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Original Article

Gonadal characterization of the Amazonian fish *Serrapinnus kriegi* (Characidae: Cheirodontinae)

Caracterização gonadal do peixe Amazônico *Serrapinnus kriegi* (Characidae: Cheirodontinae)

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Abstract

The knowledge of the testicular and ovarian morphology of a particular fish species is of paramount importance. Such analyze enables the development of studies and techniques aiming the improvement of their reproduction, management, commercialization and even their conservation. This study performed the ovarian and testicular characterization of the ornamental Amazon fish *Serrapinnus kriegi*. A total of three males and three females had their gonads analyzed by optical microscopy. Females present ovaries filled with oocytes in asynchronous development, indicating partial spawning in the species. Moreover, the micropyle and micropilar cell formation was observed in primary growing oocytes, representing a precocious oocyte development; and the zona radiata in the final vitellogenic oocytes is thicker than other related species, evidencing the development of a better protection to the embryos in function of the waters' turbulence that characterize it spawning sites in the Amazonian streams. The male specimens' present anastomosed tubular testes with unrestricted spermatogonia spread along the entire seminiferous tubules. The present data elucidate the dynamic of spermatogenesis and oogenesis of an ornamental Amazonian species, through the description of the male and female germ cells development.

Keywords: oogenesis, ornamental fish, reproductive biology, spermatogenesis, testicular type.

Resumo

O conhecimento da morfologia testicular e ovariana de uma determinada espécie de peixe é de suma importância, pois através destas análises é possível o desenvolvimento de estudos e técnicas visando o melhoramento de sua reprodução, manejo e comercialização e até mesmo auxiliar em sua conservação. Este estudo realizou a caracterização ovariana e testicular do peixe Amazônico ornamental *Serrapinnus kriegi*. Um total de três machos e três fêmeas tiveram suas gônadas analisadas através de microscopia óptica. As fêmeas apresentam ovários preenchidos por oócitos em desenvolvimento assincrônico, indicando desova parcelada da espécie. Além disso, observou-se a formação de micrópila e célula micropilar em oócitos em crescimento primário, representando o desenvolvimento precoce do oócito; a zona radiata nos oócitos vitelogênicos finais é mais espessa em comparação a outras espécies relacionadas, evidenciando o desenvolvimento de uma melhor proteção aos embriões, em função das águas turbulentas que caracterizam seu local de desova nos córregos amazônicos. Os machos apresentam testículos do tipo tubular anastomosado com espermatogônias irrestritas, espalhadas por todo o túbulo seminífero. Os dados apresentados elucidam a dinâmica da espermatogênese e oogênese de uma espécie de peixe ornamental amazônica, por meio da descrição das células germinativas masculinas e femininas.

Palavras-chave: oogênese, peixe ornamental, biologia reprodutiva, espermatogênese, tipo testicular.

1. Introduction

The knowledge of the gonadal morphology in a particular species allows the performance of studies aiming the best comprehension of its different levels of reproductive strategies (Vazzoler, 1996). The histological analyses enable the observation and determination of several gonadal characteristics, such the stage of germ

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cell development, gonadal morphology (such the testes type (Parenti and Grier, 2004)), besides the study of the reproductive cycle based on the description of the gonadal maturation phases throughout the year (Gonçalves et al., 2006; Quagio-Grassiotto et al., 2013), mainly, in species from seasonal environments, allowing the determination of parameters used in the management of fishing resources.

The number of native species whose reproductive biology was investigated by morphological characterization is still scarce and concentrated, mainly, in large-sized species, such the Tambaqui (*Colossoma macropomum*, Cuvier, 1816) (Vieira et al., 1999), the yellow peacock bass (*Cichla kelberi*, Kullander & Ferreira, 2006) (Siqueira-Silva et al., 2013), Jurupoca (*Hemisorubim platyrhynchos*, Valenciennes, 1840) (Andrade et al., 2014), Pirarucu (*Arapaima gigas*, Schinz, 1822) (Lopes and Queiroz, 2009), which are economically important animals, especially for food purpose.

Meanwhile, the market of small-sized fish is still uncertain in spite of its high lucrative potential, social inclusion, low cost of implementation among other good characteristics that enable the establishment of the aquarism as a commercial activity in Brazil. However, the enhancement of techniques aiming the improvement of small-sized fish production and the advance of the aquarist trade, still yield low profitability and a long-term financial return to the producers. In this sense, morphological studies about reproduction of small-sized fish habiting "igarapes" are scarce. Consequently, the main way of aquarism exploitation is still based on the extractivism of Amazonian species, such the fishing of the species *Symphysodon aequifasciatus* (Pellegrin, 1904) (Rossoni et al., 2014) and *Hypancystrus zebra* (Isbrücker & Nijssen, 1991), which in accordance to The Red Book of Brazilian Threatened Fauna (ICMBIO, 2018), are threatened in the categories Less Worrying (LC), which include rare taxa and taxa with restricted distribution, and critically endangered (CR), respectively.

In order to provide biological data, aiming to help the reproduction of ornamental fish in captivity and their conservation by avoidance of more animals' withdrawal from the environment, this study performed the morphological characterization of *Serrapinus kriegi* (Schindler, 1937) gonads (testes and ovary). Characterized by its small size, the presence of a black dot on its abdomen, its shoal behavior, besides to be easily handling, this species has been used in the ornamental Brazilian trade. However, to the best of our knowledge, there are no studies about its reproductive characteristics. Popularly known as "piabinha", *S. kriegi* species belongs to Characidae family, subfamily Cheirodontinae that includes 16 species, distributed in the main Cis-Andean basins of South America (Malabarba and Jerep, 2014; Eschmeyer et al., 2018; Jerep et al., 2018).

2. Material and Methods

2.1. Ethics, animals and breeding protocol

The specimens of *S. kriegi* (see Figure 1) used in this study were donated by the aquariums employ (Projeto Arapaima Importação e Exportação de Aquicultura LTDA-EPP- Belém/PA; CNPJ: 14.113.625/0001-74). In the laboratory, the animals were acclimated for two weeks in aquariums (23 x 21 cm, capacity of 13 liters of water) containing artificial pumps at room temperature (26°C in average). All of the experimental protocols and procedures described in this study were approved by the University Animal Care and Use Committee and adhere to the National Research Council's Guide for Care and Use of Laboratory Animals.

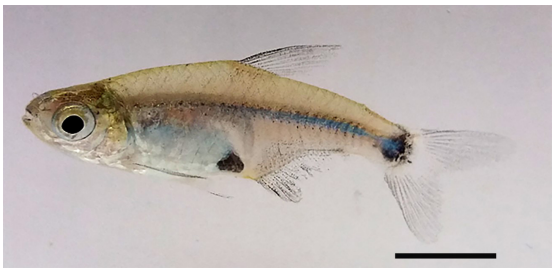

Figure 1. Specimen of *S. kriegi*. Scale bar: 1 cm.

2.2. Histological characterization

Six animals, three males and three females, were used for gonadal characterization. The analyses were performed during summer station. Initially, the animals were euthanized by excessive dose of Eugenol (Biodinâmica, Eugenol 20 ml/180 ml Ethanol 96%), followed by a ventro-longitudinal incision, performed to expose the gonads. The gonads were collected, fragmented and fixed in 2.5% Glutaraldehyde solution (Êxodo Científica®). The material was submitted to the procedure of inclusion in glycol methacrylate (Historesin, Technovich, 7100®) and sectioned at 3 µm in manual microtome (Leica, 2200®) equipped with glass blade. The slides were labeled with Hematoxylin and Eosin (H.E.) and examined on an optical microscope (Physis®). Photo documentation was performed using the camera TABLET YW5699-Android 5.1, and image analysis were conducted using the software EYE-1.6.8.

The thickness of the zona radiata was measured in fifty vitellogenic oocytes in 400x magnification, aided by the ImageJ program (bundled with 64-bit Java 1.8.0_112(70MB)). Data are presented as Mean and standard deviation.

3. Results

3.1. Ovarian morphology in S. kriegi

The ovaries of *S. kriegi* are elongated paired organs individualized through their entire extension into the coelomic cavity, posteriorly unifying in an oviduct before opening in the urogenital papilla (see Figure 2a). They are irrigated by blood vessels and are surrounded by connective tissue (albuginea tissue) that sends septa to the ovary inner, forming the lamellae. The lamellae bound the central region of the ovarian and are place of development

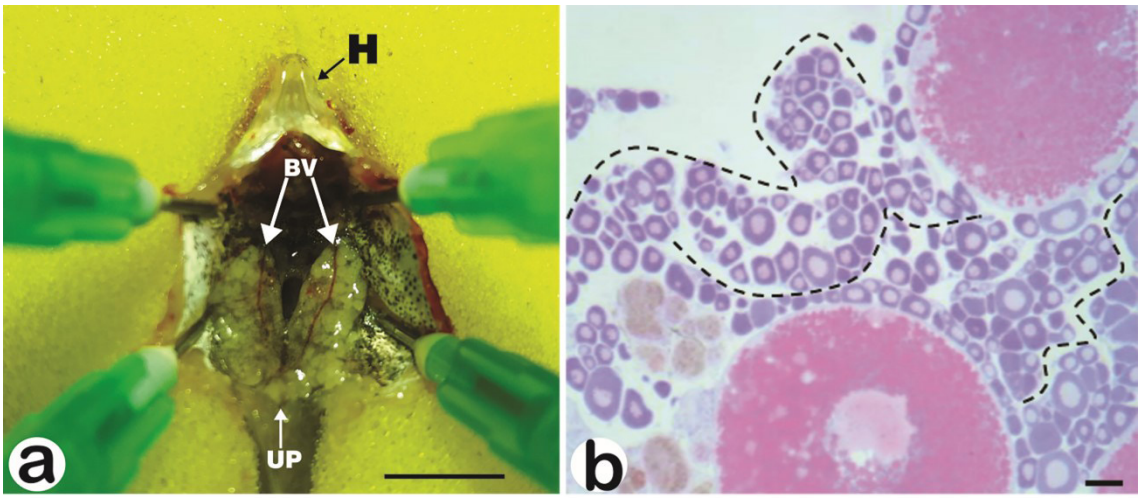

Figure 2. Ovarian structure in *S. kriegi*. a) Ovaries inside the coelomic cavity, highlighting the presence of the blood vessels (BV) and the posterior union of the ovaries to open in the urogenital papilla (UP). **b)** Dashed lines limiting the ovulligerous lamellae. **Scale bars**: a: 1 cm; b: 50 µm. **Label:** Hematoxylin/Eosin.

of the female germ cells lineage in different maturation stages (see Figure 2b).

3.2. Oogenesis in S. kriegi

3.2.1. Folliculogenesis

Two types of isolated oogonia are observed in the germinal epithelium of *S. kriegi*: The Type A undifferentiated oogonia (see Figure 3a-c) and the Type A differentiated oogonia (see Figure 3d). The Type A undifferentiated oogonia are isolated cells containing an elongated basophilic nucleus, with a few (see Figure 3a) or only one basophilic nucleolus (Figure 3b). Nuages are observed on their cytoplasm (see Figure 3a, b). These cells are associated to epithelial cells and are always bordering the ovarian lumen (see Figure 3a, b). Type A undifferentiated oogonia mitotically proliferate giving rise to nests of Type A-differentiated oogonia, which present a rounder and more basophilic nucleus (Figure 3c). Type A-differentiated oogonia are composed of a bigger and rounder nucleus, which is less basophilic and presents a single, and more prominent nucleolus than the Type A undifferentiated oogonia. The Type A differentiated oogonia are wrapped by one pre-follicle cell and their chromatin are more homogeneous and less condensed than in the Type A-undifferentiated oogonia. Nuages are still observed on their cytoplasm (see Figure 3d). Type A differentiated oogonia proliferate to give rise to Type B oogonia, which are enclosed in germline cysts formed by pre-follicular cells (see Figure 3e). The Type B oogonia present a round and more basophilic nucleus, besides one single nucleolus. Nuages are still observed in the cytoplasm of this cell type (see Figure 3e).

Type B oogonia enter in meiosis and give rise to a cyst of prophase oocytes. Those oocytes, which are in Leptotene stage, are enclosed by pre-follicle cells forming germline cell cysts (see Figure 3f). Type B oogonia present a round and basophilic nucleus with granulated chromatin. Inside the same nests can be found more than one germline cell cyst, as observed in the Figure 3f. They keep the division passing through the stages of Zygotene, Pachytene and Diplotene (see Figure 3g-j).

A diplotene oocyte becomes totally involved by pre-follicle cells that isolate them from the nest, giving rise to an ovarian follicle (see Figure 3k, l).

3.3. Primary growth

During the primary growth the oocytes develop important structures such the follicular, the theca and the zona radiata layers. The primary growth begins with a perinucleolar oocyte, which is characterized by the arrange of the nucleolus in the nucleus periphery and a strongly basophilic cytoplasm, contrasting the nucleus (see Figure 4a, b). Balbiani bodies are also observed in the cytoplasm of the oocytes in primary growth. Their presence evidence high activity of cytosol organelles (Figure 4c). At this moment the micropyle starts its development and can be visualized with the presence of a micropilar cell (see Figure 4c). As the oocytes develop, cortical alveoli are deposited and agglomerated in their cytoplasm, near to the oocyte's periphery (see Figure 4d).

3.4. Secondary growth

The secondary growth is highlighted by deposition of yolk granules in the developing oocyte cytoplasm. Those granules are deposited first in the central region of the cytoplasm, spreading to the periphery until complete filling the cytoplasm. In the beginning of yolk granules deposition, it is still possible to observe the cortical alveoli on the periphery (see Figure 4g). They became less visible as the oocytes get closer to the maturity stage, disappearing when it is Spawning Capable (see Figure 4f).

The secondary growth oocytes increased substantially in size since the primary growth step (see Figure 4h). The

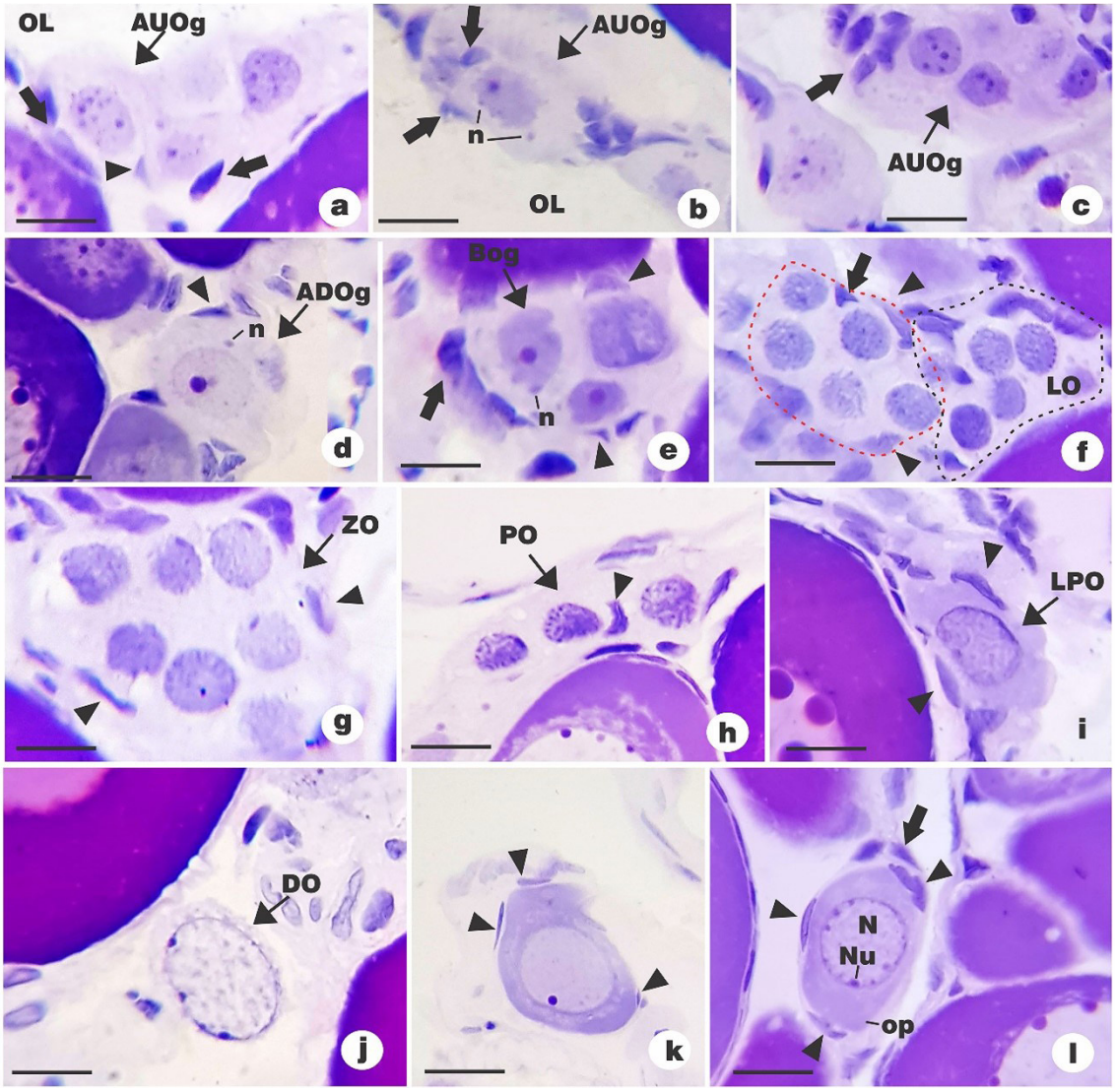

Figure 3. Folliculogenesis in *S. kriegi*. a-b) Type *A-Undifferentiated* oogonia (AUOg), n: nuages; OL: ovarian lumen; epithelial cell (arrow); Prefollicle cell (Arrowhead). **c)** Nest with Type *A-Undifferentiated* oogonia (AUOg). **d)** Type *A-Differentiated* oogonia (ADOg). **e)** Type B oogonia. **f)** Nest containing two cysts (dashed lines) of Leptotene oocytes (LO). **g)** Cyst of Zygote oocyte (ZO). **h)** Pachytene oocyte (PO). **i)** Late Pachytene oocyte (LPO). **j)** Diplotene oocyte (DO). **k)** The former prefollicle cells (Arrowhead) surround the new formed ovarian follicle. **l)** Transition of the Diplotene oocyte to primary growth. N: Nucleus; Nu: nucleolus; op: ooplasma. **Scale bars:** 10 µm. **Label:** Hematoxylin/Eosin.

zona radiata is thick (8.4 ± 3.4 µm) and presents several invaginations along its entire surface in the vitellogenic oocytes (see Figure 4e, f, h).

The secondary growth step is finished with the nucleus migration, now named germinative vesicle, in direction to the micropyle, indicating the oocyte is fully developed and ready to be ovulated (see Figure 4f, i).

3.5. Testicular morphology of S. kriegi

The testes of *S. kriegi* are elongated and individualized for all of their extension inside the celomatic cavity. They join caudally in a common spermatic duct before opening in a urogenital papilla (see Figure 5a). Microscopically they are of the anastomosed tubular type with spermatogonia spread along the entire seminiferous tubule (see Figure 5b).

3.6. Spermatogenesis in S. kriegi

The first identified germ cells in *S. kriegi* spermatogenesis are the Type A undifferentiated spermatogonia* (A*und**), which present an elongated basophilic nucleus containing one single voluminous nucleolus (see Figure 6a), and the Type A undifferentiated spermatogonia (A*und*), showing a voluminous round and heterochromatic nucleus, containing a single central nucleolus. Nuages are observed in the cytoplasm of those cells (see Figure 6b). Both, A*und** and A*und* spermatogonia are isolated in the seminiferous

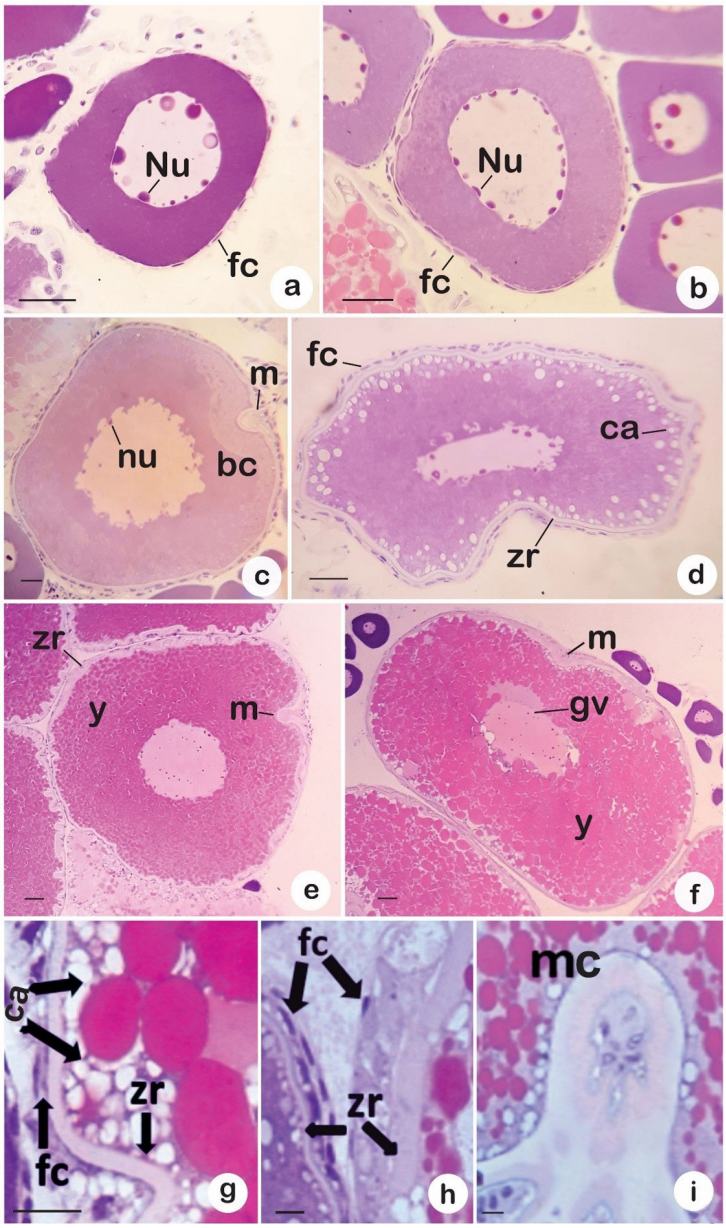

Figure 4. Oogenesis in *S. kriegi*. a-b) Oocytes in primary growth stage (perinucleolar), nu: nucleus in the oocytes' periphery. **c)** Perinucleolar oocyte with the presence of Balbiani body (b) and the initial formation of the micropyle (m). **d)** Oocytes in primary growth stage (Cortical alveolar), ca: cortical alveoli. **e)** Oocytes in secondary growth stage filled by yolk granules (y). **f)** Spawning capable oocyte with the germinal vesicle migration (gv). **g)**; Highlight of the presence of cortical alveoli (ca) in oocytes after the beginning of yolk deposition. **h)** Comparison of the zona radiata (zr) between an oocyte of primary growth and a vitellogenic one. **i)** Highlight of the micropyle and micropilar cell (mc). Abbreviations: **fc** = follicular cells **Scale bar**s: a, b, c, d, g: 50 µm; e, f, h: 100 µm; i: 20 µm. **Label:** Hematoxylin/Eosin.

epithelium. The A*und* spermatogonia differentiate in A differentiated spermatogonia (A*diff*), which are enclosed by Sertoli Cells inside cysts (see Figure 6c). By mitotic proliferation the A differentiated spermatogonia originate Type B spermatogonia. Those cells are smaller than the previous ones, presenting a more irregular and basophilic nucleus with several small nucleolus (see Figure 6d). The last mitotic division of type B spermatogonia originate primary spermatocytes (see Figure 6e-g).

The primary spermatocytes were identified in different stages of Prophase I of meiosis. Their identification is based on nucleus morphology and Leptotene/Zygotene (see Figure 6e), Pachytene (see Figure 6f) and Diplotene (see Figure 6g) spermatocytes were described. Spermatocytes in metaphase were also observed (see Figure 6h, Inset) and secondary spermatocytes, indicating the continuity of meiotic division (see Figure 6i), finally culminating in the origin of spermatids. Those cells pass by cellular

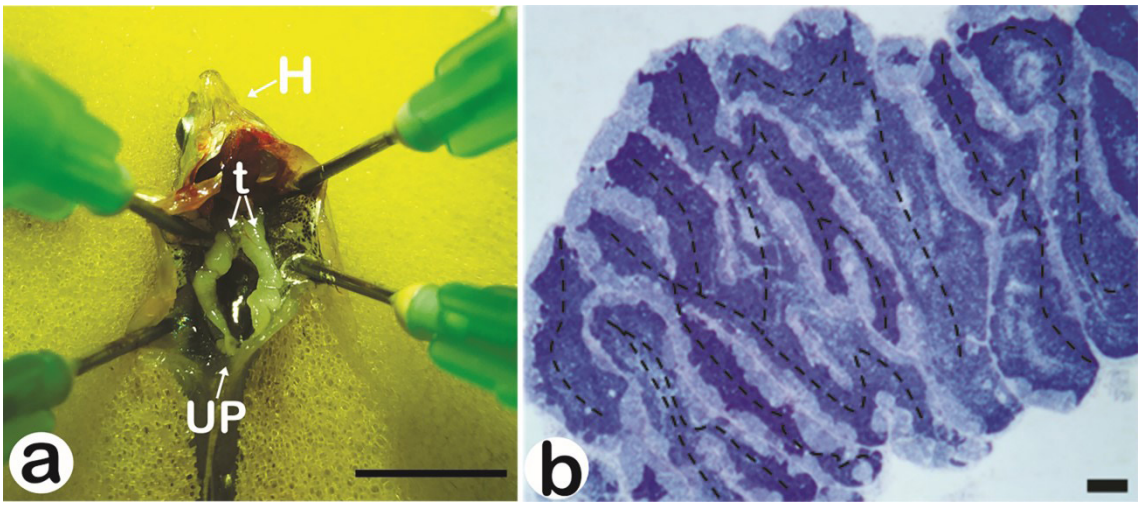

Figure 5. Testes structure in *S. kriegi.* a) Testis positioned in the coelomic cavity (t) joining posteriorly in a spermatic duct, before opening in the urogenital papilla (up). **b)** Dashed lines are highlighting the anastomosed testicular type. **Scales bar:** a: 1 cm; b: 25 µm. **Label:** Hematoxylin/Eosin.

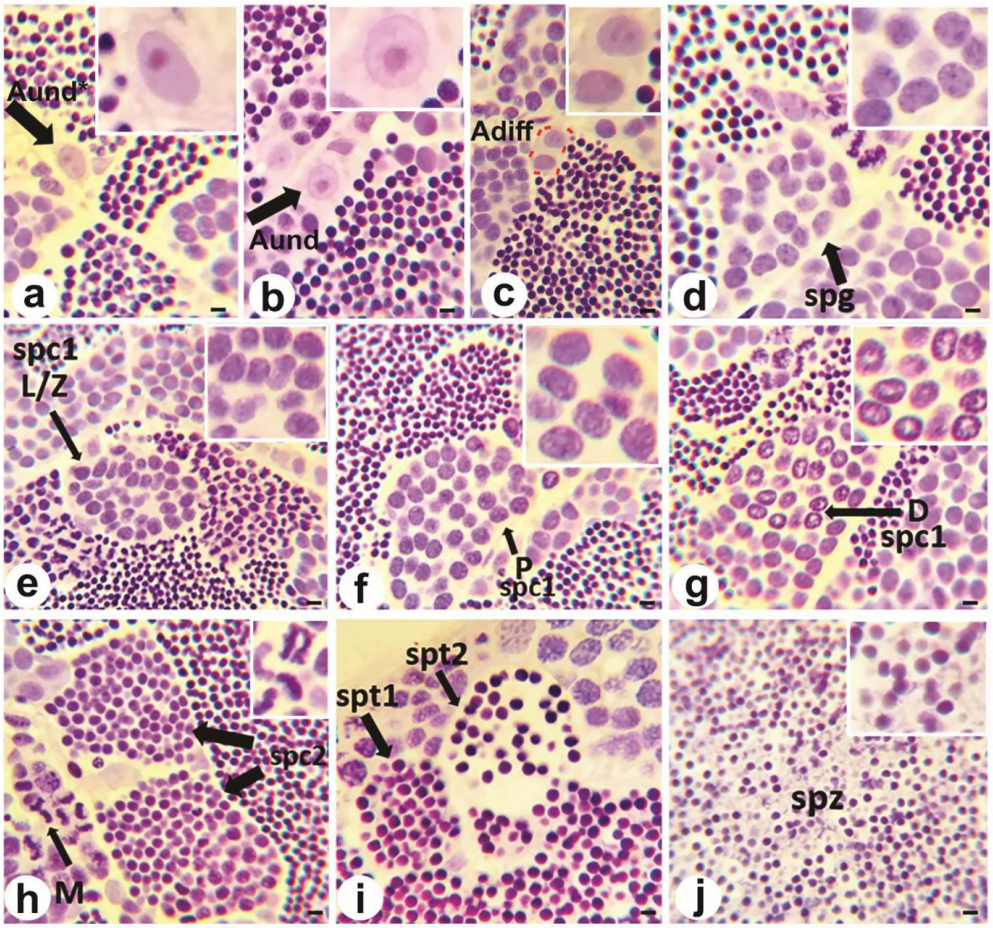

Figure 6. a) Spermatogenesis in *S. kriegi.* Type *A-undifferentiated* spermatogonia (Aund. **b)** Type *A-differentiated* spermatogonia (Adif. **c)** B spermatogonia cyst (Bspg). **d)** Cyst of primary spermatocytes in Leptotene/Zygotene stage (spc1 L/Z). **e)** Cyst of primary spermatocytes in Pachytene stage (spc1 P). **f)** Cyst of primary spermatocytes in Diplotene (Spc1 D). **g)** Cyst of secondary spermatocytes (spc2) and spermatocytes in metaphase stage (M). **h)** Cyst of initial (spt1) and final (spt2) spermatids. **i)** spermatozoa in the tubular lumen. **Insets highlight the respective cells from each image. Scale bars:** 20 µm. **Label: Hematoxylin/Eosin**

differentiation processes, being classified as initial spermatids, in which cells are very compacted in the cysts (see Figure 6i), and final spermatids, in which cells are very dispersed inside the cysts, due to the formation of cell flagellum. Their nuclei are also more basophilic in result of chromatin condensation (see Figure 6i). Those cells complete their differentiation inside the cysts until the fully formation of spermatozoa, which are then released from cysts into the tubular lumen, characterizing the spermatogenesis as cystic type (see Figure 6j).

4. Discussion

The morphological analyses of *S. kriegi* gonads allowed the confirmation of its testicular type as anastomosing tubular with unrestricted distribution of spermatogonia, which in accordance to Parenti and Grier (2004) and Uribe et al. (2015), are characteristically found in the most basal orders of Teleostei, as the Characiformes species *Astyanax altiparanae* (Garutti & Britski, 2000) (Siqueira-Silva et al., 2017), *Astyanax fasciatus* (Cuvier, 1819) (Carvalho et al., 2009), and *Astyanax scabripinnis* (Jenyns, 1842) (Veloso-Júnior et al., 2009) and the Cypriniformes *Devario aequipinnatus* (Mecclelland, 1839) (Chagas et al., 2016). Moreover, the species oocytes development is asynchronous, raising the hypothesis this species reproduces more than once a year, a very important characteristic for species with commercial value, since it enables them to produce numerous offspring batches in successive reproductive events throughout the year, such the other ornamental specie, the Amazonian Red discus, *Symphysodon discus* (Chellappa et al., 2005).

The appearance of micropyle and micropilar cell in oocytes of primary growth is not common in fish, since their development is linked to vitellogenic oocytes, such in the freshwater catfish *Pimelodus maculatus* (Lacepède, 1803) (Quagio-Grassiotto et al., 2011), which also belongs to a basal order (Siluriformes, see Parenti, Grier (2004) and Siqueira-Silva et al., (2019)), and the Characiformes *Hoplias malabaricus* (Bloch, 1794) (Quagio-Grassiotto et al., 2013), in which the micropyle only appears after yolk deposition in secondary growth oocytes. Micropilar cells were showed to remain in the post-ovulatory follicular complex after oocytes ovulation in *H. malabaricus* (Quagio-Grasiotto et al., 2013), and their presence means the oocytes are already mature and ready to be ovulated (Ricardo et al., 1996). Such precocious formation in *S. kriegi* oocytes may indicate an accelerated oocyte development during its reproductive cycle.

Besides the precocious formation of micropilar structure, the vitellogenic oocytes in *S. kriegi* present a thicker zona radiata in comparison to other small species from Characidae family, such the tetras *A. altiparanae* and *A. fasciatus*, whose zona radiata thickness were always smaller than 5 µm (Orsi, 2005). According to Riehl (1996), the zona radiata thickness is directly related to the place where species spawn, reflecting their adaptations to the ecological conditions. The animals living in seasonal environments, with constant environmental changes, or in fast waters, show vitellogenic oocytes presenting thicker zona radiata in order to increase their strength against the mechanical chock during spawn (Melo et al., 2011). That characteristic reinforces the adaptive capacity of *S. kriegi* to live in a stressful environment, due to continuous environmental changes, as the periods of flood and dry, which characterize the lotic streams from Amazonian region (Souza et al., 2011).

The morphological analyses of *S. kriegi* gonads allowed the comprehension of relevant aspects from its reproductive biology. Understanding the gametogenesis of a species is one of the first steps in order to start or even improve the success of its management in laboratory, thinking about its reproduction and, consequently conservation.

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