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Characterization of undifferentiated spermatogonia and the spermatogonial niche in the lambari fish *Astyanax altiparanae*

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ABSTRACT

Undifferentiated type A spermatogonia are the foundation of fish spermatogenesis. This cell population includes the spermatogonial stem cell population, which is able to either self-renew or differentiate into cells that will generate the male gamete — the spermatozoa. Spermatogonia stem cells are located in a specific region of the testes known as the spermatogonial niche, which regulates spermatogonial stem cell function. This study characterizes undifferentiated type A spermatogonia and their S-phase label-retaining cell properties in *Astyanax altiparanae* testes. This is a fish species of substantial commercial, environmental, and academic importance. Two types of undifferentiated spermatogonia have been described in *A. altiparanae* testis: A_{und^*} and A_{und}. Among the main differences observed, A_{und^*} spermatogonia have an irregular nuclear envelope, decondensed chromatin, one or two nucleoli, and nuages in the cytoplasm; meanwhile, type A_{und} have a round nucleus. A_{und^*} is preferentially distributed in areas neighboring the interstitial compartment, whereas A_{und} is located in the intertubular area. Finally, this study found that undifferentiated type A spermatogonia were able to retain BrdU over a long chase period, suggesting that these cells have a long cell cycle and potential stem cell candidates among them. Based on these findings, undifferentiated type A spermatogonia may be characterized as putative stem cells in *A. altiparanae* testis. This work will contribute to further studies on the stem cell biology of this promising Neotropical experimental model.

1. Introduction

Unlike in mammals, spermatogenesis in fish is cystic [1,2]. In this arrangement, cysts are formed when a single, undifferentiated spermatogonium (A_{und}) is completely surrounded by cytoplasmic extensions of Sertoli cells. When dividing, A_{und} spermatogonia create two single undifferentiated cells if self-renewal is favored, or two daughter cells connected to each other by a cytoplasmic bridge when the differentiation pathway is assumed. In the latter case, cells remain interconnected and undergo all phases of spermatogenesis synchronously: the proliferative, meiotic, and spermiogenic phases [1–3].

A_{und} cells are considered the largest cells of the germinal epithelium [3–5]. This cell population includes spermatogonial stem cells [5]. In zebrafish (*Danio rerio*), two A_{und} subtypes have been described in the testis and are referred to as A_{und^*} and A_{und} [5]. However, it is not yet known whether these cells are separated by mitosis or whether they represent different stages of the same cell cycle [3,5]. In the same study, Nóbrega et al. [5] showed "stemness" properties in this cell population (A_{und^*} and A_{und}) by transplanting these cells into a spermatogenesis-depleted testis and showing their ability to regenerate and recover recipient spermatogenesis.

In addition to morphology and transplantation studies, one approach for evaluating the presence of spermatogonial stem cell candidates in the testes is exploiting the stem cell property of retaining a S-phase label over a long period, which is known as the label-retaining cell approach [4]. In this method, spermatogonial stem cell candidates can be identified by their capacity to retain 5′-bromo-2′-deoxyuridine (BrdU) over a long period of time after a pulse-chase experiment. This is possible because stem cells are quiescent cells with a long cell cycle [4,5].

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Spermatogonia stem cells are located in a specific region of the testes known as the spermatogonial niche [6]. This niche is characterized as a highly dynamic molecular and cellular microenvironment that affects all aspects and functions of these cells, including maintenance, self-renewal, and differentiation [7]. The exact location and cellular nature of the spermatogonial niche, as well as the characterization of spermatogonial stem cells, have been the focus of recent studies [3,8,9]. From what we know so far based on mammalian testes [6,7] and the testes of the teleosts *Danio rerio* [5] and *Oreochromis niloticus* [10], spermatogonial stem cells are preferentially located in the seminiferous tubules that contact the interstitial compartment, and most frequently next to Leydig cells and blood vessels.

Among the Characidae species, *Astyanax altiparanae* is a species of great importance in several habitats: it contributes to the balance of the ecosystem and the food chain, and it is of substantial economic importance in Brazilian fish farms due to its capture volume [11]. Furthermore, this species has become a useful commercial and academic fish because of the ease associated with fish-seed production, its fast growth and maturation, and its sexual dimorphism, resistance, and simple handling [12]. This study sought to characterize undifferentiated type A spermatogonia and their stem cell capacities in *A. altiparanae* using the BrdU label-retaining cell approach. We have also identified the location of undifferentiated type A spermatogonia in the seminiferous tubules to characterize the spermatogonial niche in this species. The results of this study are important in that they provide basic knowledge for future studies on fish spermatogonial stem cell biology, such as transplantation of spermatogonial stem cells [5,13,14] and the isolation and differentiation of spermatogonia for the production of sperm in vitro [15], topics which are still undeveloped for native species.

2. Material and methods

2.1. Animal stocks and sampling

Astyanax altiparanae males were collected from the Hydrobiology and Aquaculture Station of the São Paulo Energy Company of Paraibuna (CESP-Paraibuna) located in Paraibuna, São Paulo, Brazil (23°24′51″ S, 45°35′59″ W). Specimens were kept in a cement tank with natural light and an average water temperature of 23 °C; they were fed twice a day with commercial food (Nutripiscis, 32% C.P.). These were sexually mature specimens in the regressed phase of the reproductive cycle; according to Costa et al. [16], this phase is characterized by the presentation of a large number of spermatogonia in the testis. The individuals were one year of age, with a standard length ranging from 6.5 and 9.0 cm (7.70 ± 0.67) and a total weight ranging from 7.20 to 18.84 g (11.58 ± 3.48).

The specimens were anesthetized with a sub-lethal dose of anesthetic (0.1% benzocaine solution in ethanol), in accordance with the procedure approved by the Ethics Committee on Animal Experiments of the Institute of Biomedical Sciences (ICB) of the University of São Paulo (USP), #77/2013. The spinal cord was sectioned, and the coelomic cavity was exposed for testis removal using different procedures (see below).

2.2. Histological and morphometrical analysis

The testes were fragmented and fixed in Karnovsky's fixative (2% glutaraldehyde and 4% paraformaldehyde in Sorensen buffer [0.1 M, pH 7.2]). The material was dehydrated in ethanol and embedded in glycol methacrylate resin (Historesin, Leica). Sections that were 3 μm thick were obtained and stained with periodic acid − Schiff's reactive + iron hematoxylin + metanil yellow [17]. The material was analyzed and documented using an Axioscop 2 light microscope (Zeiss Inc., USA) coupled with an AxioCam MRC5 camera (Zeiss Inc., USA) and equipped with a ZEN 2012 digital image capture device. The major length axis of the cell (CL) and the nucleus (NL) were measured in 50 spermatogonia, and the average values and standard deviations were calculated in micrometers.

The spatial distribution of the undifferentiated spermatogonia was assessed by evaluating their location in the germinal epithelium: close to the interstitial compartment or in the intertubular area, the latter being the area where two or more tubules contact each other without interstitial cells. To do so, 500 undifferentiated spermatogonia were counted per individual ($n = 5$), and their location in the germinal epithelium was expressed as a percentage of total cells as per Nóbrega et al. [5]. Comparisons between the different locations in the seminiferous tubules were made using Student's *t*-test. Statistical tests were performed in Systat 10, and the significance value was set at $P \leq 0.05$.

2.3. Ultrastructural analysis: transmission electron microscopy

For ultrastructural analysis, Karnovsky-fixed testes were washed in 0.1 M phosphate buffer, post-fixed in 1% osmium tetroxide in Sorensen buffer (0.1 M, pH 7.2), counterstained with 5% uranyl in water, dehydrated in acetone, and embedded in Epon resin. Semithin sections were obtained and used to select the area for the ultra-thin sections. Samples were sectioned with an ultramicrotome equipped with a diamond knife, and stained with saturated uranyl acetate in 50% ethanol and lead citrate. The sections were analyzed and documented using a Jeol 1010 transmission electron microscope (Jeol Inc., Japan) operating at 80 kV, equipped with a Gatan digital image capture device and with an Orius SC1000 camera attached (Gatan Inc., USA).

2.4. Label-retaining cell approach

For this experiment, another batch of animals ($n = 20$) was obtained from the CESP-Paraibuna. The specimens were acclimated in a 30-L aquarium with aeration and a water filtration system for 90 days. The water temperature was kept at 28 °C, and the animals were fed every two days. The filters were cleaned every three days. Pulses of BrdU (Sigma) at a concentration of 150 mg/kg were given to each fish for three consecutive days, with an interval of 12 h between each pulse. Five specimens were collected after four hours, one, two, and three weeks after the last BrdU pulse. Euthanasia and testis dissection were performed as described previously. Testes were fixed in methacarn, dehydrated in ethanol, diaphanized, embedded in paraplast, and sectioned to a thickness of 5 μm. For BrdU immunodetection, sections were deparaffinized in xylene, hydrated, and washed in running water and Tris-buffered saline with 0.05% Tween-20 (TBS-T pH 7.2). Antigens were retrieved using 1% periodic acid for 30 min at 60 °C, cooled in the same solution for 10 min, and then washed in TBS-T. To block endogenous peroxidase and nonspecific sites, sections were incubated with a hydrogen peroxidase block solution and a protein block solution (Kit Spring Reveal - Polyvalent Free Biotin-DAB [SPD-125]), respectively.

The slides were incubated for one hour in a humid chamber at room temperature using the primary anti-BrdU antibody (1:80; clone BU-33: B8434, Sigma). After incubation, slides were washed in TBS-T and incubated with a Spring Reveal kit (complement + conjugate), as per the manufacturer's instructions. The sections were washed again in TBS-T, revealed using 3-3′ diaminobenzidine (DAB), counterstained with Mayer's hematoxylin, differentiated with lithium carbonate, and washed with running tap water. Finally, the material was dehydrated, mounted, and analyzed using a light microscope (Axioskop 2, Zeiss Inc., USA). For

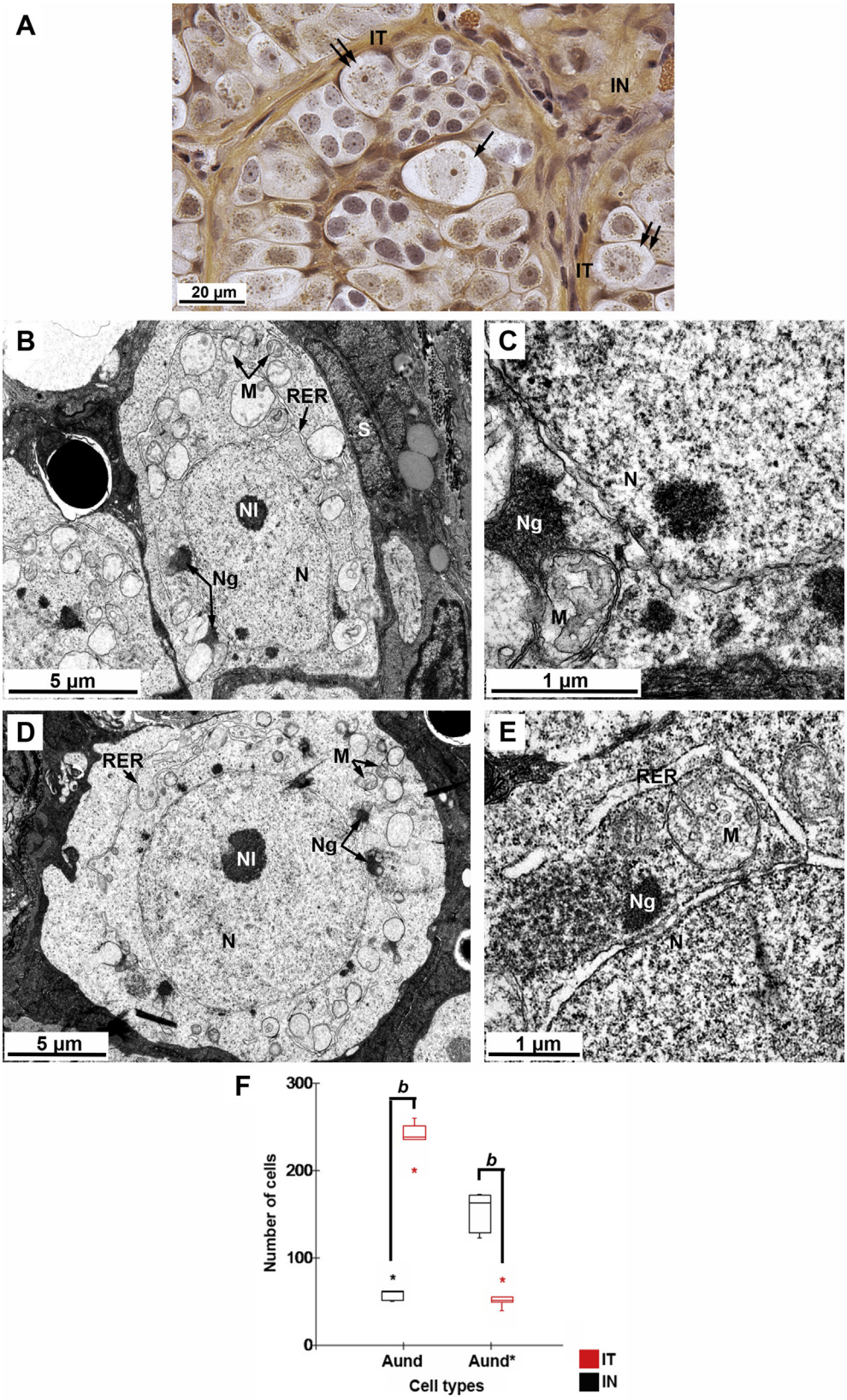

Fig. 1. Undifferentiated spermatogonia morphology and spermatogonial niche in *Astyanax altiparanae*. A) Two types of undifferentiated spermatogonia: A_{und^*} (arrow) and A_{und} (double arrow), arranged in the seminiferous tubule; B) Type A_{und^*} spermatogonia is completely surrounded by cytoplasmic extensions of Sertoli cells (S) and possesses cytoplasm with a well-developed rough endoplasmic reticulum (RER), nuages (Ng) near the nuclear envelope and/or associated with mitochondria (M), and an irregular outlined nucleus (N), loose chromatin, and one or two prominent nucleoli (Nl); C) Detail of nuages (Ng) of type A_{und^*}. Note that nuages are near the nuclear envelope (N) and/or associated with mitochondria (M); D) Type A_{und} spermatogonia have cytoplasm with well-developed rough endoplasmic reticulum (RER), nuages (Ng) near the nuclear envelope and/or associated with mitochondria (M), and a regular, round nucleus (N) with one or two prominent nucleoli (Nl); E) High magnification of A_{und} cytoplasm showing rough endoplasmic reticulum (RER) and nuages (Ng) near the nuclear envelope (N) and/or associated with mitochondria (M); F) Distribution of A_{und^*} and A_{und} in the *A. altiparanae* germinal epithelium: A_{und^*} spermatogonia are preferentially located in areas neighboring the interstitial compartment (IN) (73.7%; *b*: $P < 0.05$), while A_{und} spermatogonia are more frequent in the intertubular area (IT) (79.6%; *b*: $P < 0.05$).

the negative control, the primary antibody was omitted in some sections.

Because the type of undifferentiated spermatogonia (A_{und*} or A_{und}) could not be identified, we evaluated the total number BrdU-positive A_{und} spermatogonia without distinction between A_{und*} and A_{und}. Thus, the percentage of BrdU-positive undifferentiated spermatogonia was determined as the ratio between the number of labeled cells and the total number of A_{und} spermatogonia analyzed ($n = 100$; positive and negative to BrdU) at the different periods after the BrdU pulse. ANOVA analysis followed by Student's *t*-test were used to compare the periods evaluated. Statistical tests were performed in Systat 10, and the significance value was determined at $P \leq 0.05$.

3. Results

Two types of undifferentiated spermatogonia were identified in *A. altiparanae* testes: A_{und*} and A_{und} (Fig. 1A).

Type A_{und*} spermatogonia are the largest cells (CL = 19.707 ± 3.111 μm) in the germinal epithelium of *A. altiparanae* and are completely surrounded by cytoplasmic extensions of Sertoli cells (Fig. 1A–B). These spermatogonia have clear and abundant cytoplasm containing a well-developed rough endoplasmic reticulum (Fig. 1B), mitochondria, and nuages. The latter appear as highly electron-dense structures near the nuclear envelope and/or associated with mitochondria (Fig. 1B–C). This cell type was found to have an irregular nucleus with loose chromatin (NL = 10.745 ± 1.176 μm), which were most often eccentric, with one or two prominent nucleoli (Fig. 1A–B).

Type A_{und} are smaller than A_{und*} (CL = 16.975 ± 2.986 μm; $t = -4.480$, $P < 0.001$), and are also completely surrounded by cytoplasmic extensions of Sertoli cells (Fig. 1A and D). These cells can be found near other undifferentiated spermatogonia (Fig. 1A). These cells exhibit cytoplasm with well-developed rough endoplasmic reticulum, and nuages near the nuclear envelope and/or associated with mitochondria (Fig. 1D–E). The A_{und} nucleus is smaller than the A_{und*} nucleus (NL = 9.573 ± 1.202 μm; $t = -4.930$, $P < 0.001$). Furthermore, it has a regular outline and is more centered, with loose chromatin and one or two prominent nucleoli (Fig. 1A and D).

With regards to its distribution throughout the germinal epithelium, A_{und*} is preferentially located in areas near the interstitial compartment (73.7%; $t = 8.545$, $P = 0.001$; Fig. 1F), while A_{und} is concentrated in the intertubular area (79.6%; $t = -21.440$, $P < 0.001$; Fig. 1F).

Using BrdU immunodetection, we counted the number of BrdU-labeled A_{und} spermatogonia during different periods after the last BrdU pulse (Fig. 2) in an effort to evaluate the loss of BrdU among the progenitor cells. Interestingly, the percentage of BrdU-positive cells did not vary four hours (69%) or one week (71.6%) after the last pulse ($P = 0.211$; Fig. 2A). After two weeks (59.4%) and three weeks (54.2%), the percentage of label-retained cells decreased significantly when compared to the count at 4h (4h-2w: $t = 7.236$, $P = 0.002$; 4h-3w: $t = 4.357$, $P = 0.012$) and at one week (1w-2w: $t = 4.443$, $P = 0.011$; 1w-3w: $t = 6.206$, $P = 0.03$), but it did not vary significantly between these final weeks ($P = 0.300$; Fig. 2A).

Histologically, we found several BrdU-labeled spermatogenic cysts four hours after the last pulse, including advanced and early stages of germ cell development (Fig. 2B). Type A_{und} spermatogonia were also labeled with BrdU (see arrow and inset in Fig. 2B). As spermatogenesis progresses, BrdU was detected in A_{und} spermatogonia, spermatogenic cysts (1 week after the last-pulse), and also in spermatozoa (Fig. 2C). Over time, BrdU becomes progressively diluted and undetectable among the germ cells; only a few advanced cysts, spermatozoa, and A_{und} spermatogonia retained the label after two weeks (Fig. 2D). Three weeks after the last pulse, only A_{und} spermatogonia and some somatic cells were able to retain BrdU over a long period (Fig. 2E). Labeled spermatozoa were also found in the testicular lumen, but in a lower amount than at the previous count (Fig. 2E).

4. Discussion

Two types of A undifferentiated spermatogonia — A_{und*} and A_{und} — were characterized in *A. altiparanae* testes based on cytoplasmic and nuclear features, including their nuages, the presence of an irregular versus a regular nuclear membrane, nucleoli, and chromatin condensation. Similar criteria were used in studies on zebrafish [4,5] and tilapia [18], in which two types of undifferentiated spermatogonia with distinct morphologies were identified in the testis.

In zebrafish, undifferentiated type A spermatogonia is the largest germ cell in the testis (~677 μm^3; 8.6 μm in nuclear diameter) and is completely surrounded by cytoplasmic extensions of Sertoli cells [4]. Moreover, two types of A undifferentiated spermatogonia were described in zebrafish testis: A_{und*}, which was found to have slightly condensed chromatin and one or two prominent nucleoli with an irregular nuclear envelope and a higher nucleus/cytoplasm ratio when compared to other spermatogonia [4], and A_{und}, which was found to have a round nuclear envelope, condensed chromatin, and fewer nuages than A_{und*}. In general, nuages are characterized by their amorphous and electron-dense features, they are formed by several kinds of RNA and proteins [16,19,20] and are a general characteristic of undifferentiated spermatogonia. These structures may be required for these cells to remain undifferentiated [21]. All of these characteristics were also observed in both subtypes of undifferentiated type A spermatogonia from *A. altiparanae*.

Furthermore, we also determined the way in which A_{und*} and A_{und} were distributed in the seminiferous tubules of *A. altiparanae* and the consequent definition of the spermatogonial niche, which plays a crucial role in spermatogonial fate [7]. Moreover, the characterization of spermatogonial niche also serves as basis for understanding the spermatogenic process and the factors that regulate this process [8,9]. However, data on the spermatogonial niche and its molecular and cellular composition are incipient for both mammals and fish. In *A. altiparanae*, type A_{und*} is preferentially located near the interstitial compartment, while type A_{und} is located in the intertubular area. Similar studies in mammals [6,7,22] and also on zebrafish [5] and tilapia [18] have shown the preferential distribution of spermatogonial stem cells near the interstitium, and, in particular, close to Leydig cells, blood vessels, and endothelial cells. The distribution of A_{und*} near the interstitial compartment of *A. altiparanae* testes (the spermatogonial niche) suggests that signals from the interstitium, such as hormones (such as gonadotropins, testosterone, estradiol), growth factors, and oxygen, may regulate spermatogonial function, as demonstrated in zebrafish [5,9] and mammals [6].

However, there is no data in the literature to explain the preference on the part of A_{und*} and A_{und} for either the interstitium or the intertubular areas. In our work, we believe that hormones such as 11-ketotestosterone (11-KT) and gonadotropins (follicle-stimulating hormone, or FSH, and luteinizing hormone, or LH) may regulate the spermatogonial activity of *A. altiparanae* (self-renewal or differentiation toward meiosis) through Sertoli cells and Leydig cells, similar to the process described in zebrafish [9]. Considering the fact that 11-KT is produced by Leydig cells, and that FSH and LH are delivered to the testes through blood vessels, it is possible that A_{und*} may be located near the sources of the endocrine and paracrine signals. On the other hand, the distribution of A_{und} far from

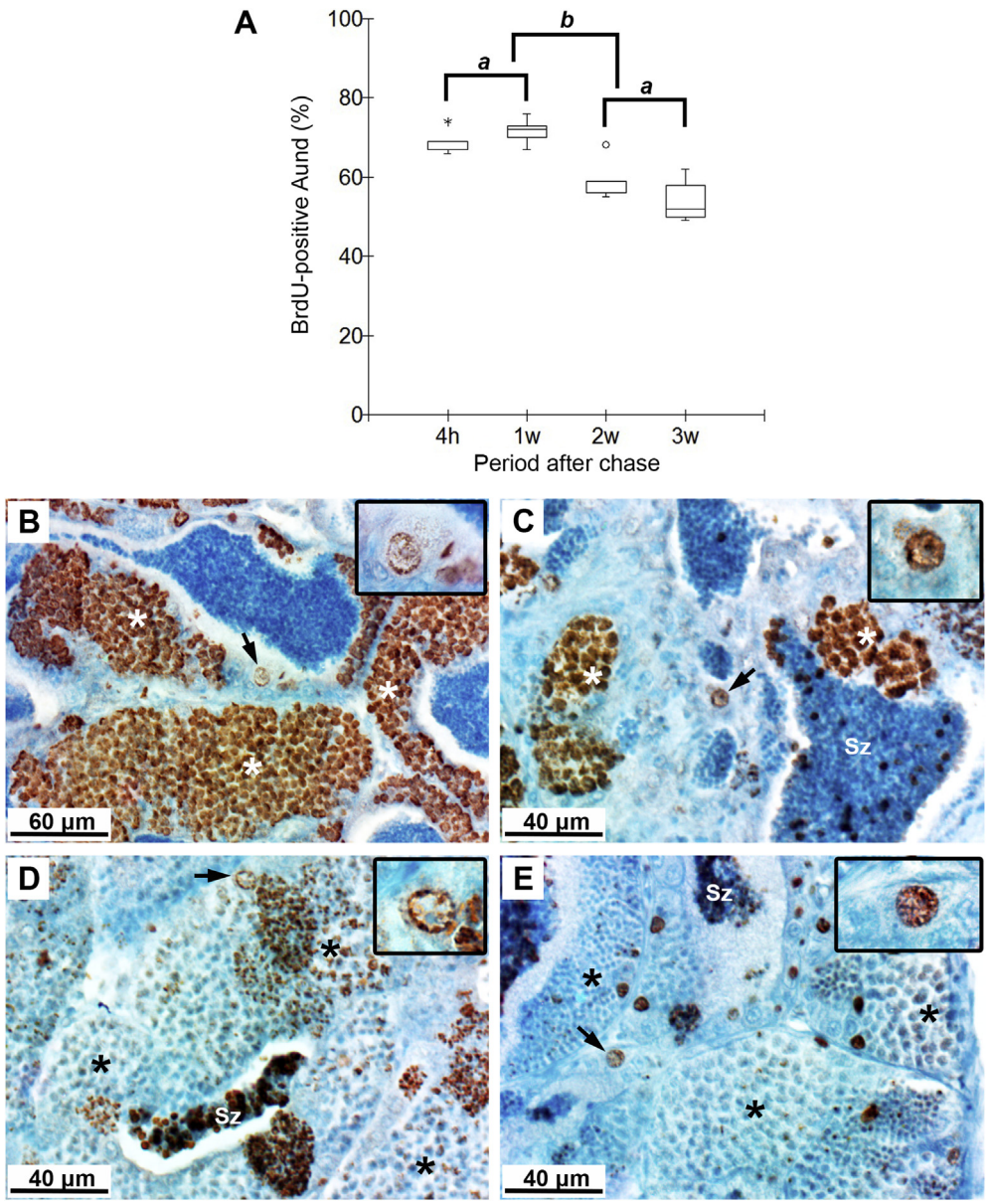

Fig. 2. Label Retaining Cell approach in *Astyanax altiparanae* testis. A) Percentage of BrdU-positive undifferentiated spermatogonia (A_{und}) four hours (4h), one week (1w), two weeks (2w), and three weeks (3w) after the last BrdU pulse. No significant differences were observed between 4h and 1w, or between 2w and 3w (*a*: $P > 0.05$). However, significant differences were found between 4h and 2w and between 4h and 3w, as well as between 1w and 2w and between 1w and 3w (*b*: $P < 0.05$); B) Specimens collected four hours after the last pulse: BrdU-positive A_{und} (arrow; inset) and spermatogenic cysts (*); C) Specimens collected one week after the last pulse: BrdU-positive A_{und} (arrow; inset), spermatogenic cysts (*), and spermatozoa (Sz); D) Specimens collected two weeks after the last pulse: undifferentiated spermatogonia (arrow; inset) and spermatozoa (Sz) were strongly labeled, and there was less BrdU retention among the spermatogenic cysts (*); E) Specimens collected three weeks after the last pulse: only undifferentiated spermatogonia (arrow; inset) were able to strongly retain BrdU over a long period. Note the less intense dilution of BrdU in the spermatogenic cysts (*). Spermatozoa (Sz) are also indicated.

the interstitial area must be better understood, since these cells may also be under regulatory mechanisms.

This study used BrdU as an S-phase label. BrdU is synthetic compound commonly used as a cell proliferation marker. It is an analog to thymidine, which is incorporated into the S phase of the cell cycle [5]. In the label-retaining cell approach, BrdU is incorporated by dividing cells and diluted among the derived descendent germ cells as spermatogenesis progresses. However, if a given cell has a long cell cycle or is quiescent, as is stem cell, then S-phase (in our case, BrdU) is retained and can be detected over a long period. These cells are referred to as labeling-retaining cells, or LRCs [3–5,23]. In *A. altiparanae*, we found that undifferentiated type A spermatogonia and somatic cells were the only cells able to retain BrdU 21 days after the last pulse. Similar results were found in a study on zebrafish [5].

This same zebrafish study evaluated BrdU loss between type A_{und*} and A_{und} [5]. A_{und} spermatogonia divided more quickly and were therefore referred to as "active" cells in which BrdU was rapidly lost among the daughter cells, while type A_{und*} spermatogonia exhibited a slower cell cycle, retaining BrdU over a long period and were therefore referred to as "reserve" cells [5]. However, in *A. altiparanae*, we were not able to differentiate type A_{und*} from A_{und} in the label-retaining cell approach. For this reason, we considered all spermatogonia to be undifferentiated type A spermatogonia. We found that the number of BrdU-positive undifferentiated spermatogonia remained constant after four hours and

after one week, and then decreased after two weeks, remaining constant until the end of the experiment (three weeks after the last pulse). Therefore, we cannot make any inferences regarding the cell cycles of A_{und*} or A_{und}, but these cells were characterized as LRCs in the testes and are considered potential spermatogonial stem cell candidates in *A. altiparanae*. Interestingly, the BrdU label was stronger in *A. altiparanae* three weeks after the last pulse when compared to the results from zebrafish testes [5]. This difference may suggest a longer cell cycle for undifferentiated type A spermatogonia in *A. altiparanae*. A long chase time would be required to clarify this aspect of spermatogonial cell cycle.

5. Conclusions

This article has characterized undifferentiated type A spermatogonia in *A. altiparanae* testes and has found two types: A_{und*}, which has an irregular nuclear envelope, loose chromatin, one or two nucleoli, and nuages in the cytoplasm; and A_{und}, which has a regular, round nuclear envelope, one or two nucleoli, and nuages in the cytoplasm. This study has also demonstrated that A_{und*} is preferentially distributed in the areas neighboring the interstitium, whereas A_{und} is located in the intertubular areas. Finally, undifferentiated type A spermatogonia were found to be the LRCs in *A. altiparanae* testes and are considered potential spermatogonial stem cell candidates. More studies are needed to show the stemness of these cells and may include tests such as transplantation assays and in vitro differentiation. This study is the first to describe the morphological characteristics of A_{und*} and A_{und} spermatogonia and the morphological characterization of the spermatogonial niche. These results provide a foundation for further studies on the biology of spermatogonial stem cells and their niche, as well as for biotechnological studies using this promising model species.

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