ORIGINAL ARTICLE

Gonadal Development of the Piau Leporinus copelandii (Characiformes, Anostomidae) in a Tropical River in South-eastern Brazil

F. G. Araújo¹*, I. D. Gomes¹, A. A. Nascimento² and A. Sales²

Addresses of authors: ¹ Laboratório de Ecologia de Peixes, Instituto de Biologia, Universidade Federal Rural do Rio de Janeiro, BR 465, Km 7, Seropédica 23.890-000, Brazil; ² Instituto de Biologia, Área de Histologia e Embriologia, Universidade Federal Rural do Rio de Janeiro, BR 465, Km 7, Seropédica 23.890-000, Brazil

*Correspondence: Tel.: +55-21-37873983; fax: +55-21-26821763; e-mail: gerson@ufrrj.br

With 3 figures and 2 tables

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Summary

Histological analysis of the gonadal development of Leporinus copelandii Steindachner, 1875, a rheophilic Characiformes species in the Paraiba do Sul River, South-eastern Brazil, was described. We expect that this species adapt gonadal development to succeed in this river basin that has its longitudinal profile blocked by several impoundments. Fishes were examined by routine macroscopic and histological techniques. Stages of oocyte and spermatocyte development were described, and gonadal maturation was proposed. Mean oocyte diameter obtained from histological observations increased from the pre-spawning (4.2–175.5 μm) to spawning (148.5–262.0 μm) phases, followed by a sharp decrease in the post-spawning (27.0–56.7 μm) phase. Based on occurrence of different oocytes phases and oocyte size distribution, this species has group-synchronic development of oocytes. Further studies are necessary to clarify the spawning grounds for L. copelandii in the Paraiba do Sul River basin, especially considering that several impoundments obliterate the natural river course and this could limit spawning grounds.

Introduction

Studies on fish reproduction may be used to support fish management and conservation programmes designed to maintain or improve fish stocks (Marques et al., 2000). For projects of this kind, an accurate portrayal of gonad morphology is required to identify and describe the phases of gonadal development, which are the basis for assessing important information related to the reproductive strategy (Vazzoler, 1996). Spawning type can be determined by analyses of the oocyte development dynamics, and the patterns found by histological techniques have significantly increased precision and accuracy for understanding the reproductive processes (Rizzo and Bazzoli, 1993; Palmer et al., 1995; Sarre et al., 1997).

Leporinus copelandii Steindachner, 1875, is a migratory species widely spread in the Paraiba do Sul River basin (20°26′–23°38′S; 41°00′–46°30′W), SE Brazil (Araújo, 1996). This river is impounded in several stretches of the longitudinal profile for hydroelectric purposes besides being used as water supplier to Rio de Janeiro Municipality. Leporinus copelandii is a medium-sized species belonging to the Anostomidae family, which includes mainly rheophilic herbivorous species that live in rivers migrating to the upper reaches or tributaries to spawn (Lopes et al., 2000; Tavares and Godinho, 1994). Despite its importance in recreational and artisanal fisheries (Santos and Jegu, 1996), there are no available studies on gonadal stages development. The aim of this study was to describe histological oocyte and spermatocyte development stages to determine gonadal maturation and to obtain some insights on the reproductive tactics developed by this species in this lotic system that has several impoundments blocking the longitudinal profile, therefore impairing fish upriver migration during the reproductive season.
Materials and methods

Fish collection and laboratory procedures
A total of 36 specimens (11 females and 25 males) were examined for histological analysis. Fish specimens were captured bimonthly by gill nets from February to May 2008 in the middle reaches of the Paraiba do Sul River (22°30’-22°36’S; 44°43’-44°56’). Size for females ranged from 157 to 370 mm TL and for males ranged from 140 to 357 mm TL. Immediately after collection, fishes were anaesthetized in benzocaine hydrochloride (50 mg/l). All individuals were measured to total length (TL, nearest 1 mm) and weighted to total body mass (TM, nearest 0.01 g). A ventral incision was made to expose gonads for determination of the sex and macroscopic gonad development stage. Gonads were removed and weighed wet (GM, nearest 0.01 g). A portion of each gonad was fixed in Bouin’s solution for histological study during 8 h, being transferred to 70% ethanol for preservation. Afterwards, the gonads were dehydrated and embedded in paraffin wax. Cross-sections, 4–6 μm thick, were made in a rotary microtome (Leica RM 2135, Wetzlar, Germany), stained with haematoxylin eosin (H&E) and mounted on glass slides for light microscopy scrutiny. Microphotographies were taken with a Nikon Coolpix 4300 digital camera coupled to an Olympus (Tokyo, Japan) B941 microscope.

Data analysis
The gonad classification was adapted from Brown-Peterson et al. (2011) and was ultimately classified as immature (juveniles and inactive) or mature (developing, spawning capable, regressing and regenerating phases) to reduce the chance of error in correctly identifying individual stages. Accordingly, all vitellogenic oocytes are secondary growth oocytes, and cortical alveolar (CA) oocytes are secondary growth oocytes. Vitellogenesis is normally a long process during which important and visible changes occur within the oocyte: oocyte size increases noticeably, yolk progressively accumulates in the cytoplasm, and several cytoplasmatic inclusions appear (vacuoles, yolk globules, etc.). In this study, vitellogenic oocytes are separated into three stages (primary [Vtg1], secondary [Vtg2] and tertiary [Vtg3] vitellogenesis) based on the diameter of the oocyte, the amount of cytoplasm filled with yolk globules and appearance of the zona radiata. For each fish, the diameter of the oocytes and its nucleus encountered were measured to the nearest 0.0001 mm millimetre using an ocular micrometer; the mean diameter of each type of oocyte was then calculated. Measurements were taken only on oocytes sectioned through the nucleus. Cellular organization of the testis and spermatogenesis was adapted from Grier (1981).

Results

Stages of oocyte development
Oogonia, chromatin nucleolar and perinucleolar stages are present in the ovary throughout the entire annual cycle and are referred to as primary growth oocytes (PG). Other stages of the oocyte development are secondary growth oocytes and atresia and appear in mature ovary phases. A description of the different stages of oocyte development follows.

Primary growth (Fig. 1a) – Oogonia: Diameter averaging 4.2 ± 0.1 μm SE (n = 64). Very large nucleus (2.5 ± 0.01 μm, n = 64). Chromatin nucleolar: Similar to oogonia, although somewhat larger (mean diameter = 12.6 ± 0.4 μm, n = 84). Large nucleus (5.7 ± 0.1 μm, n = 84). Perinucleolar: In the early stage, size increases (mean diameter = 26.7 ± 0.7 μm, n = 64). Nucleus more conspicuous (13.6 ± 1.9 μm, n = 64). Late stage exhibits rapid growth (mean diameter = 39.0 ± 0.8 μm, n = 50) and nucleus averages to 18.2 ± 0.6 μm, n = 50.

Secondary growth (Fig. 1b). Cortical alveoli formation: Oocytes in different stages of development. Small vesicles and alveoli appear in the periphery of the cytoplasm. Mean diameter of oocyte 83.4 ± 2.8 μm, n = 70 and nucleus 28.9 ± 0.6 μm, n = 70. Zona radiata visible, although not yet stained by eosin. Primary vitellogenic – Vtg1 (Fig. 1c): In early stage, yolk granules small and numerous, also called yolk spheres or yolk globules containing yolk vesicles present, occupying the entire cytoplasm. Mean oocyte diameter 143.5 ± 2.2 μm (n = 51) and nucleus 26.5 ± 0.9 μm (n = 51). Secondary vitellogenic – Vtg2 (Fig. 1d,f): Cortical alveoli increase in size and gravitate towards the periphery as the yolk granules grow. Follicular layer and zona radiata are visible, with the latter being dyed with eosin. Mean oocyte diameter 219 ± 3.8 μm, n = 51 and nucleus 37.8 ± 1.4 μm, n = 51. Tertiary vitellogenic – Vtg3 (Fig. 1c–f): Nucleus decreases in size. Yolk globules inclusions dispersed in the cytoplasm. Mean oocyte diameter 254.4 ± 1.5 μm (n = 52) and nucleus 59.6 ± 2.2 μm, n = 52.

Atresia (Fig. 1f): The cells of the granular layer migrate to the interior of the ooplasm, absorbing the yolk; at the end of this stage, the zona radiata disappears.

The structure of the post-ovulatory follicle is recognizable by its disorganized structure, abundant vacuoles and a convoluted follicular wall surrounding an irregular cavity or lumen containing only a large cell (Fig. 1c,f).

Stages of spermatocytes development
The spermatogenic cells appear in the interior of the seminiferous tubules at different stages during spermatogenesis (spermatogonia, primary and secondary spermatocytes,
spermatids and spermatozoa), forming cysts. Each cyst is bound by a layer of connective tissue and contains cells at the same stage of development. In mature testes, the seminiferous tubules are filled with spermatozoa (Fig. 2a,b).

Oocyte size distribution

Histological classification of ovaries was based upon different types of 50–84 oocytes from 11 females. The pre-spawning phase was characterized by primary growth oocytes with diameters between 2.7 and 54.0 µm. The nuclear migration phase occurs during the final of oocyte maturation (FOM) before spawning, with diameters between 48.0 and 175.5 µm, and secondary growth oocytes (primary vitellogenic and secondary vitellogenic) occurs during spawning phase, with diameters between 148.5 and 243.0 µm. When mature phase occurred just before ovulation, oocytes outgrew the standing stock of secondary growth oocytes (tertiary vitellogenic) with diameters between 243.0 and 262.0 µm. A mode of smaller-size oocytes (60 µm) was present in post-spawning phase (Fig. 3).

Fig. 1. (a–f): Photomicrographs of ovarian histology illustrating oocytes at different stages of development of *L. copelandii*. (a) Primary growth oocyte. (b, c, d, e, f) Secondary growth oocyte. pg: primary growth, ca: cortical alveolar, ow: ovarian wall, vtg1: primary vitellogenic, vtg2: secondary vitellogenic, vtg3: tertiary vitellogenic, a: atresia pof: post-ovulatory follicle complex. Scale bars 40 (a, b, e) and 100 (c, d, f) µm.

Fig. 2. (a–b): Photomicrographs of testicular histology illustrating spermatocytes at different stages of development of *L. copelandii*. (a) Male with developing phase. (b) Male with spawning capable phase. Sg2: secondary spermatogonia, Sc1: primary spermatocyte, Sc2: secondary spermatocyte, St: spermatid, Sz: spermatozoa, Cy: spermatocyst, St: spermatid, Sz: spermatozoa, L: lumen of lobule. Scale bars 10 (a) and 15 (b) µm.
Phases of gonadal maturation

Ovaries and testes were assigned to one of five developmental phases of the reproductive cycle according to macroscopic and microscopic characterization of the gonads. Macroscopic variations were related to gonadal morphology and microscopic histology according to the composition of the oocytes and spermatogenic cells (Tables 1 and 2).

Discussion

This study is the first recorded information on the description of gametogenesis based on histological techniques for *Leporinus copelandii*. The seven oogenesis and the five spermatogenesis stages, here described, seem to be a reasonable approach to detect changes in oocyte development and their respective diameters (including nucleus diameter), and are a baseline to compare with other studies on *Leporinus* species. Although most authors agree on the basic characteristics incorporated into such a system, schemes used are often too brief, or incomplete, or too complicated (Coetzee, 1983). We believe that our findings contributed to clear the patterns of gonadal development of this species.

The different stages of cells of oogenic development found in ovaries (e.g. oogonia, perinucleolar, vitellogenic and post-ovulatory follicles) indicate various phases (pre-vitellogenic, vitellogenic and post-vitellogenic) of ovary development clustered. Moreover, the nuclear migration stage had oocytes diameter ranging from pre-vitellogenic (<30 μm) to early-vitellogenic (48.0–175.5 μm) phases. These findings suggest that the development of oocyte cohorts appears to be group-synchronic. Confirming this hypothesis, we found numerous pre-vitellogenic oocytes that remain in the ovaries after spawning together with those in primary growth (early-vitellogenic) forming the oocyte stock for the next spawning season.

The diameter of mature oocytes of *L. copelandii* in the Paraíba do Sul River (1–2 mm) is smaller than other species reported as total spawning, such as *L. friderici* (Barbi-eri and Santos, 1988), *Leporinus elongatus* Valenciennes, 1850 (Brito et al., 1999), *L. taeniatus* (Thomé et al., 2005) and *L. piau* (Sampaio and Sato, 2009) that have oocyte diameter ranging from 2.2 to 2.6 mm.

The habitats selected for spawning of *L. copelandii* are unknown. The distribution of critical habitats for the life cycle of rheophilic species, such as spawning sites and nursery areas, is essential because some significant impacts, such as the existence of reaches not connected to spawning grounds or to high-quality nursery grounds, could jeopardize fish populations (Godinho and Kynard, 2009). Most *Leporinus* species have a migratory upriver migration during the reproductive season spawning in lotic environments (Lopes et al., 2000; Thomé et al., 2005). However, spawning in reservoirs (Barbi-eri and Santos, 1988) or in lentic and semi-lotic environments in the floodplain areas for the Paraná River (Agostinho et al., 1997; Vazzoler et al., 1997) has been reported for *L. friderici*, a typically migratory species. Further studies are necessary to clarify the spawning grounds for *L. copelandii* in the Paraíba do Sul River basin, especially considering that several impoundments obliterate the natural river course and this could limit spawning grounds. Mapping spawning areas for fish species, especially those with some migratory characteristics, is important, because the number of dams is rising in Brazil (Suzuki et al., 2013), and there is an increased risk of migratory species being confined to short river stretches without spawning sites or rearing grounds like floodplain lagoons.
Table 1. Description of the phases in the reproductive cycle of female of *Leporinus copelandii* in the Paraíba do Sul River

<table>
<thead>
<tr>
<th>Phase</th>
<th>Macroscopic and histological features</th>
</tr>
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<tbody>
<tr>
<td>Immature (never spawned)</td>
<td>Small ovaries, often clear, measuring 40–50 mm and weighing between 0.01 and 0.1 g, blood vessels in distinct. Only oogonia and PG oocytes present. No atresia or muscle bundles. Thin ovarian wall and little space between oocytes.</td>
</tr>
<tr>
<td>Developing (ovaries beginning to develop, but not ready to spawn)</td>
<td>Enlarging ovaries, measuring 50–60 mm and weighing 4.0–5.0 g, blood vessels becoming more distinct. PG, CA, Vtg1, and Vtg2 oocytes present. No evidence of POFs or Vtg3 oocytes. Some atresia can be present. Early developing subphase: PG and CA oocytes only.</td>
</tr>
<tr>
<td>Spawning capable (fish are developmentally and physiologically able to spawn in this cycle)</td>
<td>Large ovaries, measuring 120–190 mm and weighing 10.0–80 g, blood vessels prominent. Individual oocytes visible macroscopically. Vtg3 oocytes present or POFs present in batch spawners. Atresia of vitellogenic oocytes may be present. Early stages of OM can be present. Actively spawning subphase: oocytes undergoing late GVM, GVBD or ovulation.</td>
</tr>
<tr>
<td>Regressing (cessation of spawning)</td>
<td>Flaccid ovaries, measuring 40–50 mm and weighing 1.2–3.5 g, blood vessels prominent. Atresia (any stage) and POFs present. Some CA and/or vitellogenic (Vtg1, Vtg2) oocytes present.</td>
</tr>
<tr>
<td>Regenerating (sexually mature, reproductively inactive)</td>
<td>Smaller ovaries, measuring 50–60 mm and weighing 4.4–5.6 g, blood vessels reduced but present. Only oogonia and PG oocytes present. Muscle bundles, enlarged blood vessels, thick ovarian wall and/or late-stage (gamma/delta) atresia or old, degenerating POFs may be present.</td>
</tr>
</tbody>
</table>

Oocyte stages code: PG. Primary growth oocyte; CA, cortical alveolar oocytes; Vtg1, primary vitellogenic oocyte; Vtg2, secondary vitellogenic oocytes; Vtg3, tertiary vitellogenic oocyte; PG, primary growth oocyte; MB, muscle bundle; A, atresia; OW, ovarian wall; POF, post-ovulatory follicle complex; OW, ovarian wall; A, atresia; OM, oocyte maturation; GVM, germinal vesicle migration; GVBD, germinal vesicle breakdown.

Table 2. Description of the phases in the reproductive cycle of male of *Leporinus copelandii* in the Paraíba do Sul River

<table>
<thead>
<tr>
<th>Phase</th>
<th>Macroscopic and histological features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature (never spawned)</td>
<td>Small testes, often clear and threadlike, measuring 45–55 mm and weighing between 0.01 and 0.1 g. Only Sg1 present; no lumen in lobules.</td>
</tr>
<tr>
<td>Developing (testes beginning to grow and develop)</td>
<td>Small testes but easily identified, measuring 60–70 m and weighing between 0.04 and 2.2 g. Spermatocysts evident along lobules. Sg2, Sc1, Sc2, St, and Sz can be present in spermatocysts. Sz not present in lumen of lobules or in sperm ducts. GE continuous throughout. Early developing subphase: Sg1, Sg2, and Sc1 only.</td>
</tr>
<tr>
<td>Spawning capable (fish are developmentally and physiologically able to spawn in this cycle)</td>
<td>Large and firm testes, measuring 90–100 mm and weighing between 1.0 and 14.0 g. Sz in lumen of lobules and/or sperm ducts. All stages of spermatogenesis (Sg2, Sc, St, Sz) can be present. Spermatocysts throughout tests, active spermatogenesis. GE can be continuous or discontinuous. Actively spawning subphase (macroscopic): milt released with gentle pressure on abdomen. Histological subphases based on structure of GE. Early GE: continuous GE in all lobules throughout testes. Mid-GE: continuous GE in spermatocysts at testsis periphery, discontinuous GE in lobules near ducts. Late-GE: discontinuous GE in all lobules throughout testes.</td>
</tr>
<tr>
<td>Regressing (cessation of spawning)</td>
<td>Small and flaccid testes, measuring 65–75 mm and weighing between 0.6 and 0.9 g no milt release with pressure. Residual Sz present in lumen of lobules and in sperm ducts. Widely scattered spermatocysts near periphery containing Sc2, St, Sz. Little to no active spermatogenesis. Spermatogonial proliferation and regeneration of GE common in periphery of testes.</td>
</tr>
<tr>
<td>Regenerating (sexually mature, reproductively inactive)</td>
<td>Small testes, often threadlike, measuring 70–80 mm and weighing between 1.4 and 1.5 g. No spermatocytes. Lumen of lobule often non-existent. Proliferation of spermatogonia throughout testes. GE continuous throughout. Small amount of residual Sz occasionally present in lumen of lobules and in sperm duct.</td>
</tr>
</tbody>
</table>

Spermatogenic stages code: Sg1, primary spermatogonia; Sg2, secondary spermatogonia; Scs1, primary spermatocyte; Sc2, secondary spermatocyte; St, spermatid; Sz, spermatozoa; GE, germinal epithelium.

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