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Germ cell proliferation and apoptosis during testicular regression in a seasonal breeding fish kept in captivity

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ABSTRACT

Cell proliferation and apoptosis regulate germ cells stock and sperm production, eliminate anomalous gametes, and are essential parameters to consider in fish farming. Herein, spermatogenic activity as well as germ cell proliferation and apoptosis were assessed in *Leporinus taeniatus,* a seasonal breeding species from the São Francisco River basin, Brazil. Testes of 24 adult fishes from a farming station were sampled between December and July and processed for light and transmission electron microscopy and immunohistochemistry for PCNA and TUNEL assay. The gonadosomatic index and seminiferous tubule diameters presented higher values during the breeding season (December/January and February/March), and then significantly reduced during the regression and resting stages (April/May and June/July). Phagocytosis of spermatozoa by Sertoli cells was evident during gonadal regression, but a significant number (up to 30%) remained at the tubular lumen during the resting stage. A higher PCNA/TUNEL ratio occurred in the breeding period, leading to an elevated proportion (%) of spermatogonia (G_A and G_B) in resting. Moreover, a higher TUNEL/PCNA ratio indicates the contribution of apoptosis to the reduction of germ cells during testicular regression. Together, these results indicate a shift in the balance between cell proliferation and apoptosis that contributes to the regulation of the spermatogenic cycle and germ cells pool of *L. taeniatus* kept in captivity.

1. Introduction

Leporinus is the most diverse genus among Anostomidae, including species economically important as a food resource and small species appreciated in the ornamental fish industry (Garavello and Britski, 2003). As popularly known, piaus are widely distributed in the major South American river basins and have omnivorous feeding habits predominantly, consuming sponges, detritus, insects, and vegetal resources such as seeds, leaves, and filamentous algae (Santos and Rosa, 1998). The piau *Leporinus taeniatus* Lütken, 1875 is a benthopelagic species important in the artisanal and sport fishing activities in the São Francisco River basin, Brazil (Sato et al., 2003). The species inhabits rivers, lakes and reservoirs, reproduces seasonally in the rainy season between December and February, presenting total spawning, non-adhesive eggs, rapid embryonic development, and absence of parental care (Rizzo et al., 2002; Thomé et al., 2005). Similar to other Neotropical species, this species does not reproduce spontaneously in captivity. Females prepare for reproduction until vitellogenesis but are dependent on hormonal induction to achieve final oocyte maturation, ovulation, and spawning (Santos et al., 2005). After induced spawning, postovulatory and atretic follicles regress progressively, with apoptosis having an essential role in the elimination of the follicular cells (Santos et al., 2005). Different to females, a full spermatogenic cycle occurs when males are kept in captivity; however, hormonal induction is needed to stimulate sperm production and spermiation.

Spermatogenesis is a highly organised process in which diploid spermatogonia proliferate and differentiate to give rise to haploid spermatozoa. Three main phases have been considered during spermatogenesis in vertebrates: the spermatogonial or mitotic phase with successive generations of spermatogonia, the spermatocytary or meiotic phase with primary and secondary spermatocytes, and the spermiogenic phase when haploid spermatids differentiate into spermatozoa (Schulz et al., 2010; Uribe et al., 2014). Although fish and mammals share a similar pattern of germ cell development, spermatogenesis has striking differences between these two groups, and the following features can be highlighted in teleost fish: the influence of temperature on

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sex differentiation and gonadal maturation, a shorter duration of spermatogenesis, and a high level of developmental plasticity and sexual bipotency of the spermatogonial stem cells (Schulz et. al., 2010; Lacerda et al., 2014; Melo et. al., 2016). Besides, the fish germinal epithelium has continuous or cyclic activity and germ cells cysts develop synchronously surrounded by Sertoli cells, which proliferate in adults to support sperm production in each reproductive cycle (Miura et al., 1999; Chaves-Pozo et al., 2005; Schulz et al., 2010).

During spermatogenesis, cell proliferation and apoptosis maintain the proportion of germ and somatic cells, regulate sperm production, and prevent the formation of anomalous gametes (Baum et al., 2005; Takle and Andersen, 2007; Zupa et al., 2013). In addition to tissue homeostasis, apoptosis is also involved in the testicular remodelling stimulated by hormones (Huettenbrenner et al., 2003; Takle and Andersen, 2007; Kaptaner and Kankaya, 2013). The balance between apoptosis and cell proliferation is essential to maintain germ cells stocks in the gonads (Kimura et al., 2003; Corriero et al., 2007). In seasonal breeding species, sperm production ceases after spawning due to lack of differentiation of mitotic germ cells, and then the testes considerably decrease in volume (Domingos et al., 2013; Kaptaner and Kankaya, 2013). Remaining germ cells may be spermatogonia and residual spermatozoa only, although all spermatogenic lineage cells can be found in some species, depending on endogenous and exogenous cues (Schulz et al., 2010).

Spermatogenesis has been frequently evaluated through morphometry undernatural and experimental conditions (Almeida et al., 2008; Domingos et al., 2012, and others); however, the contribution of cell proliferation and apoptosis has been investigated in few fish species. In this sense, the aim of the present study was to assess spermatogenic activity as well as proliferation and apoptosis of germ cells during and after the breeding season of *L. taeniatus* kept in captivity.

2. Materials and methods

2.1. Fish sampling

Adult specimens of *L. taeniatus* (17.0 ± 1.4 cm total body length and 45.2 ± 11.2 g body mass) were kept in 200 m^2 tanks with an average depth of 1 m, continuous water flow, and a stocking density of 0.5 kg fish/m^2 at the Três Marias Fisheries and Hydrobiology Station, CODEVASF (18°11'58" S, 45°15'07" W), during a reproductive cycle. The fish were fed daily with pelleted food (32% crude protein) in the proportion of 2% of body weight per day. For analysis of testicular activity, a total of 24 specimens was sampled from December to July. After euthanasia (immersion in eugenol 85 mg l^{-1}), total body length (TL), body weight (BW), and gonad weight (GW) were obtained from each specimen and the gonadosomatic index (GSI = 100 GW BW^{-1}) and Fulton condition factor (K = 100 BW TL^{-3}) were calculated. Fish collection was carried out following the ethical principles established by the Brazilian College of Animal Experimentation (COBEA), and the study was approved by the Ethics and Animal Use Committee (CEUA) of the Federal University of Minas Gerais, Brazil. The main physicochemical parameters of the water (temperature, dissolved oxygen concentration, pH, and electrical conductivity) were recorded in the morning during fish samplings using a Horiba portable water quality checker model U-10 (Table 1).

Table 1
Physicochemical water parameters during stocking of *Leporinus taeniatus* in a fishery and hydrobiology station, south eastern Brazil.

	Temperature (°C)	Oxygen (mgL^{-1})	pH	Conductivity (µS/cm)
December/ January	26.3 ± 0.3	5.3 ± 0.1	6.4 ± 0.2	51.2 ± 1.2
February/ March	26.6 ± 0.3	5.3 ± 0.2	6.3 ± 0.2	49.0 ± 0.9
April/May	24.2 ± 0.2	6.3 ± 0.2	6.1 ± 0.1	47.4 ± 1.1
June/July	21.9 ± 0.5	6.3 ± 0.2	6.3 ± 0.1	50.5 ± 1.5

Values are express as mean ± standard deviation.

2.2. Histology and electron microscopy

Testis samples from each specimen were fixed in Bouin's fluid for 8 h, dehydrated progressively in ethanol, followed by clearing in xylol, infiltration, and embedding in paraffin for histological analysis. Histological sections with a 5 µm thickness were obtained using a microtome (Leica) and then stained with haematoxylin-eosin and examined on a Carl Zeiss light microscope.

For transmission electron microscopy, testis samples were fixed in modified Karnovsky's solution (2.5% glutaraldehyde and 2% paraformaldehyde) in 0.1 M phosphate buffer pH 7.3 for 10 h at 4 °C and post-fixed in 1% osmium tetroxide, reduced with 1.5% potassium ferricyanide for 2 h at room temperature. Inclusion was performed using a mix of Epon and Araldite plastic resins. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined under a Carl Zeiss transmission electron microscope.

2.3. Immunohistochemistry

Analyses of cell proliferation (PCNA, mouse monoclonal antibody, PC-10 clone, 1:100, Santa Cruz Biotechnology) were performed using serial histological sections of the testes. For antigen retrieval, the sections were treated with citrate buffer pH 6.0 at 95 °C for 30 min. In order to block nonspecific reactions and endogenous peroxidase, the samples were submitted to 2% BSA followed by 3% hydrogen peroxide. Histological sections were incubated overnight with primary antibody at 4 °C. For the secondary development system, a Dako LSAB kit (biotin-conjugated goat secondary antibody and peroxidase-conjugated streptavidin) was used. The peroxidase reaction was revealed with diaminobenzidine (DAB) and the sections were counterstained with haematoxylin. As a negative control, one of the slides did not receive the primary antibody.

2.4. In situ TUNEL assay

For analysis of apoptosis, serial histological sections of testis samples prepared for histology and PCNA were used. The sections were submitted to the TUNEL in situ assay (QIA 33 TdT-Fragel DNA fragmentation kit, Calbiochem) following the manufacturer's protocol. Briefly, for tissue permeabilization and inactivation of endogenous peroxidase, the sections were treated with proteinase K (10 µg/ml) and 3% hydrogen peroxide, respectively. Histological sections were incubated with TdT (terminal enzyme deoxynucleotides transferase) and biotin conjugated deoxynucleotides in a humid chamber at 37 °C for 1 h and 30 min. Subsequently, peroxidase-conjugated streptavidin solution was applied in a humid chamber at room temperature for 45 min. The peroxidase reaction was revealed with DAB for 2 min at room temperature and the sections were counterstained with haematoxylin. For negative control, one of the slides did not receive the mixture containing the enzyme TdT and deoxynucleotides. Apoptotic cells had a TUNEL-positive reaction and at least two of the following characteristics: condensation of chromatin in clumps around the nuclear envelope, cytoplasmic retraction, or cell fragmentation such as an apoptotic body.

2.5. Morphometry

For the quantitative analyses of testicular activity, the specimens were grouped into four periods, following gonadal maturation: 1) December and January, 2) February and March, 3) April and May, and 4) June and July. Testicular activity was assessed considering the GSI,