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The efficiency of spermatogenesis and the support capacity of Sertoli cells in Characiformes

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ABSTRACT

This stereological analysis of the types of germ cells and the number of Sertoli cells per cyst in *Astyanax altiparanae* testes during spermatogenesis is the first such report in Characiformes. Testes of 25 male *A. altiparanae* were examined. Based on the number of spermatogonia B per cyst (469.2 ± 9.92), we estimated that spermatogonia undergo at least nine mitotic divisions before differentiating into primary spermatocytes. There are four spermatogonia types: undifferentiated spermatogonia A*, undifferentiated spermatogonia, differentiated spermatogonia, and type B spermatogonia. The number of Sertoli cells increased gradually from 1.41 ± 0.51 in the single undifferentiated spermatogonium A* to 9.25 ± 0.50 in cysts of spermatocytes in the leptotene/zygotene stage, possibly related to greater complexity of cellular events during the meiotic stage. The number of germ cells rose dramatically from spermatogonia A (1.0 ± 0) to spermatogonia B (469.2 ± 9.92); however, the quantity of spermatocytes inside the cysts in the leptotene/zygotene stage decreased (300.6 ± 6.97) relative to spermatogonia B, representing a loss of approximately 36% of the former number of cells. This was probably the result of apoptosis, which promotes successful development of the remaining cells during sperm production. The support capacity of Sertoli cells increased gradually during spermatogenesis.

1. Introduction

The yellowtail tetra *Astyanax altiparanae* (Characidae), is a good biological model due to its tolerance of handling, small size, sexual maturity at four months, and intertidal spawning, especially for research associated with biotechnology and reproductive biology, making it valuable in Neotropical aquaculture [1–5].

Much available data regarding the spermatogenesis of *A. altiparanae* are concerned with morphological aspects [3,6], but interest in morphometric and stereological analysis is increasing [7–10]. Such tools allow elucidation of cellular mechanisms involved in mitotic and meiotic events and the differentiation of germ cells to form hundreds of spermatozoa from a single spermatogonium. Stereology, the three-dimensional interpretation of two-dimensional cross-sections, can provide knowledge of the interaction between germline cells and Sertoli cells during spermatogenesis.

This study aimed to characterize the dynamics of germ cell and Sertoli cell interaction with respect to cyst type and to determine the number of spermatogonial generations, the magnitude of germ cell loss, and the support capacity of Sertoli cells during spermatogenesis of *A. altiparanae*.

2. Materials and methods

2.1. Ethics

Experimental procedures were conducted in accordance with

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the guidelines for care and use of animals in the laboratory of the University Estadual Paulista (UNESP-Ilha Solteira). The Committee of Ethics and Research of UNESP approved the protocol 006/2012/CONCEA. All surgical procedures were performed after euthanasia of animals with 0.1% (w/v) benzocaine in absolute ethanol.

2.2. Animals and sampling

Twenty-five sexually mature male *A. altiparanae* reared in in-ground tanks at the Hydrobiology Station Engenheiro Souza Dias (CESP) were used. Testes were removed, cut into transverse and longitudinal sections, and fixed in 4% paraformaldehyde and 2% glutaraldehyde in Sorensen's phosphate buffer (0.1 M, pH 7.4) for at least 24 h. Samples were dehydrated in increasing concentrations of ethanol and embedded in glycol methacrylate resin (Technovit 7100/historesin; Heraeus Kulzer, Wehrheim, Germany).

The samples were sectioned at 3.0 μm and 2.0 μm on a glass blade equipped microtome LEICA RM 2145 (Leica Instruments GmbH, Nussloch Heidelberg, Germany) and stained with hematoxylin and eosin. Photography and histological analyses were completed with a Zeiss optical microscope equipped with an AxioCam-MRC5 camera (Carl Zeiss Microimaging GmbH, Göttingen, Germany).

2.3. Stereological analysis

After identification and morphological description of the germ cells present in *A. altiparanae* spermatogenesis [6], the diameter of the cell nuclei was measured using Motic Images Plus 2.0 software. Nuclei in one-hundred spermatogonia and one-hundred spermatozoa and in 4.500 of each type of spermatocyte and spermatid were measured.

The number of these cells, as well as of Sertoli cells, arising from a single type A undifferentiated spermatogonium (Aind*) in cysts of final spermatids were counted (Table 1 and Fig. 1).

Samples were cut in approximately 24 serial sections of 3.0 μm for spermatogonia and spermatocytes and 2.0 μm for spermatids. The thickness of the sections was according to preliminary morphometry based on the diameter of the nucleus of each cell type (Table 1). As the largest spermatocytes (pachytene) had mean nuclear size of 5.33 ± 0.43 μm, 3.0 μm sections ensure to cut only once in the central region of the nucleus of these cells. To assure counting each cell only once, nuclei were counted only when the central region of the nucleus was visible on the image. The same procedure was adopted for spermatids (Fig. 2). Five cysts for each of the mentioned cell types were counted per fish [11].

The selected cysts were those in which a clear delimitation of Sertoli cells was visible (Fig. 1). The analyses were conducted with an optical microscope (Axio Scope.A1; ZEISS) using Motic Images Plus 2.0 software.

Statistical analyses were conducted with one-way ANOVA followed by Tukey's test ($P < 0.05$).

3. Results

The types of germ cells in the testicular germinal epithelium of *A. altiparanae* were morphologically defined [6]. Type A undifferentiated spermatogonia, with nuclear diameter of ~8 μm, and the final spermatids at 1 μm were, respectively, the largest and smallest germ cells of the germ cell lineages (Table 1). Nucleus differences supported the identification and characterization of those cells for further stereological analysis of spermatogenesis.

Through successive mitotic division, a single spermatogonium A gave rise to cysts of spermatogonia B containing approximately 469 ± 9.92 cells that underwent at least nine divisions before their differentiation in the spermatocyte leptotene/zygotene stage. The cysts of spermatocytes at the leptotene/zygotene stage contained a smaller number of cells (300.6 ± 6.97) than did spermatogonia B (469.2 ± 9.92), representing approximately 36% fewer cells than expected (Table 1 and Fig. 1). After two meiotic divisions, spermatocytes produced spermatids, which can be separated into three types in *A. altiparanae* (initial, middle, and final). The cysts of final spermatids contained, on average, 888 cells (Table 1).

The numbers of germ cells per cyst indicated a meiotic index of ~3.0. This parameter is calculated as the ratio of the number of mature spermatids to the number of spermatocytes in the leptotene/zygotene stage. The spermatogenesis efficiency, expressed as a percentage calculated by the number of mature spermatids observed divided by the possible number, was approximately 43%.

The number of Sertoli cells on the germ cysts increased gradually from a single cell closely linked with an Aind* (1.41 ± 0.51) to approximately ten cells (9.25 ± 0.50) involving spermatocytes in the leptotene/zygotene stage. At this stage, the number of Sertoli cells per cyst peaked, at approximately seven Sertoli cells per spermatid cyst. This was used to calculate the support capacity of the Sertoli cells, defined as the ratio of the number of germ cells to the number of Sertoli cells per cyst (Table 1 and Fig. 1).

4. Discussion

Recent studies of spermatogenesis in several fish species have considered only the morphologic aspects of the process [6,12–14]. However, for a deeper understanding of the mechanisms that regulate spermatogenesis, stereological analysis is an effective means of investigating parameters such as numbers of mitotic divisions, making it possible to estimate the quantity of spermatozoa

Table 1
Stereology of germ cells in *Astyanax altiparanae*.

Types of germ cells	Germ cells per cyst (n)	Sertoli cells per cyst(n)	Nucleus diameter (μm)	Support capability of Sertoli cells
SPG Aind.*	1.0 ± 0^{a}	1.41 ± 0.51^{a}	7.67 ± 0.63^{a}	0.709^{a}
SPG Aind.	$1.0 \pm .\ 0^{a}$	1.44 ± 0.49^{a}	6.12 ± 1.16^{b}	0.694^{a}
SPG Adif.	2.4 ± 0.75^{a}	2.13 ± 0.40^{a}	4.55 ± 2.16^{c}	0.939^{ab}
SPG B	$469.2 \pm 9{,}92^{b}$ [512]	7.31 ± 0.49^{bc}	4.83 ± 0.54^{c}	64.186^{d}
SPC L/Z	300.6 ± 6.97^{c} [512]	9.25 ± 0.50^{d}	5.16 ± 0.84^{c}	32.497^{bc}
SPC P	$298.4 \pm 6{,}99^{c}$	9.71 ± 0.47^{d}	5.33 ± 0.43^{d}	30.731^{cd}
E1	910.8 ± 20.47^{b}[2048]	7.22 ± 0.55^{c}	2.54 ± 0.42^{e}	126.149^{e}
E2	898.0 ± 12.78^{b}[2048]	6.41 ± 0.50^{b}	1.38 ± 0.53^{f}	140.094^{f}
E3	888.8 ± 18.98^{b}[2048]	6.33 ± 0.49^{b}	1.31 ± 0.19^{g}	140.411^{f}
SZ			1.02 ± 0.16^{g}	

SPG Aind* - Spermatogonia A undifferentiated*; SPG Aind - Spermatogonia A undifferentiated; SPG Adif - differentiated spermatogonia A; SPG B – spermatogonia B; SPC L/Z – spermatocytes leptotene/zygotene; SPC P – spermatocytes in pachytene; E1 – early spermatid; E2-mid spermatid; E3-mature spermatid; SZ – spermatozoon.
[a-g] different letters indicate significantly different mean values ($P < 0.05$).

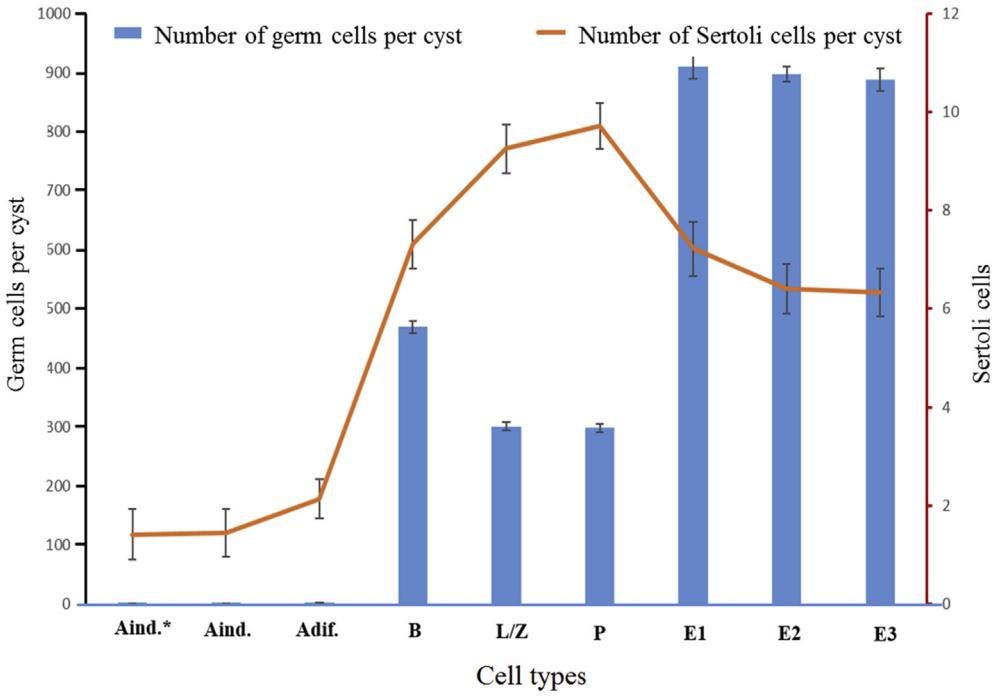

Fig. 1. Graphic comparing the number of germ cells and Sertoli cells per cyst. Aind* – undifferentiated spermatogonia A*; Aind –undifferentiated spermatogonia A; Adif – differentiated spermatogonia A; B – spermatogonia B; L/Z – spermatocytes leptotene/zygotene; P – spermatocytes in pachytene; E1–early spermatid; E2–mid-spermatid; E3 - mature spermatid.

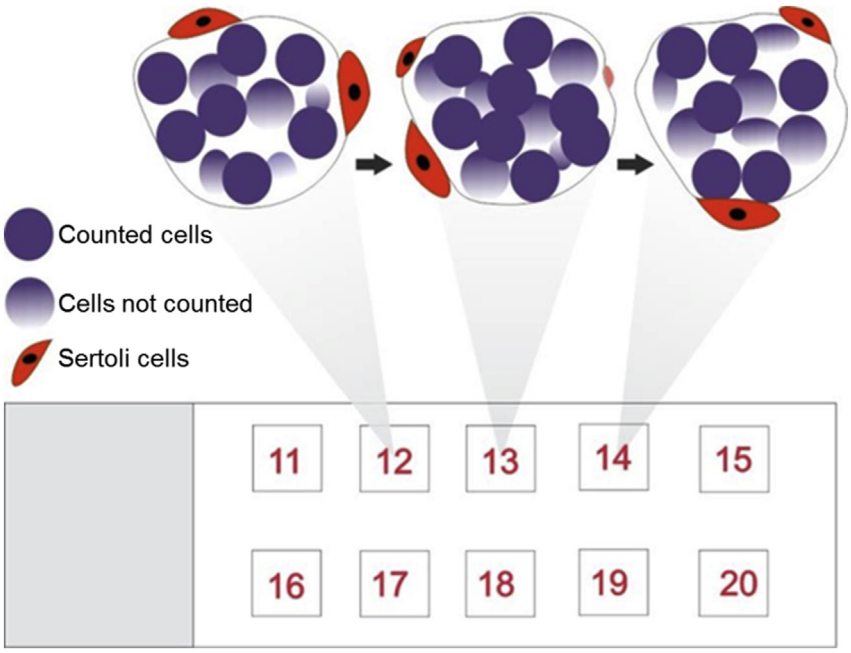

Fig. 2. Diagram illustrating stereological analysis of germ cells and Sertoli cells per cyst. Each numbered square symbolizes one section. The magnified images of sections 12, 13, and 14 represent the same cyst at different depths. The germ cells were counted only when clearly defined.

produced by cysts at the completion of spermatogenesis. It can provide information to characterize the interaction between germ and Sertoli cells and the capacity of Sertoli cells to support the different types of germ cells, as well as to investigate the reduction in cell numbers, to reveal the stage at which the greatest apoptosis occurs.

The present study is one of few to investigate these areas, and *A. altiparanae* is one of few species in which spermatogenesis has been studied by stereology [15–18], allowing the characterization of cell lineages, such as the types of spermatids and the number of spermatogonial generations, that is essential to the understanding of spermatogenesis [19]. In addition to self-renewal and differentiation in daughter cells dedicated to forming spermatozoa, spermatogonia perform functions [19–22] such as the transmission of inherited characters, allowing, in association with cryopreservation and transplantation of germ cells, and gene banking for the

recovery of threatened species and potential restoration of extinct species [23–25].

Spermatogonia lineages have been characterized by the number of mitotic divisions that the cells undergo before differentiating into spermatocytes [24,26]. This number is species-specific and seems to be related to the phylogenetic position occupied [26]. However, in contrast to the eight spermatogonial generations found in *Hoplias malabaricus* [27], which belongs to the same order as *A. altiparanae*, the number of generations in *A. altiparanae* was the same as that reported for *Danio rerio* [9] of Cypriniformes, which separated ~127 million years ago. Therefore, it is necessary to analyze a larger number of species to infer whether this variation occurs at the specific, family, or order level [26].

Sertoli cells also play a fundamental role in fish spermatogenesis. In addition to hormonal and nutritive support, their numbers regulate the quantity of germ cells during the process, limiting testes size and sperm production [28,29]. As in *O. niloticus* [7], the number of Sertoli cells per cyst in *A. altiparanae* gradually increased from the time it was closely associated with a single stem spermatogonium to the leptotene/zygotene stage of spermatocyte. In *Poecilia reticulata* and *D. rerio*, the increase in Sertoli cells continued to the beginning of spermiogenesis [9,30]. The greater number of Sertoli cells at the meiotic stage may be associated with the complexity of meiosis, a stage in which germ cells undergo multiple processes including nucleus division, genetic recombination, and apoptosis, leading to Sertoli cell involvement in phagocytosis of the resulting cell waste [26,31].

The meiotic index and the reduction in cell numbers by 57% from type B spermatogonia to the completion of spermatogenesis show the spermatogenetic effectiveness of 43% in *A. altiparanae*. The reduction in cell numbers is directly related to the support capacity of Sertoli cells and seems to be essential, positively contributing to the quality of the generated spermatozoa [7,9]. A similar observation was reported for *H. malabaricus* [27].

Stereological study contributed to the understanding of spermatogenesis in the yellowtail tetra *A. altiparanae*, showing the complexity of this process by assessing morphometric parameters including support capacity of Sertoli cells, meiotic index, spermatogenesis efficiency, and cell number reduction. Stereology is recommended for descriptive studies of gametogenesis of other teleost species.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.theriogenology.2017.07.025.

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