguillicaudatus* (Saito et al., 2006), 59.3% *Anguilla japônica* (Saito et al., 2011), 69.3% *Tinca trinca* (Linhartova et al., 2014) and 74.6% *Prochilodus lineatus* (Coelho et al., 2019). These results suggest the conservation of the sequence and functionality of this gene among more distant taxonomic species. However, for *P. mangurus* it was not possible to mark the PGCs, demonstrating that the *D. rerio* mRNA was not adequate for the species. Conserved regions in the alignment of *P. mangurus nanos3* 3'UTR and zebrafish *nanos1* and *nanos3* 3'UTRs sequences (data not shown) were not observed, as verified in the alignment of *nanos3* 3'UTR in Siluriformes (Figure S3).

The non-specificity of *nanos1* 3'UTR from *D. rerio* for *P. mangurus* PGCs led to the construction of new species-specific mRNAs using the sequence of the 3'UTR regions of *bucky ball* and *nanos3* from *P. mangurus*. The GFP-*Pm-bucky ball* 3'UTR and GFP-*Pm-nanos3* 3'UTR of the *P. mangurus* were injected into 246 and 213 one-cell embryos, respectively. The mRNA of the 3'UTR region of *bucky ball* showed no evidence of positive PGC labeling for GFP during embryonic and larval development. Only three embryos injected with GFP-*Pm-nanos3* 3'UTR mRNA presented 3 to 5 evidence of possible PGC marking in the hatching phase.

The PGC labeling is an essential step for PGC transplantation methods, such as blastomere transplantation or single PGC transplantation, for generating germline chimeras in fish. In blastomere transplantation, sometimes it is challenging to identify PGC labeled differentially from other cells, and generally is transplanted several blastomeres from microinjected zygotes, and PGC labeled are posteriorly observed in the gonadal region (Saito et al., 2010). In zebrafish, the germplasm has been visualized at early cleavage stage embryos using the microinjection of GFP-*buc* mRNA (Bontems et al., 2009). Alternatively, the microinjection of RFP-Olbuc 3'UTR mRNA in Oryzias latipes (medaka) was used for PGC labeling, validating bucky ball 3'UTR as PGC marker (Song et al., 2021). In this work, we have microinjected GFP-*Pm*-bucky ball 3'UTR mRNA aiming the PGC labeling at blastula stage intending blastomere transplantation; however, the PGC labeling was unsuccessful. The medaka *bucky ball* 3'UTR (accession code: MT622506.1) has half of the size (230 nucleotides) of the *Pm-bucky ball* 3'UTR (582 nucleotides) and its sequences are more variables between them (alignment not shown) compared to those used in the sequence alignment of the Siluriformes species (Figure S1). The PGC labeling using GFP-*nanos* 3'UTR mRNA microinjection generally permits the PGC visualization in somitogenesis, and therefore, the single PGC transplantation is possible to be performed. Moreover, the PGC ability to migrate toward the gonadal region decreased after the stage of 10 to 15 somites (Saito et al., 2008 and 2010). *P. mangurus* PGC labeled was identified after hatching, suggesting that using these could compromise the migration in the chimera.

Several studies have appointed that GFP-positive PGCs became evident in the final stages of gastrulation (Saito et al., 2006, 2011, Linhartova et al., 2014; Fernández et al., 2015) or during the early stage of somitogenesis (Coelho et al., 2019). The PGC numbers positive for GFP in these studies ranged on average from 5.2 to 43.2 between species. The level of GFP expression decreases during embryonic

development in somatic cells, but it is kept stable in germ cells, allowing the visualization of these cells *in vivo* (Mishima et al., 2006).

The *nanos3* expression observed during embryonic development until hatching using WISH suggests that this gene is involved in the differentiation of PGCs during migration and gonadal development in *P. mangurus*. This fact is reinforced by *nanos3* expression detected in the initial development stages and female gonad. The gonad of the female used was found in the vitellogenin stage, suggesting that maternal RNAs had already been deposited. *Bucky ball* and *nanos3* gene expressions during embryonic development and whole larvae using conventional PCR corroborate their higher activity in the early ontogenetic stages, as their role in PGC's differentiation, organization, migration, and maintaining, which occurs crucially in these phases (Bontems et al., 2009; Yon and Akbulut, 2015; Škugor et al., 2016). *P. mangurus bucky ball* expression was identified in the fertilized egg until blastula phases and female gonad, like as reported for sturgeon (Ye et al., 2018). *P. mangurus nanos3* expression was identical to that observed for *bucky ball*; however, in the *Oreochromis niloticus nanos3* expression can be observed in lower expression after blastula and in the testis (Jin et al., 2019). The pattern of *P. mangurus bucky ball* and *nanos3* expression appoint to similar expression.

Conclusion

This study tracked the primordial germ cells in the early embryonic development of marbled catfish *Pseudopimelodus mangurus* by the WISH method and mRNA injection. Besides, the *bucky ball* and *nanos3* expressions were identified in initial developmental phases and gonads. Improvements are still needed for descriptions of the *P. mangurus* germ cell line by *in vivo* technique to elucidate more details and effectively apply biotechnologies such as the production of germ chimeras. Our results provide helpful information for future investigation of development mechanisms and subsidies for conservation efforts of Neotropical Siluriformes species with the use of biotechnology tools.

Declarations

Funding

The authors are grateful to São Paulo Research Foundation - FAPESP (Award Grant #2016/16386-3) and AES Tietê (Research & Development Projects #0064-1052/2014 and #0064-1062/2020) for the financial support, and ICMBio/CEPTA for provided the facilities.

Competing Interests

The authors declare no competing interests.

Ethics approval

Pseudopimelodus mangurus were collected from the Mojiguaçu River (Pirassununga – SP - Brazil), according to the Brazilian law (Sisbio #60.383-1), and the experiments were conducted following the Animal Ethics Committee from National Center for Research and Conservation of Continental Aquatic Biodiversity (CEUA/CEPTA #010/2015).

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Data availability

The datasets generated during the current study are not publicly available due to the deposit of sequences being in processing but are available from the corresponding author on reasonable request.

Code availability

Not applicable.

Author contributions

GFS performed, analyzed, and wrote WISH experiments. GCZC performed, analyzed, and wrote an embryo micromanipulation experiment. LSL performed, analyzed, and wrote tissues gene expression experiments. GPP performed, analyzed, and wrote initial developmental gene expression experiments. SCAdS designed and supervised the experiments and reviewed the manuscript. JAS collected animals in the natural environment, performed, and wrote fish reproduction. PSM performed and analyzed vectors constructions and in vitro RNA synthesis experiments; reviewed and edited the manuscript. GSY designed and supervised the experiments; and reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Figures

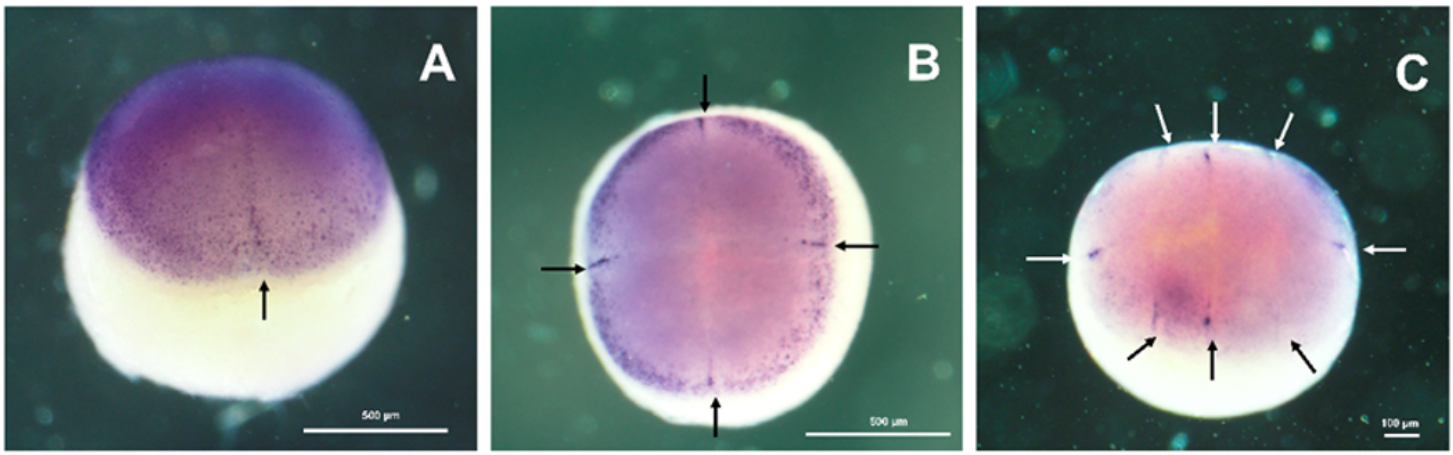

Figure 1

Image of *in situ* hybridization at the stages of 2 (**A**), 4 (**B**), and 8-cells (**C**). The signals of *nanos3* mRNA are located in the cleavage furrows.

Figure 2

Expression of *nanos3* during cleavage embryonic stages of the marbled catfish *P. mangurus*. Embryos were hybridized with a *P. mangurus nanos3* antisense probe. **A**- 16-cell stage; **B**- 64-cell stage. Arrowheads indicate *nanos3* signals.

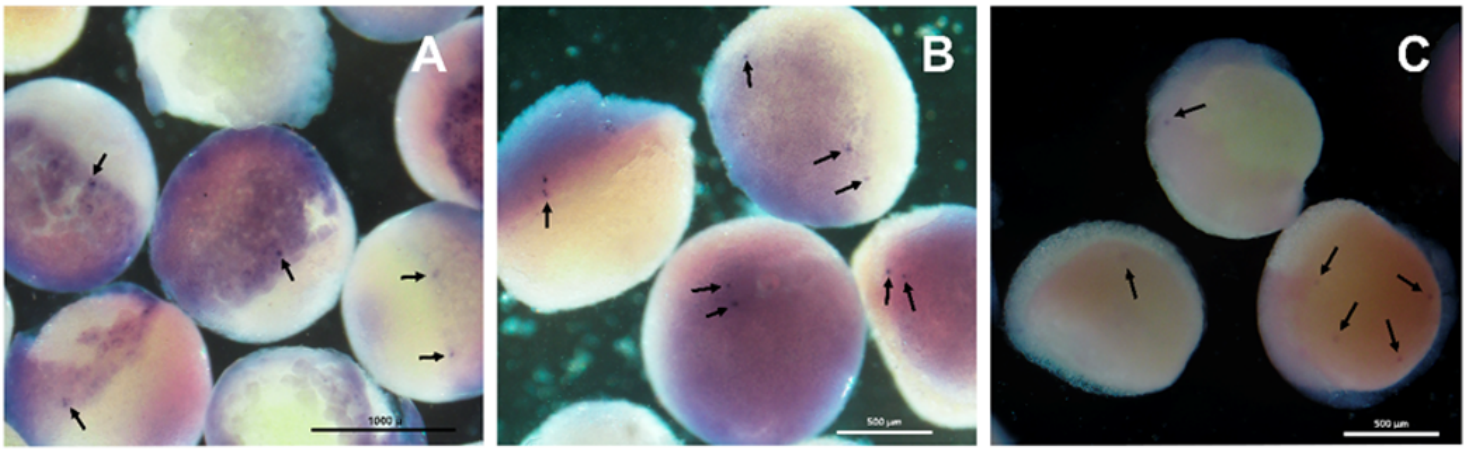

Figure 3

Expression of *nanos3* during the blastula embryonic stages of the marbled catfish *P. mangurus*. Embryos were hybridized with a *P. mangurus nanos3* antisense probe. **A-** 128-cell stage; **B-** 1k-cell stage; **C-** spherical stage. Arrowheads indicate *nanos3* signals.

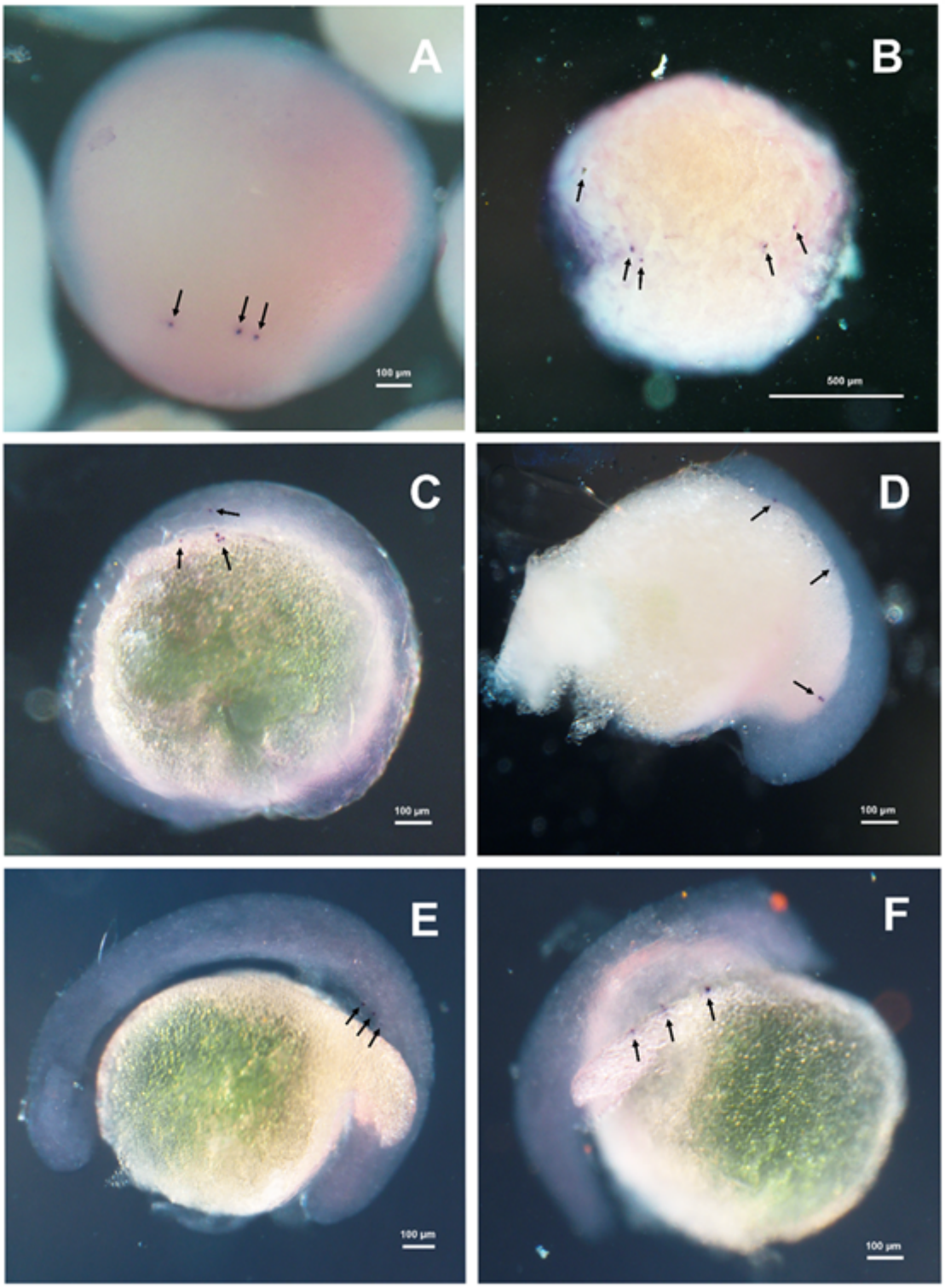

Figure 4

Expression of *nanos3* during gastrula and segmentation embryonic stages of the marbled catfish *P. mangurus*. Embryos were hybridized with a *P. mangurus vasa* antisense probe. **A-** expression of *nanos3* in *P. mangurus* embryo at 90% epiboly stage; **B-** 100% epiboly stage; **C-** neurula ~1-somite stage; **D-** 8-somite stage; **e**, 20-somite stage; **F-** 28-somite stage. Arrowheads indicate *nanos3* signals.

Figure 5

Pseudopimelodus mangurus embryos in the segmentation stage. **A, B-** Embryo injected with GFP-*nos1* 3'UTR mRNA; **C, D-** Embryo injected with GFP-*Pm-bucky ball* 3'UTR mRNA; **E, F-** Embryo injected with GFP-*Pm-nanos3* 3'UTR mRNA. **B, D,** and **F-** images captured under fluorescence from **A, C,** and **D**.

Figure 6

Embryos of *Pseudopimelodus mangurus* injected with GFP-*Pm-nano3* 3'UTR mRNA. **A-D-** Newly hatched larvae. **E, F-** Larva with one day after hatching. **B, D, F-** images captured under fluorescence from **A**, **C**, and **E**. Arrowhead, positive signals for GFP.

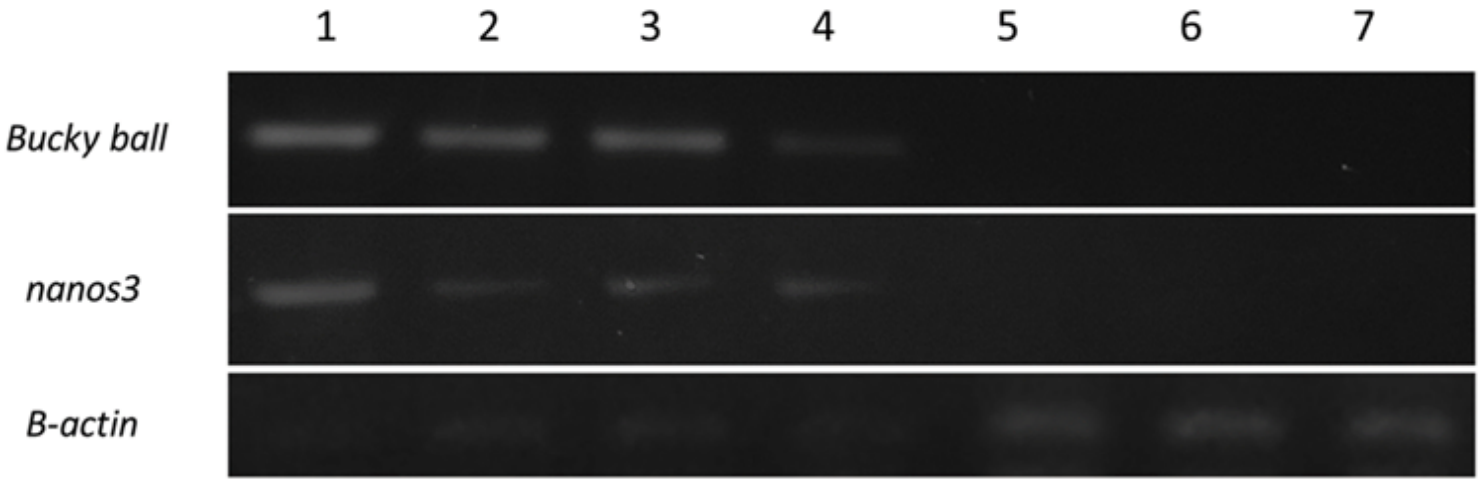

Figure 7

Bucky ball, *nanos3* and *β-actin* expression in *P. mangurus* embryos in the stages of egg (**1**), 2-cells (**2**), 8-cells (**3**), blastula (**4**), gastrula with 50% of epiboly (**5**), segmentation (**6**) and hatching (**7**). The grouping of gels was cropped from different gels and identified with horizontal white space.

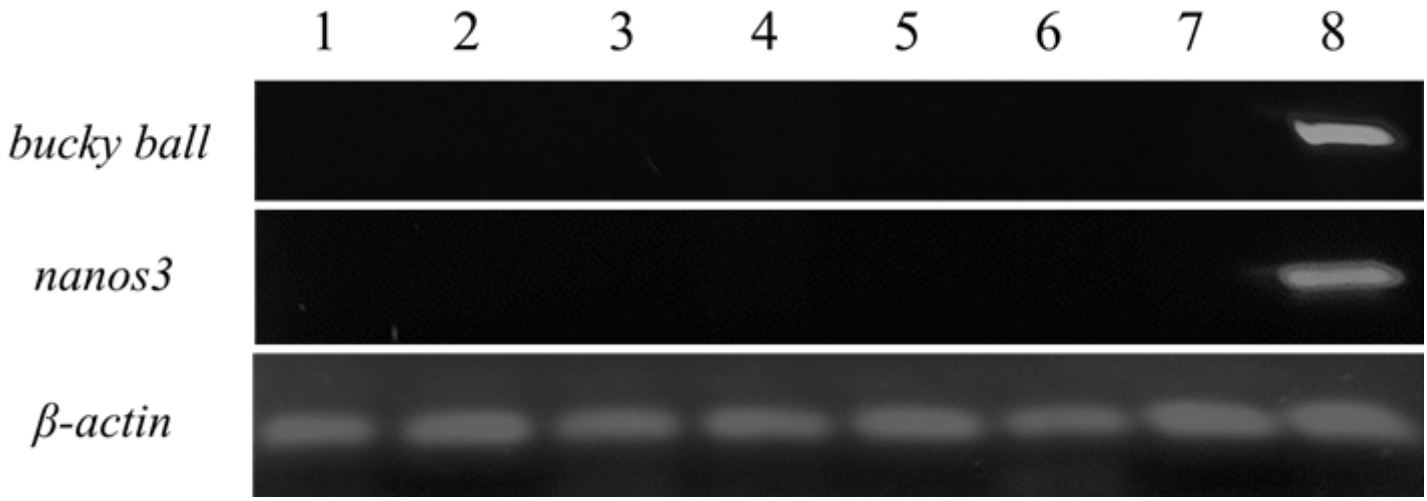

Figure 8

Bucky ball, *nanos3*, and *β-actin* expressions in tissues from adults male and female of *P. mangurus*. **1-** muscle; **2-** liver; **3-** kidney; **4-** brain; **5-** hypophysis; **6-** spleen; **7-** testicle; **8-** ovarium. The grouping of gels was cropped from different gels and identified with horizontal white space.

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