General and Comparative Endocrinology 273 (2019) 249–259

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Duration of spermatogenesis and identification of spermatogonial stem cell markers in a Neotropical catfish, Jundiá (*Rhamdia quelen*)

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ARTICLE INFO

Keywords:
Rhamdia quelen
Testis
Duration of spermatogenesis
Spermatogonia
Stem cell gene expression
plzf
pou5f3

ABSTRACT

Spermatogenesis is a process driven by stem cell, where germ cell cycle is under the control of a specific genotype species. Considering that Jundiá (*Rhamdia quelen*) is a Neotropical catfish with great economical importance and useful experimental model, little information is available on basic aspects of its reproductive biology, especially on spermatogenesis. As a result, this study aimed to characterize the male germ cells, estimate the duration of spermatogenesis and evaluate the expression of selected stem cell genes in Jundiá testis. Similar to other fish species, our results showed a remarkable decrease of germ cell nuclear volume during Jundiá spermatogenesis, particularly from type A undifferentiated to late type B spermatogonia and from diplotene to late spermatids. Using a S-phase marker, bromodeoxyuridine (BrdU), the combined duration of meiotic and spermiogenic phases in this species was estimated in approximately 7 days. This is considered very short when compared to mammals, where spermatogenesis last from 30 to 74 days. Selected stem cell genes were partially sequenced and characterized in Jundiá testis. Expression analysis showed higher *plzf* and *pou5f3* mRNA levels in the cell fractions enriched by type A undifferentiated spermatogonia. These results were further confirmed by *in situ* hybridization that showed strong signal of *plzf* and *pou5f3* mRNA in type A undifferentiated spermatogonia. Altogether, these information will expand our knowledge of the reproductive biology of this species, contributing to improve its production and management, and also for biotechnological applications, such as germ cell transplantation.

1. Introduction

Rhamdia quelen, also known as Jundiá, is a Neotropical catfish considered an important source of protein for human consumption in some regions of Brazil (Sampaio and Sato, 2006). Although *R. quelen* cultivation is increasing in Brazil (Schenone et al., 2011; Valladão et al., 2018), several reproductive parameters are missing or dispersed in literature, specially those related to testis function and spermatogenesis. In this context, studies on the reproductive biology of *R. quelen* are essential for better use of this species in fish farming besides contributing to conservation and genetic improvement programs.

Spermatogenesis is a stem cell-driven process, where a single, diploid and undifferentiated spermatogonium, known as spermatogonial stem cell (SSC) produces many haploid and motile gametes (Lacerda et al., 2014; Oatley and Brinster, 2012). This process relies on the activity of the SSCs, which are capable to either self-renew (to produce more stem cells) and differentiate into daughter cells committed to spermatogenesis (Russell et al., 1990). In this context, SSCs are remarkable adult stem cells that can transmit the genetic information to the next generation (Russell et al., 1990). Altogether, these properties make SSCs as valuable tools for species conservation by SSC transplantation means (Brinster and Zimmermann, 1994; Lacerda et al., 2010). In this technique, SSCs from a donor species are transplanted into recipient testes, which previously had their spermatogenesis depleted. The recipient can either be of the same species (syngeneic) or not (xenogenic) (Lacerda et al., 2010; Silva et al., 2016). The transplanted SSCs restore spermatogenesis in recipient testes and produce gametes with donor genetic characteristics (Brinster and Zimmermann, 1994; Lacerda et al., 2010). This methodology has been effective in the preservation of economically relevant lineages and endangered species

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https://doi.org/10.1016/j.ygcen.2018.10.018
Received 21 March 2018; Received in revised form 23 October 2018; Accepted 26 October 2018
Available online 01 November 2018
0016-6480/

(Nóbrega et al., 2009; Rana and Mcandrew, 1989; Silva et al., 2016). Although xenogenic germ cell transplantation using Jundiá and Nile tilapia has been established (Silva et al., 2016), there are some limitations in this technique, such as the reduced number of SSCs obtained prior transplantation. This is attributed partly to the absence of molecular markers for identification of these cells in fish (Lacerda et al., 2014; Nóbrega et al., 2010; Panda et al., 2011).

Among vertebrate testes, spermatogenesis can be cystic, such as in anamniotes (fish and amphibians), or non-cystic, as seen in amniotes (reptiles, birds and mammals) (França et al., 2015, 2016; Schulz et al., 2010). In cystic spermatogenesis, a dynamic group of Sertoli cells surround a single, undifferentiated spermatogonium, thus, forming a morpho-functional unit, called cyst (Uribe et al., 2014; França et al., 2015, 2016; Schulz et al., 2010). In this type of spermatogenesis, Sertoli cells support one single germ cell clone derived from a single SSC, while in the amniote testis at least 5 different germ cell clones/generations in different stages of development are supported by a single Sertoli cell (França et al., 2015). Except for the spermatogonial phase, in which the number of spermatogonial mitotic divisions/generations can vary among species, the other two phases (meiotic and spermiogenic phases) show similarities between cystic and non-cystic spermatogenesis (França et al., 2015; Leal et al., 2009; Schulz et al., 2010; Vilela et al., 2003).

It is well established in the literature that the duration of spermatogenesis is important for determining the speed of spermatozoa formation and consequently the spermatogenic efficiency and reproductive strategies (Hess and França, 2007). According to studies developed in rodents, using xenogenic germ cell transplantation (rats to mice), it was found that the duration of spermatogenesis is determined by germ cell genotype (França and Russel, 1998). In most mammalian species already investigated, the total duration of spermatogenesis takes from 30 to 75 days (Hess and França, 2007). In fish, however, data from literature show that the duration of this process is usually much faster (Lacerda et al., 2006; Leal et al., 2009; Nóbrega et al., 2009; Vilela et al., 2003). Moreover, in fish and amphibian there are strong evidences that abiotic factors, such as temperature, affect the duration of spermatogenesis (Lacerda et al., 2006; Morgan, 1979).

The present study aimed to investigate important aspects of testis function and spermatogenesis of *R. quelen*, focusing on the spermatogenesis duration and characterization of SSC markers in their testes, using histological, morphometrical and molecular tools. Altogether, it is expected that these information will expand knowledge of *R. quelen* reproductive biology. Our data can be useful for production, management, and biotechnology applications.

2. Materials and methods

2.1. Animals

The experiments were developed in two different universities. In experiment 1, ten sexually mature *R. quelen* (mean body weight = 200 g) were used to estimate the duration of spermatogenesis and to perform germ cell histomorphometric analysis during the spawning season (September–March). These fish were obtained from commercial farmers located nearby to Belo Horizonte city (Minas Gerais state, Southeastern Brazil) and maintained in 250 L plastic tanks at the Laboratory of Cellular Biology, Department of Morphology, Institute of Biological Science, Federal University of Minas Gerais. Water temperature was kept at 30 °C using aquarium heaters, and photoperiod was fixed at 14 L:10D. All procedures used followed approved guidelines for the ethical treatment of animals and national laws (CEUA; protocol 89/2012). In experiment 2, for the molecular analysis, *R. quelen* (n = 11 adults [7 males and 4 females]; n = 12 embryos and n = 4 larvae) were obtained from commercial stocks and kept in 500 L water tanks under proper photo-thermal conditions in the aquarium of the Reproductive and Molecular Biology Group, Department of Morphology, Institute of Bioscience of Botucatu, São Paulo State University (São Paulo state, Southeast Brazil). All molecular procedures followed the foundations of the animal ethical treatment and were approved by the Ethics Committee for the Use of Animals of São Paulo State University – UNESP, Institute of Biosciences of Botucatu, São Paulo (CEUA; protocol 666).

2.2. Experimental designs and tissue preparation

2.2.1. Experiment 1: histology, histomorphometrical analysis and spermatogenesis duration

After 1 week of acclimation, the specimens were kept at a 30 °C water temperature, which is considered the optimal temperature for this fish species to reproduce in captivity conditions (Gomes et al., 2000). After that, the males received a single intracelomic injection of BrdU (Sigma), a S-phase marker of proliferating cells. The fish were weighed and euthanized at 1 h and 3, 5, 6 and 7 days after injection. Subsequently, testis samples were fragmented (2–3 mm in thickness) and routinely fixed and embedded as described briefly below. To estimate the duration of spermatogenesis, testis fragments were fixed in Bouin's solution (Merk, Kenilworth, New Jersey, United States) and embedded in Paraplast® Tissue Embedding Media (Sigma-Aldrich, St. Louis, Missouri, United States). Five-micrometer-thick sections were subjected to antigen retrieval (1% [v/v] citrate buffer at 98 °C for 15 min) and peroxidase blocking (1% [v/v] H_2O_2 in PBS for 10 min). Subsequently, slides were incubated at room temperature during 1 h using mouse anti-BrdU [1:100 (BD Biosciences, San Jose, CA) diluted in PBS containing 1% (w/v) bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO)], and then incubated with secondary biotinylated antibody [horse anti-mouse, 1:100 (Vector Laboratories, Burlingame, CA) diluted in phosphate buffered saline (PBS) containing 1% (w/v) BSA] for an additional hour. Detection of immunostaining was done using avidin-biotin complex incubation for 1 h (Vector Laboratories) followed by diaminobenzidine (Dako, Glostrup, Denmark) substrate for 20 s. Nuclei were counterstained with Harris's hematoxylin (Sigma-Aldrich) for 30 s. For negative control, the primary antibody (mouse anti-BrdU) was omitted. After these procedures, the most advanced BrdU labeled germ cells at each time period considered was evaluated.

For histology and histomorphometrical analysis, testis fragments were fixed by immersion in 4% buffered glutaraldehyde, embedded in glycol methacrylate (Leica, Wetzlar, Germany) and sectioned accordingly. In order to evaluate germ cell nuclear volume, 50 nuclei were measured for each germ cell type per fish (n = 10). Since the nucleus is spherical or nearly round, the volume was estimated using the formula $4/3\ \pi R^3$, where R = nuclear diameter/2. This data allowed to estimate the germ cell nuclear changes during the spermatogenic process, from type A undifferentiated spermatogonia (A_{und}) to spermatids. Images were captured through Cell F software (Olympus) using 40× and 100× oil immersion objectives in an Olympus BX60 Microscope.

2.2.2. Experiment 2: expression analysis (RT-PCR and qPCR) and in situ hybridization

The expression analysis were carried out by RT-PCR (reverse transcription polymerase chain reaction) or qPCR (reverse transcription polymerase chain reaction quantitative real time) in (1) adult tissues (brain, gills, heart, liver, muscle, gut, gonads) from male (n = 4) and female (n = 4); (2) during different stages of *R. quelen* development (n = 3 for each stage of development – embryogenesis, larval development and adult) and (3) A_{und} enriched fractions obtained after discontinuous Percoll density gradient centrifugation (n = 3 males). In this study we evaluated the expression of genes commonly expressed in stem cells or SSCs of mammals and fish, such as *gfra1* (GDNF family receptor alpha-1 – SSC marker); *klf4* (Kruppel-like factor 4 – SSC and stem cell marker); *nanog* (Homeobox protein Nanog, a transcription factor – SSC and stem cell marker); *plzf* (promyelocytic leukaemia zinc finger – SSC marker); *pou5f3* (POU domain class 5 transcription factor

Table 1
Primers used for RT-PCR, qPCR and *in situ* hybridization.

Gene	Sequence 5′-3′	Product Size (pb)	Usage
gfra1a	Forward: TGGCTCCCATCTATTCAGGTG Reverse: TGGAGACACGCTGATCTTGG	533	RT-PCR
klf4	Forward: AAGACTGCATCCTGACAGAG Reverse: GTGTGTTTCCTGTAGTGACG	500	RT-PCR
nanog	Forward: CCTGGAGAGATCGAGACTAT Reverse: GTTCTCTCACCTGAGGGTTA	500	RT-PCR
plzf	Forward: GATGGCGATAAGAGCCGTGA Reverse: TGGATGAAGCTCTGTGGCAG	317	RT-PCR
pou5f3 (*Oct4*)*	Forward: CTGAGGAAGAGGAGACTCTG Reverse: CCATACACGCACTACATCTC	500	RT-PCR
sox2	Forward: AAGTCCGAGTCTAGTTCGAG Reverse: AACTGTGTGGAGTTCCTACC	500	RT-PCR
sycp3l	Forward: AGGAAGCGCCTGGAATGTTT Reverse: TCCCTGGAGGAAAGTCTCGT	333	RT-PCR
β-actin	Forward: TGACCTGACTGACTACCTCA Reverse: AGCTCATAGCTCTTCTCCAG	179	RT-PCR
*gfra1**	Forward: ATCAAACACCGCTTGGGCTA Reverse: GTGTCATTCAGGTTGCATGCTT	90	RT-qPCR
klf4	Forward: TGTTTAGCGAACCATTGCGG Reverse: AAATCCCAGGAGGGGTGAGG	83	RT-qPCR
nanog	Forward: GGAGCAACGGGTCTAACGTA Reverse: CCAACTACTGTCTCTTTGATGCC	90	RT-qPCR
plzf	Forward: CGATAAGAGCCGTGATGGGC Reverse: CCACCGTCTTCACGCACATA	90	RT-qPCR
pou5f3 (*Oct4*)*	Forward: GTGGCTGTGTCTAGCCTACTC Reverse: AGTCTCGACCAGTTGAGCAATA	91	RT-qPCR
*sycp3l**	Forward: AGGAAGCGCCTGGAATGTTT Reverse: TTCTGCCTCTGGCTATGCTG	95	RT-qPCR
β-actin	Forward: TACAGCTTCACCACCACAGC Reverse: CCCATCTCCTGCTCGAAGTC	92	RT-qPCR
plzf	Forward T3#: GGGCGGGTGTTATTAACCCTCACTAAAGGATGGCGATAAGAGCCGTGA Reverse T7#: CCGGGGGGTGTAATACGACTCACTATAGTGGATGAAGCTCTGTGGCAG	414	*in situ* hybridization
pou5f3 (*Oct4*)*	Forward T3#: GGGCGGGTGTTATTAACCCTCACTAAAGCGCACTGCTCTCGAGTCATA Reverse T7#: CCGGGGGGTGTAATACGACTCACTATAGAGCCTGTTTGAAGACCTCCG	417	*in situ* hybridization

* Degenerate primers.
\# Underlined the insertion of the T3 and T7 promoters in the primers for *in situ* hybridization.

3, aka Oct4 – SSC and stem cell marker) and *sox2* (SRY -sex determining region Y-box 2 – stem cell marker). As control (gonad specific expression) or to detect the presence of meiotic cells, we evaluated the expression of *sycp3l* (synaptonemal complex protein 3) in some cases.

Total RNA from tissue screening and developmental stages was extracted with TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's recommendations. For the testicular cell fractions obtained after Percoll (see below), total RNA was extracted using the PureLink™ RNA Mini Kit (Ambion – Life Technologies). To avoid genomic DNA contamination, the RNA samples were treated with DNase I and RNase-free kit (Invitrogen, Carlsbad, CA, USA), prior to cDNA synthesis. The cDNA synthesis was performed with random hexamers using Supercript® II (Invitrogen™, Carlsbad, CA, USA), according to standard protocols (Nóbrega et al., 2010). RT-PCR and qPCR were conducted using species-specific and degenerate primers (Table 1). For RT-PCR, the amplified products were separated on 1% agarose gel and the expected bands were compared to the molecular weight of the ladder. For qPCR, Cq values were determined using SYBR Green kit (Invitrogen) in 20 µl reaction, 900 nM for each primer and cDNA using a StepOne Plus (Applied Biosystems). Each reaction was performed in duplicate. Relative gene expression profiles were calculated according to the ΔΔCt method as described previously (Vischer et al., 2003). β-actin endogenous expression was used as control in all qPCR reactions.

To separate the different testicular cell fractions by discontinuous Percoll density gradient centrifugation, adult testes (n = 3) were collected, washed in Hanks' balanced saline (HBSS) containing 0.1% penicillin–streptomycin (10.000 UI/10 mg/mL) and subjected to enzymatic dissociation (Bellvé et al., 1997; Lacerda et al., 2006) with collagenase 0.2% (Gibco, Life Technologies) in Dulbecco Modified Eagle medium/Ham F-12 medium (DMEM/Ham's F12 – Gibco, Life Technologies) for 3 h at 28 °C, under gentle stirring. The dispersed testicular tissue was then incubated with 0.25% trypsin/1mM EDTA (Life Technologies) and 0.03% DNase I (Invitrogen™, Life Technologies) for 30 min under similar conditions. An equal volume of fetal bovine serum (FBS, Gibco, Life Technologies) was used to inactivate the trypsin. The cell suspension was filtered through a 60 µm mesh, centrifuged at 200 × *g* for 10 min and re-suspended in DMEM/F12. The obtained cells were purified by discontinuous Percoll density gradient centrifugation (Percoll – Nutricell, Brazil) using Dulbecco's Modified Eagle Medium (DMEM/F12) and 0.75% Bovine Serum Albumin (BSA – Sigma Aldrich). Three columns were prepared, each with four gradient concentrations of Percoll: 10%, 25%, 30% and 40% (Fig. 6A). Each gradient was carefully and sequentially, from 40% to 10%, placed in a 15 mL conical tube (Fig. 6A). To help the visualization of the Percoll bands, trypan blue solution (Gibco, Life Technologies) was added in the

10 and 30% gradients. Subsequently, the cell suspension was deposited at the top of the column and centrifuged for 30 min (800 × *g*) at 25 °C, as described by Lacerda et al. (2006). After the centrifugation, the cells were observed in the different interfaces of the solutions, being possible the identification of distinct bands, which were individually collected for gene expression (above) and morphology (see below) (Fig. 6B).

For light microscopy, the Percoll bands were fixed in 4% paraformaldehyde in Sorensen buffer (0.1 M, pH 7.2) for at least 24 h, dehydrated in a graded ethylic series, embedded in Historesin (HistoResin), sectioned (3 μm thickness) and stained with toluidine blue for counting germ cells at different stages of development. Five hundreds to one thousand testicular cells were counted in each band. In the first analysis, the percentage of A_{und} was determined in the different Percoll bands (n = 3) (Fig. 6C). In the second analysis, the percentage of testicular cells (A_{und}; spermatocytes; spermatids; spermatozoa and cell debris) was determined in the cell suspension after enzymatic digestion (n = 1) and in the different Percoll bands (n = 1) (Fig. 7A). Since it was difficult to distinguish types A_{diff} and B spermatogonia in each cell fraction, we did not count these cells, although it was possible to identify them in the total cell suspension stained with toluidine blue (Supplemental Fig. 1). The histological sections were examined and documented using Leica DMI6000 microscope (Leica).

To confirm the most expressed transcripts in A_{und} enriched fractions were potential SSC markers, we performed *in situ* hybridization as described previously (Nóbrega et al., 2015). Biotin-labeled sense and antisense cRNA were synthesized from specific PCR product generated with primers containing T3 or T7 RNA polymerase promoter sequences at 5′-ends (Table 1). The expected PCR product was extracted, purified with Zymoclean DNA recovery kit (Synapse D4001) and transcribed using biotin-16-UTP and RNA T7 polymerase (anti-sense) or T3 (sense) from the Biotin RNA Labeling Mix Roche kit (Roche 11685597910). The *in situ* hybridization was performed with adaptations of Thisse and Thisse (2008). Briefly, tissues were fixed overnight in 4% phosphate-buffered paraformaldehyde (pH 7.4) in RNase-free conditions, dehydrated, diaphanized, embedded in paraffin (Paraplast®, Sigma) and sectioned with 5 μm thickness. Subsequently, the sections were rehydrated and washed with PBT (Tris buffer phosphate, pH: 7.4) and TrisHCl buffer (0.05 M, pH: 7.5). The material was treated with proteinase K (20 mg/ml) at 37 °C for 5 min and incubated with hybridization solution containing either sense or antisense RNA probe at 70 °C overnight. The slides were incubated with anti-streptavidin-AP primary antibody (anti-streptavidinalkaline phosphatase conjugated) diluted 1:2000 in the same blocking solution at 4 °C overnight. Tissues were washed and incubated with staining solution NBT/BCIP (nitro-blue tetrazolium chloride/5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt) (Thermo Scientific Pierce), and the slides were photographed using the microscope Leica DMI 4000B. Histological sections of Jundiá testes stained with hematoxylin and eosin (H&E) were used to support the germ cell identification in the *in situ* hybridization signal.

2.3. Statistical analysis

The results were expressed as mean ± standard error (SE). The differences between two groups were identified using Student's unpaired *t*-test ($p < 0.05$). Comparisons between the means (more than two groups) were performed using one-way ANOVA, followed by Student-Newman-Keuls method ($p < 0.05$). The GraphPad Prism software, version 4.0 was used for all of the statistical analyses.

3. Results

3.1. Histology and histomorphometrical analysis

Histological analysis of testes from different stages of the reproductive cycle are shown in Fig. 1. In the regressed stage (Fig. 1A), the germinal compartment is composed of cysts of A_{und}. At the developing stage (Fig. 1B), germ cell cysts at different phases of spermatogenesis and presence of spermatozoa in the tubular lumen are observed. Jundiá testes at mature stage show abundant amounts of spermatozoa in the testicular lumen whereas, fewer and scatterd cysts of A_{und} are observed in the germinal epithelium (Fig. 1C).

According to morphological characteristics, A_{und} are single cells surrounded by Sertoli cells and are the largest germ cell observed in Jundiá testis (Figs. 1 and 2). A_{und} also present a large nucleus with poorly condensed chromatin, one compact nucleolus and irregular nuclear envelop (Figs. 1D and 2). Type A differentiated spermatogonia (A_{diff}) show denser and smaller nucleus than A_{und} and are found in pairs or small groups inside the cyst (Figs. 1E and 2). In comparison with A_{und} and A_{diff}, type B spermatogonia are drastically smaller and present an elliptical/round nucleus with one or two small nucleoli and an evident larger amount of heterochromatin (Figs. 1F–H and 2). Type B spermatogonia have several generations, and their number per cyst increases remarkably, whereas their size continually decreases (Fig. 1F–H). Early (B_{early}), intermediate (B_{int}) and late (B_{late}) type B spermatogonia were identified in Jundiá testes (Fig. 1F–H).

All together, spermatogonial cells, from A_{und}, A_{diff} to type B, compose what is morpho-functionally defined as spermatogonial phase of spermatogenesis. After the large expansion of the number of spermatogonial cells, the last generation of type B spermatogonia forms the preleptotene spermatocytes (Pl), whose size and morphology resemble their predecessor cells (Fig. 1I). The PI progress through meiosis and grow continually forming leptotene/zygotene (L/Z), pachytene, (P) and diplotene (D) spermatocytes (Fig. 1J–L). The morphological characteristics of these cells are very similar to those usually described in vertebrates. Also, pachytene spermatocytes are the most frequent meiotic germ cells observed in the seminiferous tubules. At the end of meiosis I, diplotene spermatocytes divide to form secondary spermatocytes (S) (Fig. 1M), a much smaller cell type that quickly goes through meiosis II and divide forming haploid spermatids (Figs. 1N–P and 2).

In the spermiogenic phase, the haploid spermatids undergo a remarkable reduction in the cellular volume (Fig. 1N–P). Based on the gradual chromatin compact, round spermatids are classified as initial (E1), intermediate (E2) and final (E3) spermatids (Figs. 1N–P and 2). The morphological criteria used to characterize the different germ cell types in this study was based on Schulz et al. (2010).

3.2. Spermatogenesis duration

The most advanced BrdU-labeled germ cells after injecting different times apart are shown in Fig. 3. As it can be noted, the BrdU-labeled cells were preleptotene spermatocytes (1 h; Fig. 3A), pachytene spermatocytes (3 days; Fig. 3B), diplotene spermatocytes (5 days; Fig. 3C), spermatids (6 days; Fig. 3D), and spermatozoa in the tubular lumen (7 days; Fig. 3E). Based on these observations, the combined duration of meiotic and spermiogenic phases (from preleptotene to spermatozoa) in Jundiá kept at 30 °C water temperature was estimated in approximately 7 days (Fig. 3F).

3.3. Tissue distribution and ontogenic analysis

RT-PCR analysis showed broad expression of the evaluated transcripts *gfra1*, *klf4*, *nanog*, *plzf*, *pou5f3* and *sox2* mRNA in both adult male and female of *R. quelen* (Fig. 4). As control, *sycp3l* which is involved in the synaptonemal complex formation during the meiotic process was found only in the gonads (Fig. 4). Further analysis evaluated the expression of these selected genes by qPCR during early embryogenesis [0 h post-fertilization (hpf); blastulation (7 hpf) and gastrulation (10 hpf)], larval phase [somitogenesis (15 hpf)] and adult life (testes were used for the expression analysis) (Fig. 5). The *gfra1*, *klf4*, and *nanog* mRNA levels did not change along the sampled periods (Fig. 5). On the other hand, *pou5f3* transcripts are decreased in the adult testis when compared to its levels during early embryogenesis and

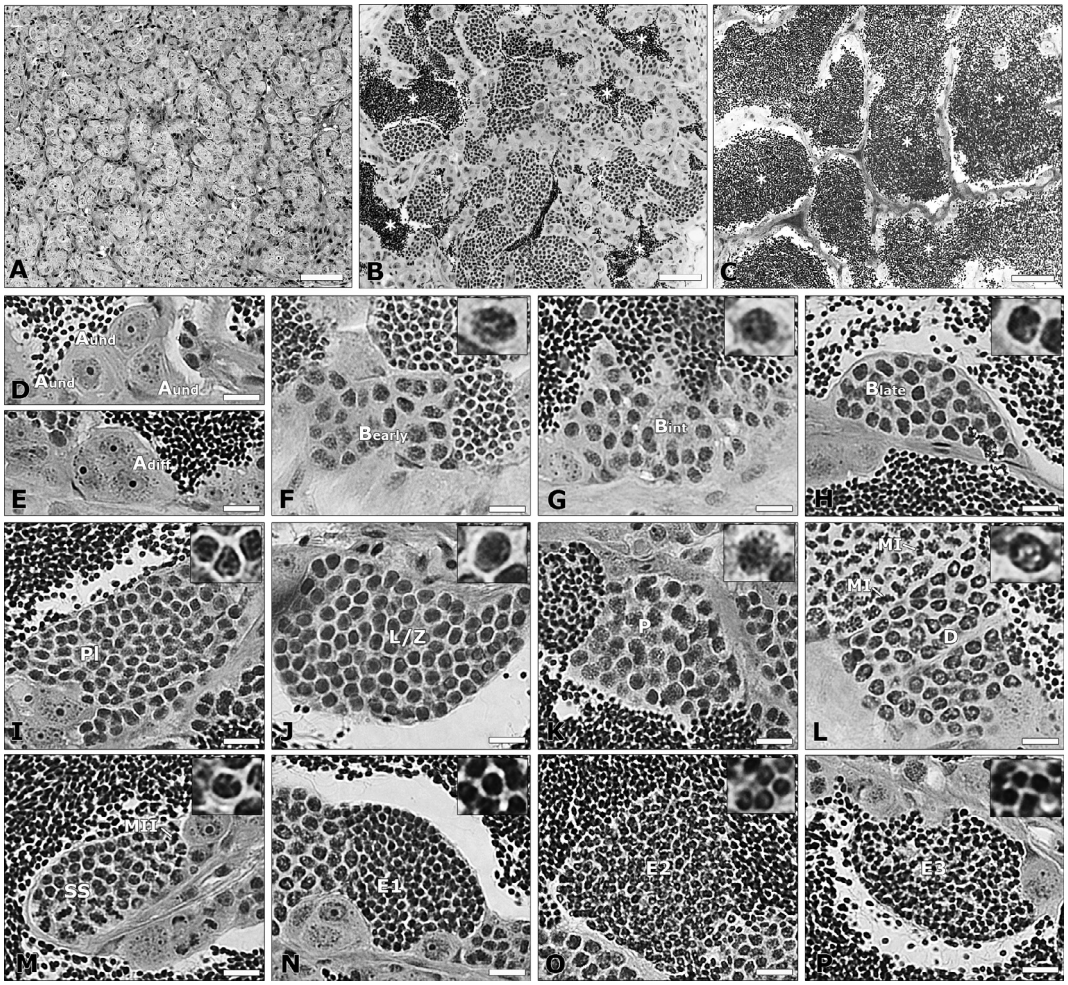

Fig. 1. Histology of the different reproductive stages of Jundiá testes. (A) Regressed stage: testes with predominance of early spermatogonia (type A undifferentiated spermatogonia - A_{und}). (B) Developing stage: germ cell cysts at different stages of spermatogenesis are observed; spermatozoa in the tubular lumen (asterisk) can also be noticed. (C) Mature stage: tubules are fulfilled of spermatozoa. The morphology of the different germ cell types are shown: A_{und} (D); type A differentiated spermatogonia (A_{diff}) (E); early type B spermatogonia (B_{early}) (F); intermediate type B spermatogonia (B_{int}) (G); late type B spermatogonia (B_{late}) (H); primary spermatocytes (I-L) in preleptotene (Pl), in the transition from leptotene to zygotene (L/Z), pachytene (P), diplotene (D), and meiotic figures from the first (MI) and second (MII) meiotic division; secondary spermatocytes (SS) (M); spermatids (N–P) at the initial (E1), intermediate (E2), and final (E3) steps of spermiogenesis. Bar A–C = 50 μm; Bar D–P = 10 μm.

somitogenesis (Fig. 5). Concerning *plzf*, lower mRNA levels were found in gastrula, while a significant increase of this transcript was found during somitogenesis and adult testis (Fig. 5).

3.4. Expression analysis of spermatogonial enriched cell fraction from Percoll density gradient centrifugation

First, we have established a methodology to isolate and enrich Jundiá spermatogonial cells (A_{und}) (Fig. 6). The enzymatic digestion of Jundiá testes resulted in a heterogeneous cell suspension, which were submitted to a discontinuous Percoll density gradient centrifugation (Fig. 6A). After this procedure, 5 bands were obtained (Fig. 6B). The histological and morphometrical analysis showed different germ cell types along the bands (Fig. 7), but only band 3 exhibited an increased proportion of A_{und} (Figs. 6B and C and 7A and E). Spermatocytes, spermatids, spermatozoa and many cell debris were found in practically all bands (Fig. 7).

When evaluating the expression of the selected transcripts, *klf4*, *nanog* and *sycp3l*mRNA did not vary along the evaluated bands (Fig. 8B, C, F). *gfra1* mRNA levels were slightly increased in band 5 in comparison to bands 1, 3 and 4 (Fig. 8A). On the other hand, *plzf* was significantly higher in band 3, in contrast to its reduced levels seen in bands 1, 4 and 5 (Fig. 8D). With regards to *pou5f3*, an increased expression of this transcript was found in bands 3 and 4 (Fig. 8E).

3.5. Cellular localization of plzf and pou5f3 mRNA expression in Jundiá testes

Identification of specific cell types expressing the Jundiá *plzf* and *pou5f3* mRNA was performed by *in situ* hybridization. A strong *plzf* transcript signal was found mainly in A_{und} (Fig. 9C and D). *plzf* signal was also found in cysts of two or three A_{diff}, although less intense than the one found in A_{und} (Fig. 9C and D). On the other hand, *plzf* mRNA was not found in more advanced cysts, such as type B spermatogonia (Fig. 9D). No signal was observed when sections were hybridized with the sense cRNA *plzf* probe (Fig. 9D-inset). Similar pattern of cellular localization was reported for *pou5f3* mRNA; *pou5f3* is mainly expressed in A_{und} and A_{diff} with no expression in type B spermatogonia (Fig. 9E–G). Specific signal is shown by sense cRNA *pou5f3* probe *in situ* hybridization (Fig. 9E-inset). Histological sections of Jundiá testis stained with H&E (Fig. 9A and B) were used to support the *in situ* hybridization detection.

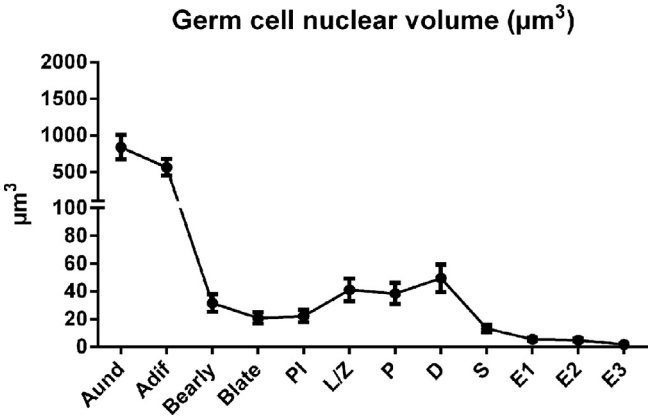

Fig. 2. Germ cell nuclear volume during *R. quelen* spermatogenesis. Type A undifferentiated spermatogonia cells (A_{und}) are the largest germ cell in the testis. During the progression of spermatogenesis, the germ cell nuclear volume shows a remarkable reduction. A_{und}, type A undifferentiated spermatogonia; A_{diff}, type A differentiated spermatogonia; B_{early}, early type B spermatogonia; B_{late}, late type B spermatogonia; Pl, preleptotene spermatocyte; L/Z, transition from leptotene to zygotene spermatocyte; P, pachytene spermatocyte; D, diplotene spermatocyte; S, secondary spermatocyte; E1, initial spermatid; E2, intermediate spermatid; E3, final spermatid.

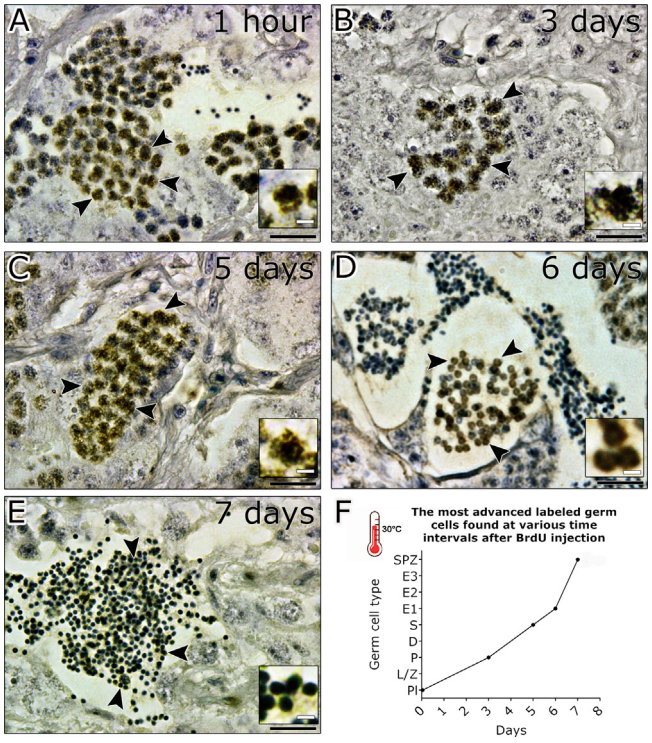

Fig. 3. Most advanced labeled germ cell at different time periods after BrdU injection in *R. quelen* kept at 30 °C water temperature. The most advanced BrdU-labeled cells (arrowheads) were: (A) preleptotene spermatocytes (1 h post-injection); (B) pachytene spermatocyte (3 days post-injection); (C) secondary spermatocyte (5 days post-injection); (D) initial spermatids (6 days post-injection) and (E) spermatozoa (7 days post-injection) observed in the lumen of the seminiferous tubules. The inset in (F) shows the kinetics of the most advanced labeled germ cells during Jundiá spermatogenesis. Black bars = 30 µm and white bars = 5 µm.

4. Discussion

Knowledge of germ cell differentiation steps and duration of spermatogenesis are very useful for understanding the reproductive biology,

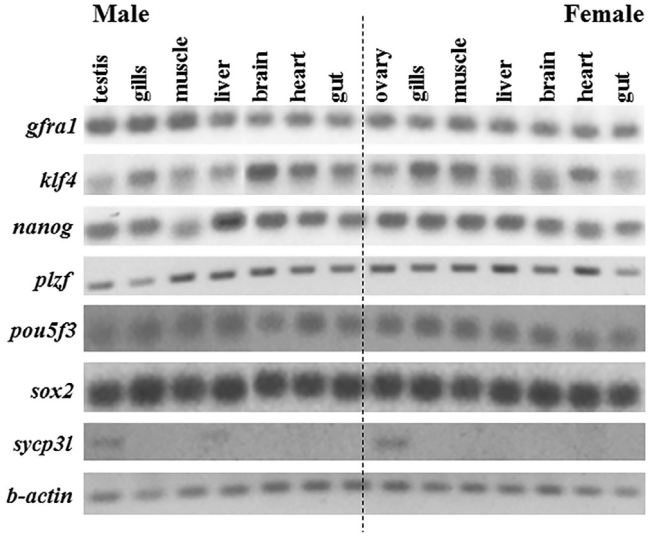

Fig. 4. Tissue distribution of *R. quelen gfra1*, *klf4*, *nanog*, *plzf*, *pou5f3*, *sycp3l* and *sox2* mRNA in adult male (M) and female (F). Gills, muscle, liver, brain, heart, gut, testis and ovary were used. *β-actin* was amplified at the same conditions and used as a positive control for each sample.

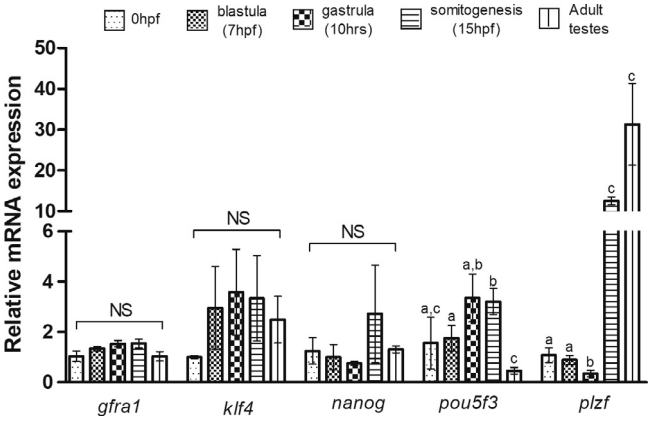

Fig. 5. The expression of *R. quelen gfra1*, *klf4*, *nanog*, *plzf* and *pou5f3* mRNA during early embryogenesis, larval phase (somitogenesis) and adult phase (testes). RNA was extracted from whole embryos at different stages of development from 0 h post-fertilization (hpf), 7 hpf - blastulation, 10 hpf - gastrulation, 15 hpf - somitogenesis until adult phase, where testes were used. The level of expression was determined by qPCR and normalized to *β-actin* expression. Data are expressed as mean ± SEM (n = 3) relative to the expression levels of each gene at 0hpf. Different letters denote significant differences among the groups ($p < 0.05$). NS, Not significant.

in particular the functional and regulatory mechanisms of spermatogenesis and reproductive strategies from different species (Nóbrega et al., 2009). Jundiá catfish cultivation has been steadily increasing in South America, particularly in southern Brazil (Baldisserotto and Radünz Neto, 2004; Carneiro and Mikos, 2005; Canton et al., 2007). However, because several biological parameters for this species are not available, including those related to reproductive physiology, Jundiá production is still far below its potential (Carneiro and Mikos, 2005). Here, for the first time, we described germ cell morphology, duration of spermatogenesis and stem cell gene expression in the testis of this species.

Although some specific molecular markers for germ cells have been described in fish (reviewed by Lacerda et al., 2014; Bellaiche et al., 2014a,b; Ozaki et al., 2011), these cells are mainly characterized by morphological criteria (Nóbrega et al., 2010; Uribe et al., 2014). Overall, the morphological characteristics of the different Jundiá germ

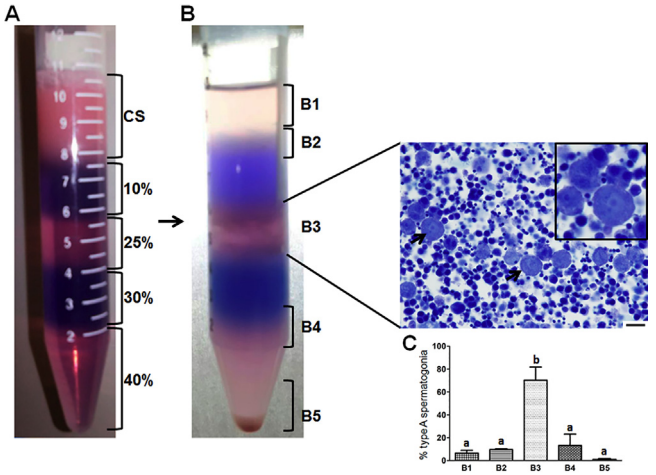

Fig. 6. (A) Jundiá cell suspension (CS) obtained from testicular enzymatic digestion was placed in 15 mL falcon tube, which was previously prepared with different concentrations of Percoll: 10%, 25%, 30% and 40%. (B) After centrifugation, 5 bands containing different germ cell types were formed: B1 (band 1), B2 (band 2), B3 (band 3), B4 (band 4) and B5 (band 5). Histological sections stained with toluidine blue showed higher concentration of type A undifferentiated spermatogonia (A_{und}) in band 3, as indicated by black arrows. Scale bar: 10 μm. (C) Percentage of type A undifferentiated spermatogonia (A_{und}) in each Percoll band relative to the total number of A_{und} found in the analysis. B1: band 1, B2: band 2, B3: band 3, B4: band 4 and B5: band 5. Different letters denote significant differences among the groups ($p < 0.05$).

cell types are very similar to those observed for other teleost species already investigated (Billard, 1990; Grier, 1981; Grier and Neidig, 2000; Leal et al., 2009; Selman and Wallace, 1986; Le Gac and Loir, 1999; Miura, 1999; Schulz et al., 2010). In addition, type A_{und} spermatogonia, including the SSCs, are the largest germ cell observed in Jundiá, and a remarkable decrease in the germ cell nuclear diameter was observed from A_{und} to late type B spermatogonia, as well as from pachytene/diplotene to final spermatids. This trend is quite similar to other fish species investigated in this aspect (Almeida et al., 2008; Melo et al., 2014), including data obtained in our laboratory for tilapia (Schulz et al., 2005) and zebrafish (Leal et al., 2009).

Literature shows that Jundiá is capable of adjusting to a wide range of environmental temperatures (Lermen et al., 2004). However, it has been found that in captivity conditions, this species presents a better development and higher growth rate in temperatures around 30 °C (Andrews and Stickney, 1972; Andrews et al., 1972; Gomes et al., 2000). Under this condition, in the present study we found that the combined duration of meiotic and spermiogenic phases of spermatogenesis was rather fast and lasted approximately one week. This value was similar to those found for other fish species investigated in our laboratory, such as the Nile tilapia kept in temperatures between 27 °C and 30 °C (Silva and Godinho, 1983; Vilela et al., 2003) and zebrafish kept at 20 °C (Nóbrega et al., 2010). Particularly in Nile tilapia kept at 35 °C, the meiotic and spermiogenic phases of spermatogenesis last 5–6 days (Lacerda et al., 2006).

Several environmental factors are known to influence fish sperm production and spermatogenesis (Alvarenga and França, 2009; Schulz et al., 2010). However, temperature is one of the most prevalent

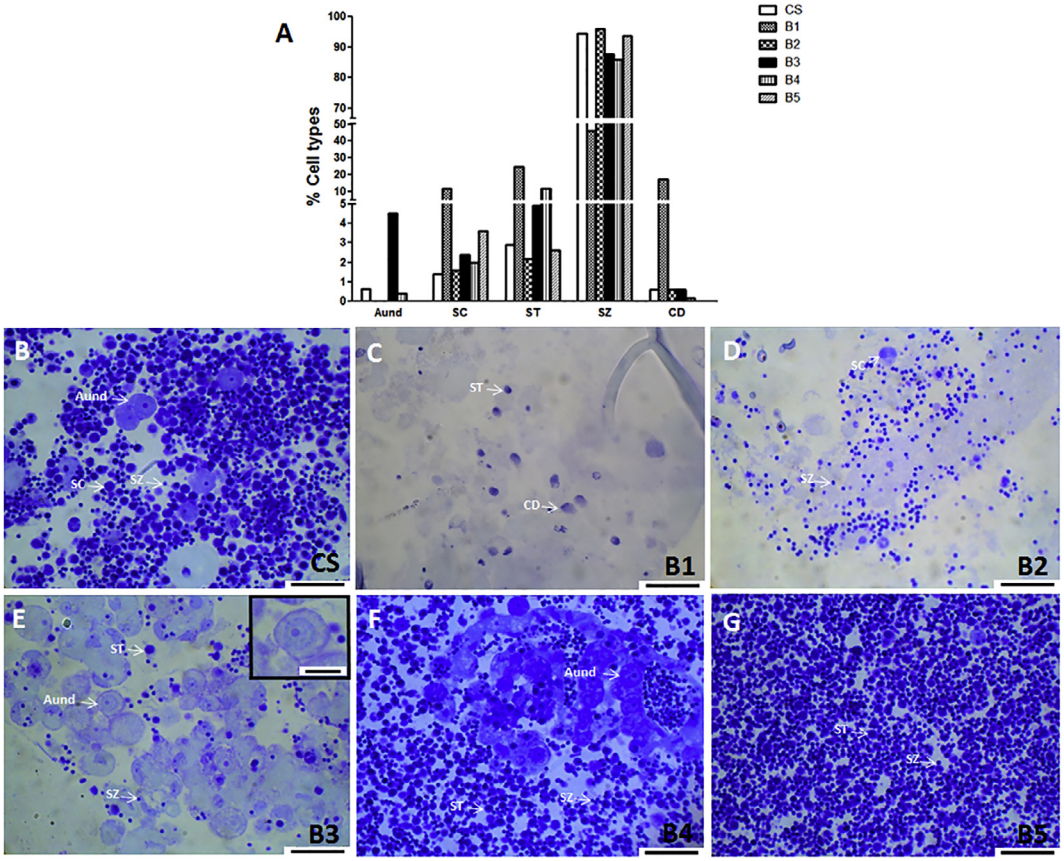

Fig. 7. (A) Percentage of germ cell types in Percoll bands relative to the total number of germ cells found in the analysis. B1 (band 1), B2 (band 2), B3 (band 3), B4 (band 4), B5 (band 5) and CS (total cell suspension) are indicated. Type A undifferentiated spermatogonia (A_{und}); spermatocytes (SC); spermatids (ST); spermatozoa (SZ) and cell debris (CD). (B–G) Histological section stained with toluidine blue. Type A undifferentiated spermatogonia (A_{und}); spermatocytes (SC); spermatids (ST); spermatozoa (SZ) and cell debris (CD). The inset in Figure E shows a higher magnification of type A undifferentiated spermatogonia found in band 3. Scale bars: 25 μm.

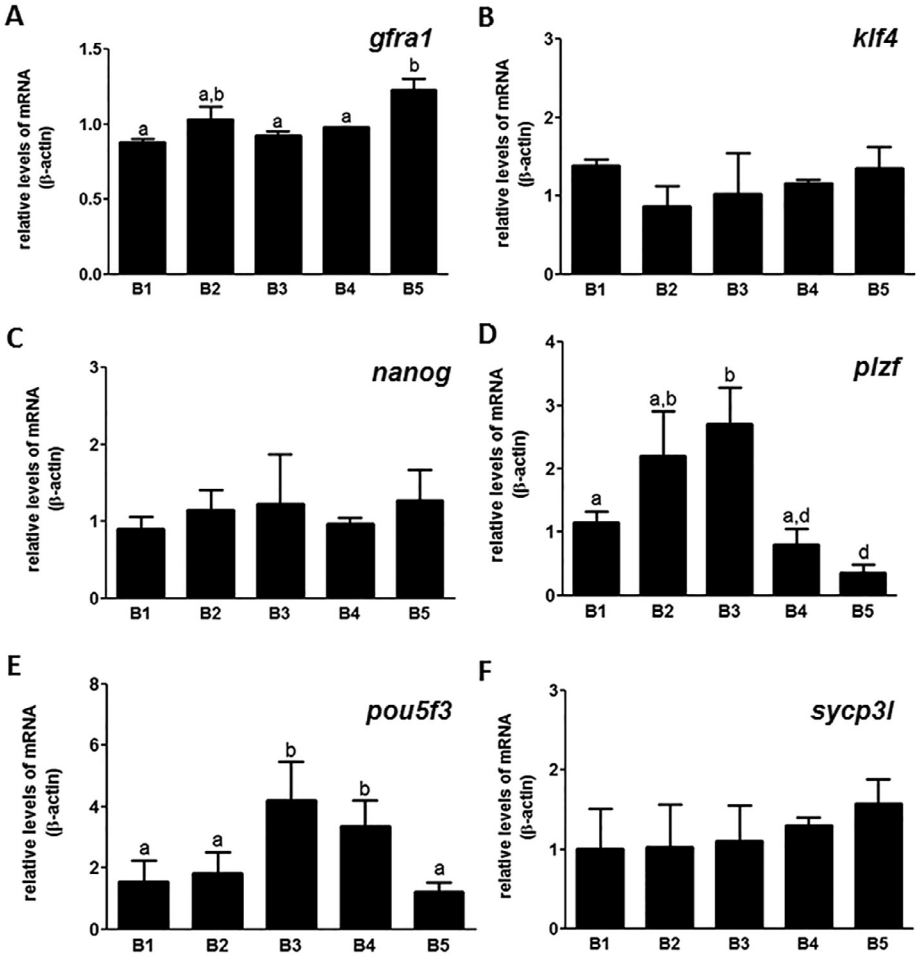

Fig. 8. Expression of *R. quelen gfra1*, *klf4*, *nanog*, *plzf*, *pou5f3* and *scyp3l* mRNA in the different Percoll bands (A-F). B1: band 1, B2: band 2, B3: band 3, B4: band 4, B5: band 5. The expression levels were normalized to the expression of *β-actin* (reference gene). Values represent mean ± SEM (n = 3) relative to the average expression in all bands. Different letters indicate significant differences among the groups ($p < 0.05$).

environmental factors capable of affecting reproductive physiology of aquatic wildlife (Pankhurst and Munday, 2011). For comparative purpose, a data compilation related to the duration of spermatogenesis (meiotic and spermiogenic phases) of several fish species kept at different temperatures is shown in Table 2. It is noteworthy that the duration of spermatogenesis is much longer in fish exposed to lower temperatures (< 10 °C) (group A), lasting more than 4 weeks. On the other hand, fish kept in temperatures ranging from 20 to 25 °C (group B), the time period from the appearance of early spermatocytes to the formation of sperm is about 2–3 weeks. At higher temperatures (27–30 °C; group C), the time required to form spermatozoa, including Jundiá investigated in the present study, is in the range of 1–2 weeks. Whereas, in temperatures around 35 °C (ex: Nile tilapia; group D), the time required to form sperm is less than 1 week. Therefore, independent of the fish species considered, water temperature exerts a high influence on the spermatogenesis speed, either accelerating or delaying the process. Taking advantage of this knowledge and showing a practical application of these findings, in order to deplete endogenous spermatogenesis before germ cells transplantation, in association with busulfan treatment, we experimentally manipulated (increased) the water temperature in which the tilapias and zebrafish were kept (Lacerda et al., 2006; Nóbrega et al., 2010). In these conditions, spermatogenesis was accelerated and the germ cells became more vulnerable to the cytotastic drug used (Alvarenga and França, 2009; Lacerda et al., 2006; Nóbrega et al., 2010; Silva et al., 2016). These approaches were also used in other fish species (Majhi et al., 2009, 2014).

Using Nile tilapia as an experimental fish model we also demonstrated that higher temperatures (30 °C and 35 °C) resulted in germ cell differentiation, as well as faster and larger sperm production (Alvarenga and França, 2009). In contrast, lower temperature (20 °C) triggered self-renewal of type A spermatogonia, Leydig cell and Sertoli cell proliferation, apoptosis of more advanced germ cells, and arrest of spermatogenesis (Alvarenga and França, 2009; Vilela et al., 2003). These findings again illustrate the effects of higher temperatures on the acceleration of spermatogenesis and provide some clues for reproductive strategies. However, it is interesting that there is a physiological limit for this acceleration. Because, in comparison to a water temperature of 30 °C, in which only two days is enough to form sperm in zebrafish, the temperature of 35 °C promotes spermatogenic arrest in this species (Table 2) (Nóbrega et al., 2010), whereas such arrest in Nile tilapia occurs at a low temperature (20 °C; Vilela et al., 2003). In order to better understand the molecular mechanisms involved in the determination of the germ cell pace, more studies are necessary.

SSCs are responsible for transmitting genetic contents of an individual over generations, making these cells as valuable tools for species conservation (Brinster and Zimmermann, 1994). Nevertheless, the molecular signature for SSCs in many vertebrate species is still unknown. In fish, the SSC molecular markers are restricted to fewer teleosts (reviewed by Lacerda et al., 2014; Ozaki et al., 2011), and there is no information in this regards for Neotropical species. In this context, this work also evaluated gene expression of some selected specific stem cell and SSC genes in Jundiá.

The *plzf* (promyelocytic leukemia zinc finger protein) is responsible for self-renewal and maintenance of SSCs in mammals (Costoya et al.,

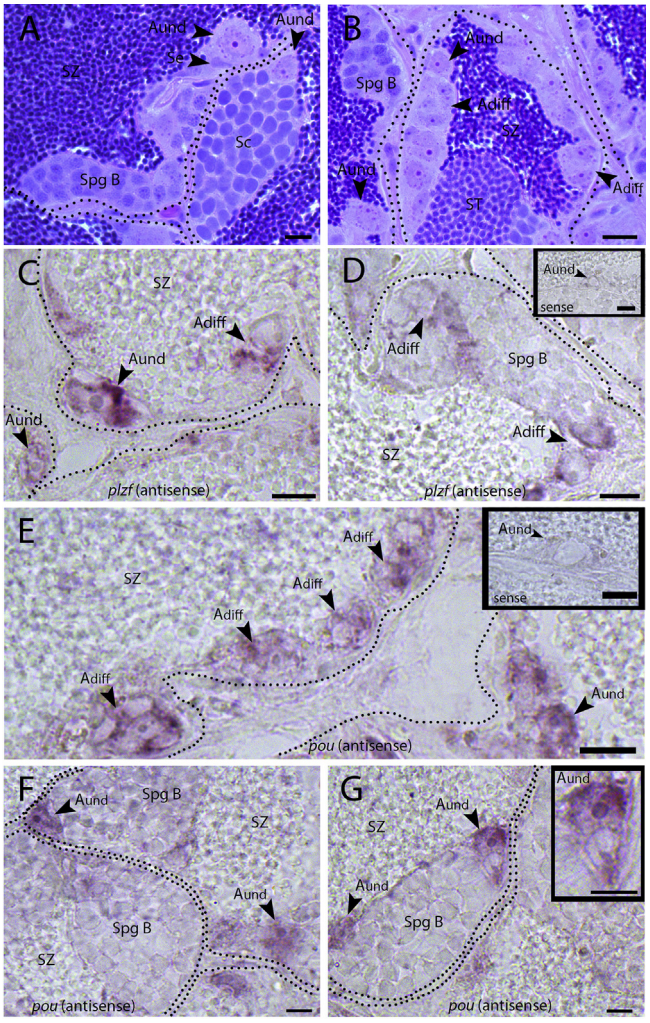

Fig. 9. Cellular localization of *plzf* and *pou5f3* in Jundiá testes. (A–B) Histological sections stained with hematoxylin and eosin (H&E). Type A undifferentiated spermatogonia (A_{und}); type A differentiated spermatogonia (Adiff), type B spermatogonia (Spg B), spermatocytes (Sc), spermatids (ST); spermatozoa (SZ); Sertoli cells (Se). The interstitial compartment is delimited by dashed lines. Scale bars: 10 μm. (C–D) The antisense cRNA probe for *plzf* showed a strong signal in type A undifferentiated spermatogonia (A_{und}), and weak staining in cysts of 2 or 3 germ cells of type A differentiated spermatogonia (A_{diff}). Note that *plzf* is not expressed in more advanced germ cell cysts, such as type B spermatogonia (Spg B). The interstitial compartment is delimited by dashed lines. Spermatozoa (SZ) are also indicated in the figures. Inset in B shows the sense cRNA probe; note the absence of specific staining. (E–G) The antisense cRNA probe for *pou5f3* showed a strong signal in type A undifferentiated spermatogonia (A_{und}). *pou5f3* could also be detected in type A differentiated spermatogonia (A_{diff}), but not in type B spermatogonia (Spg B). Inset in C shows sense cRNA probe; no signal was detected. Interstitium is also delimited by dashed lines. SZ, Spermatozoa. Scale bars: 10 μm.

2004; Mohapatra and Barman, 2014). In teleost fish, *plzf* seems to have similar function and has been associated with the maintenance of SSCs in carp rohu (*Labeo rohita*) (Panda et al., 2011), being also considered a SSC marker in zebrafish (*D. rerio*) (Mohapatra and Barman, 2014; Ozaki et al., 2011). In the present study, *plzf* was detected in the Jundiá testes. Interestingly, when analyzing gene expression among the Percoll bands, higher levels of *plzf* were found in bands enriched with A_{und}. Similar data was found by Shang et al. (2015), who demonstrated the specific expression of *plzf* in SSCs of channel catfish (*Ictalurus punctatus*) and blue catfish (*I. furcatus*). In addition, Nayak et al. (2016) found *plzf* positive SSCs in walking catfish (*Clarias batrachus*). To support our expression analysis, *in situ* hybridization was performed in Jundiá testes and showed that *plzf* is strongly expressed in A_{und}. Moreover, *plzf* signal was also detected in A_{diff}, although less intense than A_{und}, and no expression was observed in more advanced germ cells. Altogether, our results indicate that *plzf* is a marker of SSCs in Jundiá testes.

The Kruppel-like factor 4 (*klf4*) is a zinc-finger transcriptional factor involved in the regulation of cell proliferation, differentiation and apoptosis (Shields et al., 1996). Wang et al. (2011) found an important participation of this gene in the maintenance of undifferentiated state of SSCs in medaka (*Oryzias latipes*). Recent studies suggested that *klf4* is a tumor suppressor in certain cancer types, including colorectal cancer (El-Karim et al., 2013). Therefore, this factor is considered a regulator of pluripotency, acting not only in the reproductive organs but also in different tissues. In this study, the expression of *klf4* remain constant during the Jundiá developmental stages, as well as among the different Percoll bands. This fact and the low expression of *klf4* in the Jundiá testis indicates that *klf4* might be a somatic cell transcription factor rather than a marker of SSCs in this species.

GFRa1 is the GDNF receptor (glial cell-derived neurotrophic factor), and nowadays it is considered a SSC molecular marker in the rodent testis, being responsible for SSC maintenance (Meng et al., 2000). Recently, GFRa1 was identified as a molecular marker for SSCs in Nile tilapia, rainbow trout and dogfish (reviewed by Lacerda et al., 2014). In the present work, *gfra1* was not differentially expressed among the Percoll bands, which suggests that *gfra1* is not a SSC molecular marker in Jundiá.

The *nanog* and *pou5f3* mRNAs are expressed in SSCs of mammals and some fish, such as medaka, zebrafish, Nile tilapia and rainbow trout (Bellaiche et al., 2014b; Lacerda et al., 2013; Froschauer et al., 2013; Sánchez-Sánchez et al., 2011; Wang et al., 2011). Both transcripts (*nanog* and *pou5f3)* are important for maintenance and self-renewal of undifferentiated and pluripotent cells (Baumann et al., 2013; Lengner et al., 2007). In the medaka testis, *nanog* and *pou2* (aka oct4) mRNA are restricted to early spermatogonia (Wang et al., 2011; Froschauer et al., 2013). In our study, *nanog* was not differentially expressed among the Percoll bands. However, *pou5f3* showed higher expression in bands 3 and 4. Considering that band 3 is enriched by A_{und}, we performed *in situ* hybridization to localize the cellular expression sites of *pou5f3* mRNA in Jundiá testes. The results showed that *pou5f3* transcripts were mainly expressed in A_{und}, although expression could also be detected in A_{diff}, which explains the detection of *pou5f3* transcripts in band 4.

In conclusion, the present study characterized Jundiá spermatogenesis and its duration, as well as some important stem cell genes for the first time. We expect that the present data will open new possibilities for a better understanding of germ cell biology in this species, allowing an improvement and development of reproductive biotechnologies such as germ cell transplantation, SSC cryopreservation and *in vitro* spermatogenesis in this economically important species.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq); ERMM (FAPESP, 13/22778-3 and CNPq, 449289/2014-1), ILDDM (FAPESP, 13/22778-3) and RHN (FAPESP, 14/07620-7 and 12/00423-6). Technical help from Mara Lívia Santos is highly appreciated. The assistance from the local farmers, José Geraldo da Silva and Marco Túlio Diniz Peixoto, was of great value. The authors are grateful to MSc. Maira S. Rodrigues for the technical assistance in the in situ hybridization panel and we also thank MSc. Juliana M. B. Ricci for providing Jundiá embryo cDNA and histological sections. The scholarships awarded to M.A.S. and the financial support from the FAPEMIG and CNPq are fully appreciated.

Table 2
Comparative duration of spermatogenesis in various fish species kept at different temperatures.

Group	Species	Water temperature	Spermatogenic duration*	References
A	Rainbow trout (*Oncorhynchus mykiss*)	09 °C	6–12 weeks	Whitehead et al. (1978)
	Roach (*Rutilus rutilus*)	10 °C	30 days	Billard (1986)
B	Nile tilapia (*Orechromis niloticus*)	20 °C	spermatogenic arrest	Vilela et al. (2003)
	Zebrafish (*Danio rerio*)	20 °C	6 days	Nóbrega et al. (2010)
	Black molly (*Poecilia sphenops*)	21–25 °C	21 days	De Felice and Rasch (1969)
	Climbing perch (*Anabas testudineus*)	25 °C$^{\Delta}$	21 days	Sinha et al. (1983)
	Guppy (*Poecilia reticulata*)	25 °C	14 days	Billard (1969)
	Nile tilapia (*Orechromis niloticus*)	25 °C	14 days	Lacerda et al. (2006)
C	Zebrafish (*Danio rerio*)	27 °C	4 days	Nóbrega et al. (2010)
	Medaka (*Oryza latipes*)	27 °C	12 days	Kuwahara et al. (2003)
	Colisa fasciata (*Trichogaster fasciata*)	27 °C$^{\Delta}$	9, 7 days	Sinha et al. (1979)
	Nile tilapia (*Sarotherodon niloticus*)	27 °C	7 days	Silva and Godinho (1983)
	Nile tilapia (*Orechromis niloticus*)	30 °C	7 days	Vilela et al. (2003)
	Jundia Catfish (*Rhamdia quelen*)	**30 °C**	**7 days**	**Present study**
	Zebrafish (*Danio rerio*)	30 °C	2 days	Nóbrega et al. (2010)
D	Nile tilapia (*Orechromis niloticus*)	35 °C	5–6 days	Lacerda et al. (2006)
	Zebrafish (*Danio rerio*)	35 °C	spermatogenic arrest	Nóbrega et al. (2010)

* From spermatocytes until spermatozoa formation.
$^{\Delta}$ Assumed temperature.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.10.018.

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