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Sertoli Cell Proliferation in the Adult Testis—Evidence from Two Fish Species Belonging to Different Orders[1]

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ABSTRACT

Germ cell survival and development critically depend on the cells' contact with Sertoli cells in the vertebrate testis. Fish and amphibians are different from mammals in that they show a cystic type of spermatogenesis in which a single germ cell clone is enclosed by and accompanied through the different stages of spermatogenesis by an accompanying group of Sertoli cells. We show that in maturing and adult testes from African catfish and Nile tilapia, Sertoli cell proliferation occurs primarily during spermatogonial proliferation, allowing the cyst-forming Sertoli cells to provide the increasing space required by the growing germ cell clone. In this regard, coincident with a dramatic increase in cyst volume and number of germ cells per cyst, in Nile tilapia, the number of Sertoli cells per cyst was strikingly increased from primary spermatogonia to spermatocyte cysts. In both African catfish and Nile tilapia, Sertoli cell proliferation is strongly reduced when germ cells have proceeded into meiosis, and stops in postmeiotic cysts. We conclude that Sertoli cell proliferation is the primary factor responsible for the increase in testis size and sperm production observed in teleost fish. In mammals, Sertoli cell proliferation in the adult testis is not observed under natural conditions. However, on the level of the individual spermatogenic cyst—similar to mammals—Sertoli cell proliferation ceases when germ cells have entered meiosis and when tight junctions are established between Sertoli cells. This suggests that fish are valid vertebrate models for studying Sertoli cell physiology.

adult testis, fish, morphometry, proliferation, Sertoli cells, spermatogenesis, testis

INTRODUCTION

The development of germ cells is critically dependent on the presence of somatic cells of the testis. In this regard, Sertoli cells in mammals, and probably in all other vertebrates, play a pivotal role in the differentiation and development of a functional testis [1, 2]. In all mammalian species investigated to date, no Sertoli cell proliferation has been observed after puberty [2–4], and postnatal Sertoli cell mitotic activity ceases during the first wave of spermatogenesis when primary spermatocytes are actively proliferating [5–7]. These events coincide with the formation of the Sertoli cell barrier, tubular lumen, and an elaborated cytoskeleton, which are morphological and functional markers of Sertoli cell differentiation [5, 7]. However, although under normal conditions Sertoli cells enter a nonmitotic status before puberty in adult mammals [8, 9], recent in vitro studies have shown that in rats, terminally differentiated Sertoli cells that overexpress the helix-loop-helix inhibitor of differentiation proteins (ID1 and ID2) are able to reenter the cell cycle and undergo mitosis [10]. Also, in adult photoinhibited Djungarian hamster, Sertoli cells are able to proliferate under proper hormonal stimulation [11]. In stallions, seasonal variation in the total number of Sertoli cells has also been observed [12].

In mammals, it is well established that FSH is the major mitotic factor for Sertoli cells [6, 13]. Because each Sertoli cell is able to support only a relatively fixed number of germ cells in a species-specific manner [4, 14, 15], the Sertoli cell number per testis ultimately dictates the magnitude of testis size and sperm production [4, 15, 16].

Histomorphometric investigation is an adequate approach to better understanding the spermatogenic process and testis function [9, 17, 18]. Such an evaluation allows an estimate of the spermatogenic efficiency for each species through, for example, the determination of the number of spermatogonial generations, the magnitude of germ cell loss that normally occurs during spermatogenesis, and Sertoli cell efficiency [4, 17].

Very little information is available regarding Sertoli cell proliferation in teleosts, the most numerous group of vertebrates. In general, fish spermatogenesis takes place in cysts within the seminiferous tubules. The spermatogenic cysts form when Sertoli cells enclose a single primary spermatogonium [19]. The germ cells derived from a single primary spermatogonium then divide synchronously to constitute an isogenic germ cell clone that is bordered by the cytoplasmic extensions of a single layer of Sertoli cells. Hence, in cystic spermatogenesis, a Sertoli cell is usually in contact with only a single germ cell clone that is accompanied through the different stages of spermatogenesis by its associated group of Sertoli cells. Spermiation, the release of mature germ cells by Sertoli cells, is achieved by opening the cysts. In some fish species studied in this regard, spermiation is associated with the degeneration of at least some of the Sertoli cells [20, 21], so that Sertoli cells might have to be replaced in part to maintain the capacity for

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supporting subsequent waves of spermatogenesis. Moreover, in many species, developmental cycles with more than 50-fold changes in testis weight occur during successive annual reproductive seasons [22]. This might be associated with yearly recurrent waves of Sertoli cell proliferation. Finally, many fish species grow throughout life, which might require Sertoli cell proliferation in adults as a basis for allometric testis growth.

The main objectives of the present work were to perform a comprehensive morphometric study of different types of spermatogenic cysts and to investigate whether Sertoli cells and other somatic cells of the testis are mitotically active in the sexually mature Nile tilapia (*Oreochromis niloticus*, Cichlidae, Perciformes) and the African catfish (*Clarias gariepinus*, Clariidae, Siluriformes). These species are representatives of two nonrelated teleost orders.

MATERIALS AND METHODS

Experimental Animals, Sampling, and Surgery

Nine sexually mature Nile tilapia were obtained from a commercial fishery station and maintained at approximately 25°C. The experiments were conducted according to the institutional animal care protocols at the Federal University of Minas Gerais, Brazil, and followed approved guidelines for the ethical treatment of animals. After the animals were killed, testis fragments of approximately 3 mm in thickness and from several different segments of the testis were taken transversally and longitudinally by hand with a razor blade. The fragments were fixed by immersion in 5% glutaraldehyde, 0.1 M phosphate buffer pH 7.3, and embedded in glycol methacrylate according to conventional techniques. Testis sections were obtained in a Reichert-Jung automatic microtome and stained with 1% toluidine blue for histological and morphometric analyses with an Olympus BX41 light microscope.

Animal culture and experimentation using African catfish were consistent with Dutch national regulations; the Life Science Faculties Committee for Animal Care and Use approved all experimental protocols. Catfish were bred and raised in the laboratory as described before [23], except that an extract from pituitaries of adult African catfish, instead of hCG, was used to induce ovulation (one pituitary equivalent per kilogram of body weight).

Previous work has shown that in our aquarium facility, male African catfish commence spermatogonial proliferation at 10–12 wk of age, and that most males have completed the first wave of spermatogenesis at 20 wk of age [24]. For a study on Sertoli cell proliferation in maturing testis, we therefore collected tissue from fish at 11, 13, 14, 15, 17, and 21 wk of age. To study adult testis, tissue was collected from fish at 12 mo of age.

Number of Nile Tilapia Germ Cells and Sertoli Cells Per Cyst

The total number of germ cells and Sertoli cells per spermatogonial, spermatocyte, and spermatid cysts was obtained in five Nile tilapias using serial sections taken at 3 and 2 μm thickness [25]. This evaluation was performed after selecting the respective cysts whose area was entirely encompassed by the serial sections. Based on the section thickness and the nuclear diameter of the germ cell type analyzed, care was taken not to count one nucleus twice. At least 12 cysts were analyzed per each germ cell type characterized per fish. Spermatids were arbitrarily classified according to the degree of nuclear condensation as initial, intermediate, and final (mature). The Sertoli cell efficiency was estimated from the ratio of germ cells to Sertoli cells, per each cyst type.

Nile Tilapia Germ Cell Morphometry and Cyst Volume

Germ cell morphometry was also performed on the testis of five adult Nile tilapias. The individual germ cell volume (in cubic micrometers) was obtained from the nucleus volume and the proportion (the percentage) between nucleus and cytoplasm of each germ cell type evaluated. Because the nucleus of all germ cells investigated in Nile tilapias is spherical or nearly round, their nucleus volume was estimated using the mean nuclear diameter. Fifty nuclei were measured for each germ cell type investigated per fish. Individual nuclear volume was expressed (in cubic micrometers) using the formula $4/3\ \pi\ R^3$, where R = nuclear diameter/2. To estimate the proportion between nucleus and cytoplasm, a 441-point square lattice was placed over the sectioned material at 1000× magnification. From 100 to 2500 points over each germ cell type were counted for each animal, depending on the cyst size and the type of germ cell cyst evaluated. The cyst volume, excluding Sertoli cells, was calculated according to the germ cell volume and the total number of germ cells per cyst. Because intercellular spaces were observed in cysts of intermediated and final (mature) spermatids, their percentage and volume were also estimated and added in the total cyst volume. The morphometric data obtained allowed us to estimate the changes in nucleus, cytoplasm, and cell volumes, and possible germ cell loss that occur during the spermatogenic process, from primary spermatogonia to mature spermatids.

Germ cell loss (apoptosis) was analyzed qualitatively and quantitatively, and is expressed as a percentage of apoptotic cells per cyst. These apoptotic cells were morphologically characterized as darker stained cells, as it is typical in this condition for cells embedded in plastic and stained with toluidine blue [26, 27].

Sertoli and Germ Cell Proliferation Analysis

To study Sertoli cell proliferation in African catfish testis, 6 h before tissue sample the animals received one injection of 50 mg of 5-bromo-2′-deoxyuridine (BrdU; Sigma, St. Louis, MO) per kilogram of body weight. BrdU was dissolved in physiological salt solution and administered between the epaxial, dorsal musculature using a 26-gauge needle and a volume of 0.05 to 0.5 ml, depending on the body size. After determination of the body weight the fish were killed rapidly by decapitation, and the testes were removed, weighed, and fixed in Bouin fixative in which the picric acid concentration was lowered by using warm (~40°C) saturated, instead of boiling saturated, picric acid solution. After overnight fixation at room temperature, the tissue was dehydrated in graded alcohol and embedded in paraffin wax according to conventional techniques.

For immunocytochemical detection of BrdU on 5-μm paraffin sections of African catfish testis tissue, a cell proliferation kit was used according to the manufacturer's guidelines (Cell Proliferation Kit RPN20; Amersham Biosciences, Piscataway, NJ). After BrdU detection, the sections were counterstained with hematoxylin.

In the developmental study, testis tissue from seven males aged 11–21 wk was analyzed, as well as tissue from five adult males. After localizing BrdU-positive Sertoli cells, we recorded the germ cell type in the cyst with which the labeled Sertoli cell was associated, and whether these germ cells were BrdU-positive. In two animals (17 and 21 wk old, both showing spermiation in some tubules), we determined the labeling index of apparently single, large (i.e., early) spermatogonia, and clonal groups of small (i.e., late) spermatogonia.

Four sexually mature Nile tilapias were used to investigate the pattern of proliferation of somatic cells in the testis. These fish received one i.p. injection of ^{3}H-thymidine (thymidine [methyl-^{3}H]; specific activity 82.0 Ci/mmol; Amersham Biosciences). Tritiated thymidine (1 μCi) was injected per gram of body weight using a hypodermic needle. All fish were killed at approximately 2 h after thymidine injection, and the testis fragments were fixed and embedded as already described for Nile tilapias.

To perform autoradiographic analysis, unstained testis sections (4 μm in thickness) were dipped in autoradiography emulsion (Kodak NTB-2; Eastman Kodak Company, Rochester, NY) at 45°C. After drying for approximately 1 h at 25°C, sections were placed in sealed black boxes and stored in a refrigerator at 4°C for 3–4 wk. Subsequently, testis sections were developed in Kodak D-19 solution at 15°C [28] and stained with 1% toluidine blue. Analyses of these sections were performed by light microscopy to detect labeled cells. Cells were considered labeled when four to five or more grains were present over the nucleus in the presence of low-to-moderate background (i.e., very few grains per histological field observed under oil immersion). The somatic cells evaluated were Sertoli, Leydig, and peritubular myoid cells, and undifferentiated/undetermined somatic cells present in the intertubular compartment. Specifically for labeled Sertoli cells, we recorded the germ cell type with which they were associated, or whether the labeled Sertoli cells were isolated (i.e., apparently not associated with any particular type of germ cell). Approximately 150 labeled somatic cells were counted for each animal. Also, the labeling index of Sertoli cells was estimated from a total of approximately 1000 Sertoli cells analyzed per fish in testis fragments sectioned longitudinally.

Labeled somatic cells were also evaluated according to their location in the testis. For this purpose, transversally sectioned testis fragments were divided proportionally into three different regions that were designated as distal (close to the tunica albuginea), intermediate, and proximal region

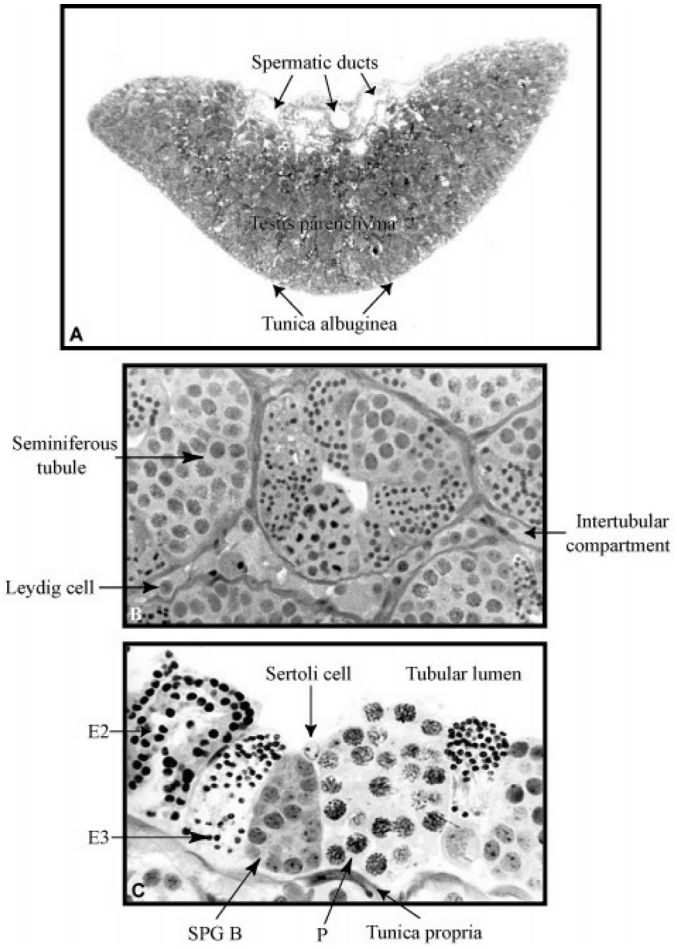

FIG. 1. Transversal section from Nile tilapia testis (**A**) showing tunica albuginea, spermatic ducts, and the testis parenchyma (**B**), where the tubular compartment and the intertubular compartment with Leydig cells can be observed. Germ cell cysts at different stages of development are shown in the higher magnification of a longitudinal section of a seminiferous tubule (**C**). Also shown in this figure are the tunica propria comprised of peritubular myoid cells and basal lamina, Sertoli cell, and tubular lumen. B, secondary spermatogonia; P, pachytene primary spermatocyte; E2 and E3, intermediate and final (mature) spermatids cysts, respectively. Magnification ×80 (**A**), ×900 (**B**), and ×1230 (**C**).

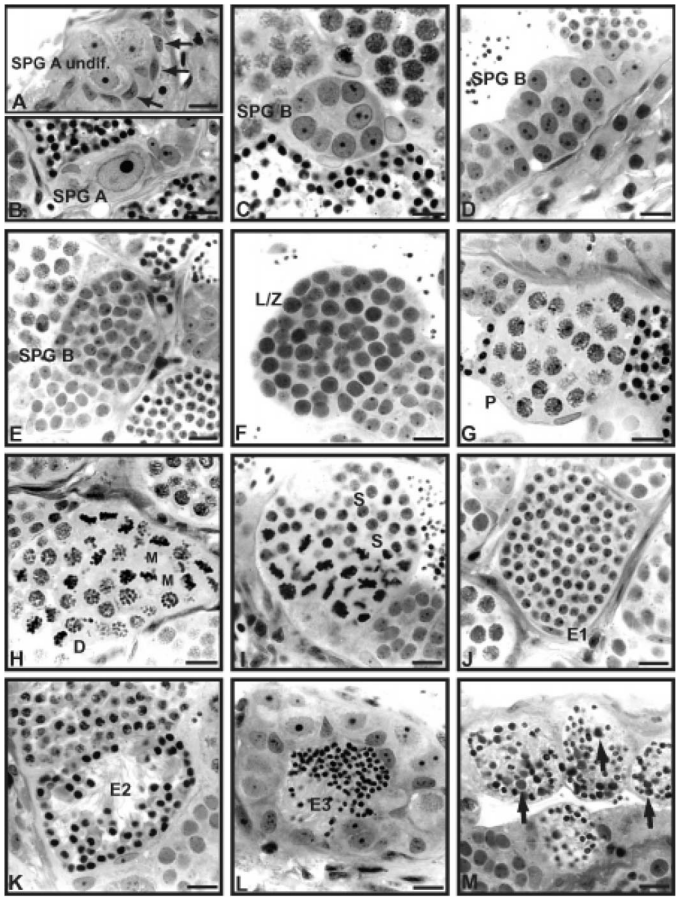

FIG. 2. Different germ cell cysts observed during the spermatogenic process in Nile tilapia. **A**) Type A immature spermatogonia and Sertoli cells (arrows); (**B**) SPG A, type A mature or differentiated (primary) spermatogonia; (**C**–**E**) SPG B, different generations of secondary spermatogonia; (**F**–**H**) primary spermatocytes in the transition from leptotene to zygotene (L/Z), pachytene (P), diplotene (D), and meiotic figures from the first meiotic division (M); (**I**) secondary spermatocytes (S); (**J**–**L**) spermatids at initial (E1), intermediate (E2), and final (E3) steps of development; (**M**) apoptotic spermatids (arrows). Bar = 10 μm.

(close to the spermatic ducts). In this part of the work, approximately 160 labeled somatic cells were counted for each animal.

Data Analysis

The data were processed by analysis of variance (Newman-Keuls test) using Statistica 3.11 for Windows software (StatSoft Inc., Tulsa, OK). All data in figures and tables are presented as the mean ± SEM. $P < 0.05$ was considered significant.

RESULTS

Spermatogenesis and Sertoli Cell Proliferation

The testis structure in sexually mature Nile tilapias is shown in Figure 1. Seminiferous tubules with different types of germ cell cysts (from spermatogonia to spermatids) and apoptotic spermatids are depicted in Figure 2. Although immature (undifferentiated) spermatogonia were present in other regions of the testis parenchyma (Fig. 2A), they were observed predominantly close to the tunica albuginea. Primary or mature (differentiated) spermatogonia (Fig. 2B) were also found more often close to the tunica, but showed a less restricted distribution pattern than immature spermatogonia. Compared to the cells in mammals, these cell types could be considered, respectively, as mature (differentiated) and immature (undifferentiated) type A spermatogonia [29].

All morphometric data related to spermatogenic cysts are presented in Table 1. The total number of Sertoli cells per cyst increased significantly ($P < 0.05$) after the second mitosis of differentiated spermatogonia that produced a clone of four B2 spermatogonia. The Sertoli cell number kept increasing up to leptotene/zygotene spermatocyte cysts, showing a strong trend toward stabilization ($P > 0.05$) from pachytene spermatocytes to mature spermatids. The number of germ cells per cyst and cyst volume increased from primary spermatogonia to early spermatid cysts, showing a significant reduction ($P < 0.05$) in intermediate and final spermatid cysts. The percentage of intercellular spaces present in cysts of intermediate and mature spermatids was 4.8% ± 0.8% and 7.0% ± 0.8%, respectively. In contrast to the germ cell number per cyst, the opposite trend was observed for nuclear diameter, and for nucleus, cytoplasm, and cell volumes. However, gradual increases ($P < 0.05$) for these parameters occurred from preleptotene to pachytene spermatocytes. The data obtained for the number of spermatogonia and early spermatocytes per cyst allowed us

TABLE 1. Morphometry of the different germ cell cysts in Nile tilapia (mean ± SEM).

Cyst type[a]	No. of Sertoil cells per cyst	No. of germ cells per cyst	Germ cells: Nuclear diameter (μm)	Nuclear volume (μm^3)[b]	Cytoplasm volume (μm^3)[c]	Cell volume (μm^3)	Cyst volume (μm^3)[d]	No. of germ cells (cyst) per Sertoil cell
SPG A	1.4 ± 0.2	1.0 ± 0.0	10.1 ± 0.8	548 ± 20 (0.24)	1,712 ± 20 (0.76)	2,261 ± 82	2,237 ± 82	0.7 ± 0.1
SPG B1	1.6 ± 0.3	2.0 ± 0.0[e]	7.8 ± 0.1[e]	251 ± 9 (0.25)[e]	740 ± 9 (0.75)[e]	992 ± 35[e]	1,983 ± 69	1.3 ± 0.3[e]
SPG B2	1.9 ± 0.3[e]	3.9 ± 0.1[e]	7.5 ± 0.1	218 ± 6 (0.29)[e]	537 ± 6 (0.71)[e]	755 ± 20[e]	2,938 ± 76[e]	2.0 ± 0.5[e]
SPG B3	2.4 ± 0.2[e]	8.0 ± 0.5[e]	7.2 ± 0.1	197 ± 7 (0.34)	376 ± 7 (0.66)[e]	572 ± 20[e]	4,368 ± 155[e]	3.4 ± 0.3[e]
SPG B4	2.4 ± 0.3	16.0 ± 1.0[e]	6.3 ± 0.1[e]	131 ± 4 (0.35)[e]	238 ± 4 (0.65)[e]	369 ± 11[e]	6,393 ± 184[e]	6.4 ± 1[e]
SPG B5	2.8 ± 0.3	32.0 ± 2.0[e]	6.0 ± 0.1[e]	113 ± 7 (0.39)[e]	174 ± 7 (0.61)[e]	287 ± 19[e]	9,505 ± 623[e]	11 ± 1[e]
SPG B6	2.9 ± 0.3	63.0 ± 4.0[e]	5.7 ± 0.2	98 ± 9 (0.47)	109 ± 9 (0.53)[e]	207 ± 19[e]	13,256 ± 1,209[e]	22 ± 3[e]
SPG B7	3.6 ± 0.3[e]	114.0 ± 7.0[e]	5.3 ± 0.1	79 ± 4 (0.50)	79 ± 4 (0.50)	158 ± 8[e]	18,845 ± 981[e]	32 ± 3[e]
P1	5.0 ± 0.4[e]	217.0 ± 12.0[e]	4.8 ± 0.2[e]	58 ± 7 (0.52)[e]	53 ± 7 (0.48)[e]	111 ± 13	24,254 ± 2,799	43 ± 5[e]
L/Z	5.6 ± 0.3	200.0 ± 15.0	5.4 ± 0.1[e]	86 ± 6 (0.50)[e]	84 ± 6 (0.50)[e]	170 ± 14[e]	34,437 ± 2,920[e]	36 ± 1
P	6.1 ± 0.1	217.0 ± 10.0	6.5 ± 0.1[e]	146 ± 5 (0.50)[e]	149 ± 5 (0.50)[e]	295 ± 9[e]	64,783 ± 4,872[e]	36 ± 2
D	6.1 ± 0.1	201.0 ± 9.0	5.8 ± 0.1[e]	102 ± 7 (0.40)[e]	151 ± 7 (0.60)	253 ± 23	50,660 ± 3,914[e]	33 ± 1
S	6.1 ± 0.1	389.0 ± 23.0[e]	4.3 ± 0.1[e]	43 ± 2 (0.41)[e]	64 ± 2 (0.59)[e]	107 ± 7[e]	41,745 ± 4,220	64 ± 4[e]
E1	5.9 ± 0.2	738.0 ± 54.0[e]	3.4 ± 0.05[e]	20 ± 1 (0.38)[e]	32 ± 1 (0.63)[e, f]	52 ± 3[e]	40,118 ± 341	137 ± 9[e]
E2	5.9 ± 0.1	684.0 ± 52.0[e]	2.5 ± 0.02[e]	8.5 ± 0.2 (0.34)[e]	17 ± 0.2 (0.66)[e, f]	25 ± 2[e]	17,624 ± 731[e]	111 ± 12[e]
E3	5.9 ± 0.1	551.0 ± 28.0[e]	2.0 ± 0.03[e]	4.4 ± 0.2 (0.23)[e]	15 ± 0.2 (0.77)[f]	19 2[e]	11,189 ± 1,256[e]	91 ± 4

[a] SPG A, primary spermatogonia; SPG B1-SPG B7, different generations of secondary spermatogonia; P1, preleptotene primary spermatocytes; L/Z, leptotene/zygotene primary spermatocytes; P, pachytene spermatocytes; D, diplotene spermatocytes; S, secondary spermatocytes; E1, initial spermatids; E2, intermediate spermatids; E3, final (mature) spermatids.
[b] Values in parentheses are the percentage of nucleus in the cell.
[c] Values in parentheses are the percentage of cytoplasm in the cell.
[d] Sertoli cells not included.
[e] $P < 0.05$ vs. previous value.
[f] Flagellum included.

to estimate that at least eight generations of differentiated (mature) spermatogonia are present in Nile tilapias—one generation of primary spermatogonia (mature type A spermatogonia) and seven generations of secondary spermatogonia (type B spermatogonia). Because the nuclear morphology among the different generations of secondary spermatogonia was very similar, the criteria used to distinguish each generation of this cell type were the cell size and the number of spermatogonia per cyst. Approximately 100 mature spermatids were found per each Sertoli cell, and germ cell loss during spermiogenesis was around 30% ($P < 0.05$).

Labeled somatic cells were frequently found in the testis parenchyma 2 h after ^{3}H-thymidine injection (Fig. 3). Most of these cells were Sertoli cells (Fig. 4), which were associated primarily with spermatogonial cysts (Fig. 5). Labeled Sertoli cells were also found to be associated with spermatocytes and spermatid cysts, but at much lower percentages (Fig. 5). From approximately 1000 cells analyzed per fish, 1.5% ± 0.2% of Sertoli cells were labeled.

In African catfish, Sertoli cells in the S-phase of the cell cycle (i.e., BrdU-positive) were found in all testis samples analyzed, including those from adult males. Most of the BrdU-positive Sertoli cells were observed in association with mature, single, primary spermatogonia (63%; Figs. 6 and 7, A and B), which is similar to the observation in Nile tilapia, but in contrast to Nile tilapia testis, these Sertoli cells appeared to be randomly distributed throughout the testis. In addition, secondary spermatogonial cysts regularly showed proliferating Sertoli cells (33%; Fig. 6). Spermatocyte cysts (Fig. 7C) only rarely showed labeled Sertoli cells, whereas none were found to be associated with postmeiotic cells in the catfish testis.

Not unexpectedly, proliferating germ cells have been observed much more frequently than dividing Sertoli cells. It was interesting to note, however, that when a Sertoli cell was BrdU-positive, the germ cell(s) in the spermatocyst associated with this Sertoli cell were usually (91%) BrdU-negative. When BrdU-positive Sertoli cells were found associated with BrdU-positive germ cells, these Sertoli cells were predominantly (87.5%) associated with secondary spermatogonia, and only rarely with primary spermatogonia. The latter also differed from clonal groups of secondary, smaller spermatogonia by their BrdU-labeling index, as determined in tissue sections from two males of 17 and 21 wk of age. Counting 667 primary large spermatogonia, we found that 58 (8.7%) were BrdU-positive, whereas 1099 (46.3%) of the 2048 secondary spermatogonia were BrdU-positive. The testes grew rapidly during this period as reflected by the gonadosomatic index (gonad weight expressed as a percentage of body weight) being 0.04% and 0.27% in 17- and 21-wk-old fish, respectively.

Studying labeled somatic cells in different regions of Nile tilapia testis, we observed that approximately 75% of labeled Sertoli cells were present at the distal (blind end) segment of the seminiferous tubules located near the tunica albuginea (Fig. 8). In general, these cells appeared to be more morphologically immature (i.e., spindle-shaped nucleus, more heterochromatin, and smaller nucleolus), and the vast majority were associated with immature and mature, primary type A spermatogonia. The opposite trend was observed for labeled Leydig cells and undifferentiated/undetermined somatic cells that were preferentially located in the region close to the spermatic ducts (Fig. 8). No clear trend was found for the location of peritubular myoid cells in the three different regions of the testis investigated (Fig. 8).

DISCUSSION

The literature contain very few quantitative data regarding germ cells during the evolution of spermatogenesis in teleosts. The pattern found for Nile tilapia in the present work was similar to that observed for nuclear diameter in

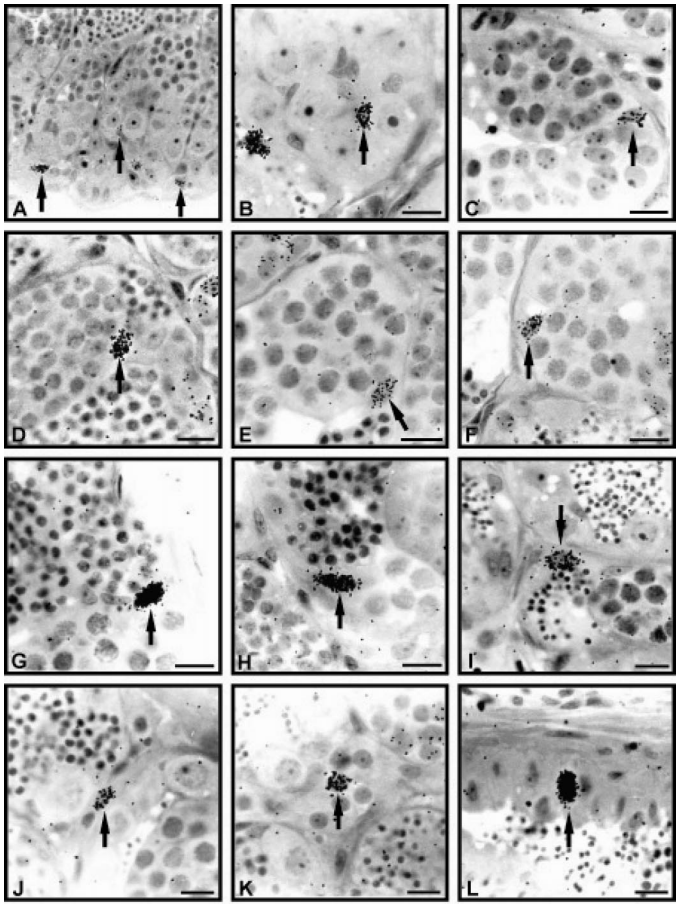

FIG. 3. Labeled somatic cells (arrows) 2 h after ^{3}H-thymidine injection. **A**) labeled Sertoli cells (arrows) located at the distal (blind end) region of the seminiferous tubules; these cells are usually associated with type A undifferentiated (immature) spermatogonia; (**B**–**I**) labeled Sertoli cells (arrow) associated with different germ cell cysts characterized as follows: type A mature or primary spermatogonia (**B**); secondary spermatogonia (**C**–**D**); primary spermatocytes in the transition from leptotene to zygotene (**E**) and pachytene (**F**); spermatids at initial (**G**), intermediate (**H**) and final (**I**) steps of development. Also shown (arrow) are a labeled peritubular myoid cell (**J**), a Leydig cell (**K**), and a cell located in the spermatic duct (**L**). Magnification ×350 (**A**). Bar = 10 μm.

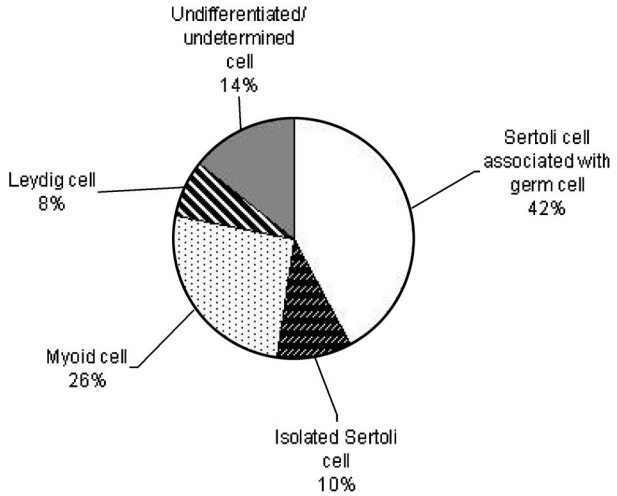

FIG. 4. Mean percentages of labeled somatic cells in the testis of Nile tilapia, killed 2 h after thymidine injection (n = 4; the SEM values ranged between 6% and 17% of the means).

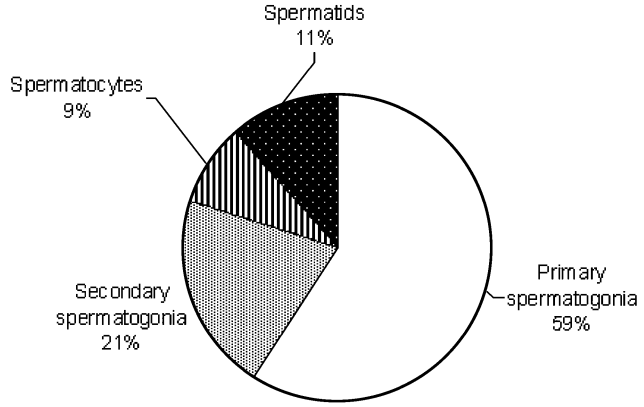

FIG. 5. Mean percentages of labeled Sertoli cells associated with different germ cell cysts in Nile tilapia, 2 h after thymidine injection (n = 4; the SEM values ranged between 8% and 14% of the means).

the guppy (*Poecilia reticulata*) [30]. The maximum nuclear diameter for spermatocytes in guppies was observed in germ cells characterized as zygotene instead of pachytene, in testis fragments embedded in paraffin. In general, the pattern of germ cell growth during spermatogenesis is similar in teleosts and mammals [4, 9]. However, in contrast to teleosts, in all mammalian species investigated to date, diplotene spermatocytes, anticipating the two rapid meiotic divisions, are the germ cells that present the biggest nuclear and ground substance volumes [4, 9]. It remains to be investigated why diplotene spermatocytes do not show the largest size in teleosts.

To our knowledge, the present work is the first study to investigate the number of Sertoli cells per cyst during the spermatogenic process in a teleost fish presenting external fertilization. In the guppy, a fish showing internal fertilization, the number of Sertoli cells per cyst increased markedly (from ~4 to ~130 cells per cyst) from primary spermatogonia to early spermatid cysts [30]. Stabilization of the number of Sertoli cells per cyst was observed in guppies only in cysts of more mature spermatids, which is different from the observation in Nile tilapia. Junctions between Sertoli cells, including tight junctions, are formed in pachytene spermatocytes in the Nile tilapia [31] or at the beginning of spermiogenesis in guppies [32] and several other teleost species [33–35]. The quantitative data obtained for Nile tilapias and guppies suggest that the formation of the Sertoli

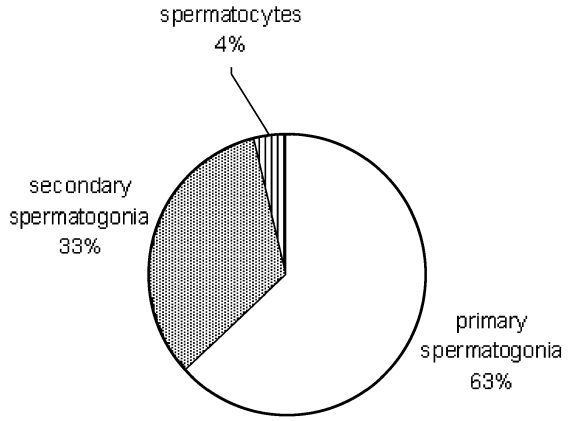

FIG. 6. Mean percentages of labeled Sertoli cells associated with different germ cell cysts in African catfish 6 h after BrdU injection (n = 5; the SEM values ranged between 5% and 17% of the means).

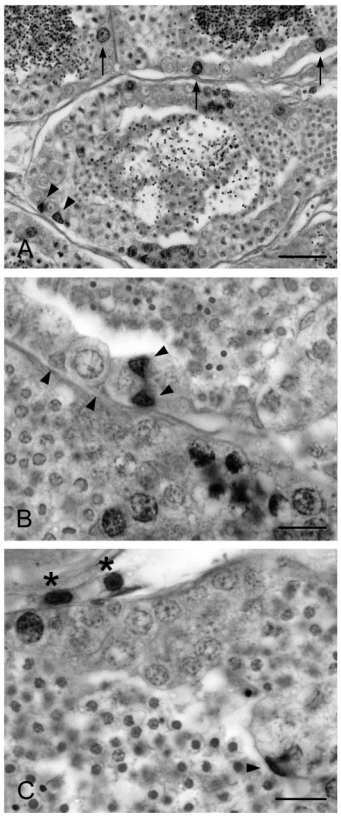

FIG. 7. Proliferating cells in sections from adult African catfish testis tissue; BrdU was detected immunocytochemically and sections were counterstained with hematoxylin. BrdU-positive single spermatogonia (arrows) or Sertoli cells (arrowheads) are readily detected; bar = 25 μm (**A**). Sertoli cells (arrowheads) that did or did not incorporate BrdU were found close to each other, suggesting that different spermatogenic cysts behave as independent functional units. Most BrdU-positive Sertoli cells were found in association with spermatogonia. Germ cell nuclei are usually spherical but show different diameters, while Sertoli cell nuclei do not seem to undergo major changes in volume and display a triangular or elongated shape; bar = 10 μm (**B**). Proliferation of interstitial cells can be observed (asterisks). Rarely, proliferating Sertoli cells (arrowhead) were found associated with spermatocyte cysts; bar = 10 μm (**C**).

cell barrier in teleost fish is coincident with the stabilization of the Sertoli cell number per cyst. Also, in African catfish, we found a strong tendency for reduced Sertoli cell proliferation once the germ cells entered meiosis.

Cyst volume was not obtained in the study on guppies [30], however, the orthogonal diameter of each cyst type was measured. Coincident with the stabilization in the number of Sertoli cells per cyst, the larger cyst diameter in guppies was found at the beginning of spermiogenesis. Although the highest cyst volume in tilapias occurred at an earlier phase of spermatogenesis compared to guppies, this event was also coincident with the stabilization in the number of Sertoli cells per cyst. In this regard, these observations suggest that the stabilization in the number of Sertoli cells per cyst, probably linked to the formation of the Sertoli cell barrier, represents a critical step for the spermatogenic process in teleosts. As expected, the striking reduction of cyst volume during spermiogenesis in Nile tilapias is coincident with the evident nuclear condensation and the elimination of cytoplasm that normally occurs during this phase of spermatogenesis [4, 9, 36–38].

Compared to Nile tilapia, the maximum number of Sertoli cells per cyst is more than 20-fold higher in guppies [30]. However, Sertoli cell efficiency inferred from the number of early spermatids per each Sertoli cell is only about 20% higher in guppies, showing a similar efficiency for Sertoli cells in these two teleost species. This finding indicates that the number of Sertoli cells per testis is one of the most important factors in determining the magnitude of sperm production in teleosts, which is similar to the finding in mammals [4, 13, 15, 16]. This suggests that a relatively constant number of germ cells is also associated with a Sertoli cell in fish, although particularly in the center of more advanced spermatogenic cysts, not all germ cells are in direct contact with a Sertoli cell. Compared to mammals investigated to date [4, 13, 39], Sertoli cell efficiency is 10- to 20-fold higher in Nile tilapias and in guppies, strongly suggesting that the cystic arrangement of spermatogenesis in teleosts is more efficient than the tubular arrangement observed in mammals. In mammals only 2 to 3 spermatozoa are formed out of 10 spermatic cells that are theoretically expected from each mature A1 type of spermatogonium [4, 29]. In Nile tilapias and in guppies, this figure is approximately 5.5, so that the overall rate of spermatogenesis is 100% higher in these two teleost species than in mammals. In mammals, germ cell loss occurs predominantly during the spermatogonial and meiotic phases of spermatogenesis; however, in teleosts, apoptotic germ cells are observed particularly during spermiogenic phases ([30] and present study).

Spermatogonial cells usually give rise to spermatocytes after a fixed number of cell divisions, which is character-

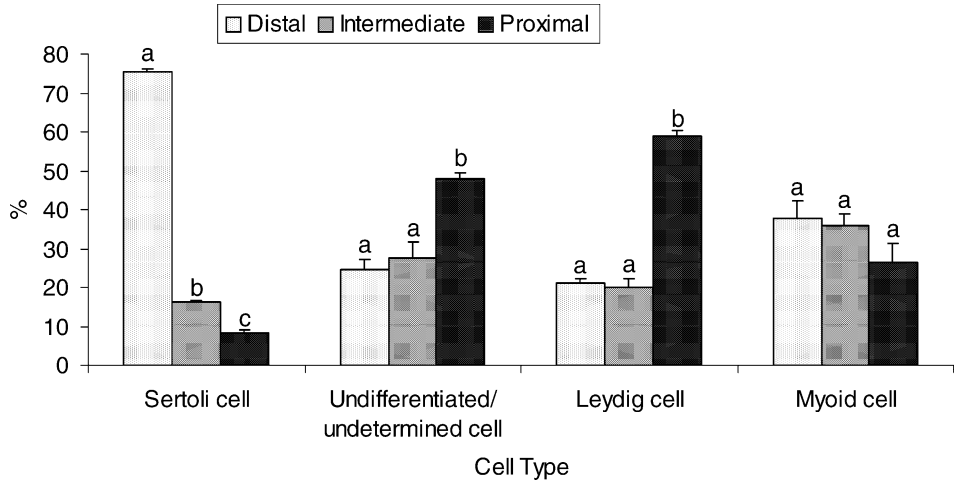

FIG. 8. Nile tilapia, killed 2 h after thymidine injection (n = 4, bar heights and error bars represent the mean ± SEM). Different letters for the same cell type indicate statistically significant differences ($P < 0.05$; Newman-Keuls test).

istic of each species; two to six mature spermatogonial generations are observed in mammals [4, 29]. Knowledge of the number of spermatogonial generations is an essential basis for understanding the regulatory mechanisms of spermatogenesis [29]. The data in the literature show that the number of spermatogonial generations in teleosts ranges from 5 to 6 in zebrafish [40], to 14 in guppies [30], whereas intermediate figures are observed for other teleost species [41–43], including the Nile tilapia (8 spermatogonial generations; present work).

In mammals, postnatal Sertoli cell proliferation occurs primarily when only spermatogonia are present in the seminiferous epithelium and ends when primary spermatocytes are proliferating [5, 7]. Although Sertoli cells proliferate in adult Nile tilapia and African catfish, our studies show that their mitotic activity is highest when they are associated with type A spermatogonia. However, Sertoli cells present in spermatocyte and spermatid cysts still can divide, suggesting that they are not yet fully differentiated or that differentiated Sertoli cells preserve their mitotic competence.

The fate of Sertoli cells after spermiation in teleosts is still a controversial issue. Our findings suggest strongly, however, that the distal (blind end) region of seminiferous tubules is the main source of new Sertoli cells in Nile tilapia. In this region, labeled immature Sertoli cells close to the tunica albuginea and associated predominantly with immature spermatogonia are frequently observed. This restricted distribution of immature spermatogonia is typically found in certain teleost orders, including perciform fish (Nile tilapia), but not in siluriform fish (African catfish). That almost all Sertoli cells located close to the tunica albuginea are immature and have a high proliferation potential suggests that Sertoli cell precursors or stem Sertoli cells are present in the Nile tilapia and are probably preferentially located in this region. It is possible that the high proliferation activity here is related to the formation of new spermatogenic cysts, the basic structural unit and prerequisite for cystic spermatogenesis to occur [16]. In fish that show an unrestricted distribution of immature spermatogonia, such as African catfish, the interaction between immature germ and Sertoli cells to form cysts seems to occur throughout most of the testis. This may explain that proliferating Sertoli cells were found throughout the testis in the latter species.

The precise origin of the somatic cells present in the testis of teleosts has not yet been established [44], which is similar to the finding in mammals [45, 46]. In contrast to Sertoli cells, labeled Leydig cells in tilapias were observed predominantly in the testis parenchyma close to the spermatic ducts, where more advanced spermatogenic cysts with differentiated Sertoli cells are predominant. The similar pattern of distribution observed for labeled Leydig cells and undifferentiated/undetermined cells suggest that among these latter cells might be Leydig cell precursors, because this has been observed in rodents during postnatal testis development [47]. Recently, an orthologue of antimüllerian hormone (AMH) has been identified in eel [48]. In flounder, it was found to be expressed predominantly by Sertoli cells contacting spermatogonia [49]. In mammals, AMH inhibits Leydig cell proliferation and maturation [50]. Assuming a similar effect of AMH in Nile tilapia testis would explain the spatial separation of Sertoli vs. Leydig cell proliferation.

Peritubular myoid cells are functionally very important for the development of the spermatogenic process, and together with Sertoli cells are responsible for the formation of basal lamina [51] or for the production of specific Sertoli cell modulatory factors that are necessary for spermatogenesis [52]. Although peritubular myoid cells showed fairly good proliferation activity, probably to adjust to the intense seminiferous tubule growth that occurs in Nile tilapias, no clear trend was observed for the distribution of labeled peritubular myoid cells in the three different testis parenchyma regions investigated. Perhaps the higher plasticity of this cell type in relation to its capacity to stretch in the tubular wall could influence its mitotic behavior. For instance, the higher proliferation activity of spermatogonia located in the distal blind ending region of the seminiferous tubules requires more and newly formed Sertoli cells necessary for the formation of new spermatogenic cysts. Probably for this reason, compared to peritubular myoid cells and to other regions of the testis parenchyma investigated, Sertoli cells specifically located in the distal region show a much higher mitotic activity.

In summary, our findings strongly suggest that Sertoli cell proliferation is the primary factor responsible for the increase in testis size and sperm production observed in most teleosts investigated thus far. This proliferation is regularly observed in the adult testis, which is different from the situation in most mammals, whereas similar to the mammalian testis, Sertoli cell proliferation predominates during the period that the germ cells in contact with the Sertoli cells are mitotically active. These observations render fish a valuable model for studying Sertoli cell functions in vertebrates.

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