Acta Zoologica

Published for The Royal Swedish Academy of Sciences and The Royal Danish Academy of Sciences and Letters

Acta Zoologica (Stockholm) doi: 10.1111/j.1463-6395.2011.00508.x

Spermatogenesis and changes in testicular structure during the reproductive cycle in *Cichlasoma dimerus* (Teleostei, Perciformes)

Graciela Rey Vázquez[1], Rodrigo H. Da Cuña[1], Fernando J. Meijide[1,2] and Graciela A. Guerrero[1]

[1]Laboratorio de Embriología Animal, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires C1428EHA, Argentina; [2]CONICET, Rivadavia 1917, Buenos Aires C1033AAJ, Argentina

Keywords:
spermatogenesis, testicular structure, Cichlidae, reproductive cycle, biflagellate spermatozoa

Accepted for publication:
20 March 2011

Abstract

Rey Vázquez, G., Da Cuña, R.H., Meijide, F.J., and Guerrero, G.A. 2011. Spermatogenesis and changes in testicular structure during the reproductive cycle in *Cichlasoma dimerus* (Teleostei, Perciformes). —*Acta Zoologica* (Stockholm) **00**:1–13.

The present study aimed at analyzing spermatogenesis and the changes occurring in the testis throughout the reproductive cycle in the South American perciform fish *Cichlasoma dimerus*. Testes were studied using light and electron microscopy techniques. This species has an unrestricted lobular testis composed of two compartments: germinal, containing Sertoli and germ cells, exhibiting a cystic mode of spermatogenesis; and interstitial, composed of Leydig cells and connective tissue elements. Spermatozoa belong to the anacrosomal type I aquasperm and possess two flagella, this being the first report of an externally fertilizing cichlid exhibiting biflagellate sperm. Under laboratory conditions, *C. dimerus* proved to be a multiple spawner throughout the year, with a season of higher reproductive activity extending from September to March, during which fish spawned on average every 29.4 days. Changes in the germinal epithelium and the germ cell stages present allowed the description of five reproductive classes: regressed, early, mid-, and late maturation, and regression. During the high reproductive season, each cycle went through the first four classes. The regressed class overlapped with the late maturation class, because sperm was being released while spermatogonia were proliferating. The regression class occurred solely in sexually inactive males during the period of low reproductive activity.

Graciela Rey Vázquez, Laboratorio de Embriología Animal, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, C1428EHA, Argentina.
E-mail: grarey@bg.fcen.uba.ar

Introduction

Fishes constitute more than half the total number of living vertebrate species. Fish behavior is as diverse as fish morphology (Nelson 2006). Diversity is also reflected in the different gonadal structures, reproductive strategies, and modes of parental care found in this group (Nagahama 1983; Pudney 1995, 1996; Nakatani *et al.* 2001). In addition, in many teleost species, the seasonal breeding patterns are governed by environmental factors such as photoperiod, temperature, salinity, rainfall, water flow, predators, and population density among others (Bromage *et al.* 2001; Cinquetti and Dramis 2003; Alvarenga *et al.* 2006; Batlouni *et al.* 2006; Godinho 2007; Fiszbein *et al.* 2010; Singh *et al.* 2010).

Despite the highly variable reproductive strategies present in this group, the basic organization of the testis is notably conserved in fish. These organs are constituted by the germinal and interstitial compartments, separated by a basement membrane. The former comprises a seminiferous epithelium composed of germ cells and somatic Sertoli cells. The latter is composed of connective tissue containing Leydig cells, fibroblasts, collagen fibers, myoid cells, blood cells, blood vessels, and amyelinic fibers (Grier 1993; Cinquetti and Dramis 2003; Lo Nostro *et al.* 2004; Grier and Uribe Aranzábal 2009).

In teleost fishes, spermatogenesis occurs in the testicular germinal epithelium within spermatocysts, synchronous clones of maturing germ cells surrounded by Sertoli cell processes (Callard 1991; Grier 1993; Schulz *et al.* 2009). This complex process initiates with the mitotic proliferation of spermatogonia, proceeds through two meiotic divisions, and concludes with spermiogenesis, during which the haploid spermatids transform into motile, flagellated genome vectors, the spermatozoa (Nóbrega *et al.* 2009; Schulz *et al.* 2009).

Arrangement of the testicular germinal epithelium varies among osteichthyans: basal taxa have an anastomosing tubular testis, whereas derived teleosts, including Atherinomorpha, exhibit a lobular testis, which can be divided into two types based on distribution and arrangement of spermatogonia. In atherinomorph fishes, the testis corresponds to a restricted lobular type, in which lobules show an orderly progression of developing germ cells, with spermatogonia confined to the distal termini. By contrast, non-atherinomorph neoteleosts possess an unrestricted lobular testis, where spermatogonia and spermatocysts at various stages of development are distributed throughout the length of the lobules (Grier 1981; Parenti and Grier 2004; Grier and Uribe Aranzábal 2009).

In some teleost species, histological changes in the morphology of the testicular germinal epithelium have been used to document a sequence of five reproductive classes during the annual reproductive cycle: regressed, early maturation, mid-maturation, late maturation, and regression. This classification is based on the germ cells stages present and the alternation between a continuous and discontinuous germinal epithelium (Grier and Taylor 1998; Taylor *et al.* 1998; Grier 2002; Lo Nostro *et al.* 2003; Parenti and Grier 2004). Among Perciformes, these classes have been described in males of common snook, *Centropomus undecimalis* (Taylor *et al.* 1998), spotted sea trout, *Cynoscion nebulosus* (Brown-Peterson 2003), cobia, *Rachycentron canadum* (Brown-Peterson *et al.* 2002), and the freshwater goby, *Padogobius bonelli* (as *Padogobius martensi*, Cinquetti and Dramis 2003).

The South American cichlid fish *Cichlasoma dimerus*, a perciform teleost, is common in quiet shallow waters of the Paraguay and most of the Paraná River basins (Kullander 1983). This freshwater species acclimates easily to captivity and shows notable reproductive features and acceptable survival rates, providing an appropriate model for reproductive and developmental studies (Pandolfi *et al.* 2009). Polder (1971) provided a general description of testis structure in this species (previously refereed to as *Aequidens portalegrensis*). However, this report was limited to light microscopy observations that were not considered within the context of current knowledge on male reproduction. The purpose of the present study was to analyze spermatogenesis using light and electron microscopy techniques in the testis of *C. dimerus*, a multiple spawning fish representative of the ichthyofauna of the La Plata River basin. In addition, it aimed at comparing the testicular germinal and interstitial compartments among the five reproductive classes by light microscopy.

Materials and Methods

Adult specimens of *C. dimerus* were captured in Esteros del Riachuelo, Corrientes, Argentina (27°25′S, 58°15′W), by local fishermen and transferred to the laboratory, where they were kept in community aquaria. Room temperature was regulated so that water temperature ranged from 22 to 26 °C from April to August and from 25 to 30 °C during the rest of the year. Photoperiod conditions were set at 12:12 h overlapping with the natural occurring light–dark cycle. Fish were fed pelleted commercial food (TetraCichlid® food sticks) occasionally supplemented with *Tubifex* worms. Reproductive pairs (n = 6) were isolated in well-aerated 45-L aquaria provided with a layer of gravel and smooth stones for egg deposition on the bottom. Pair behavior was observed daily and spawning events were registered monthly during one (three pairs) or 2 years (three pairs). Fertilization was verified by larvae hatching.

Males (n: 70; standard length: 9.5–12 cm; weight: 30–80 g) belonging to community aquaria were sacrificed throughout the year. Fish were anesthetized with Fish Calmer (active ingredients: acetone, dimethylketone, alpha methyl quinoline; Jungle Laboratories, Cibolo, TX, USA), weighed, and measured. Males then were sacrificed by decapitation, and the testes were quickly removed and weighed to determine the gonadosomatic index (GSI). To histologically evaluate testis characteristics during the reproductive cycle of the species, testes from 30 males were processed according to conventional histological techniques.

For light microscopy, samples were fixed in Bouin's liquid, gradually dehydrated, and embedded in paraffin. Six-micrometer-thick sections were stained with hematoxylin–eosin or Masson's trichrome. To identify proliferating cells within the testis, portions of each organ were fixed in 10% neutral buffered formalin and proliferating cell nuclear antigen (PCNA) immunodetection was performed in serial sections according to Ortego *et al.* (1994), using a monoclonal mouse antibody anti-PCNA (1 : 100 dilution; Enzo Diagnostics, Syosset, NY, USA) and a biotinylated horse anti-mouse IgG secondary antibody (1 : 300 dilution; Sigma, St Louis, MO, USA). Slides were examined and subsequently photographed with a Nikon Microphot FX microscope.

For transmission electron microscopy (TEM), 2-mm-thick sections of the testes were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h at 4 °C. Sections were then rinsed in 0.1 M phosphate buffer, postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 2 h at room temperature, subsequently rinsed in distilled water, dehydrated in a graded alcohol series and acetone, and embedded in Spurr resin. Ultrathin sections were obtained with a Sorvall MT2-B ultramicrotome, stained with aqueous uranyl acetate and lead citrate, and examined with a Philips EM 301 microscope. Semithin sections stained with toluidine blue were used for orientation.

Testes of three additional males were dissected and individually macerated in 2 mL of saline solution. Resulting suspension was placed in slides and whole-mount spermatozoa were observed and photographed under the light microscope.

Results

Testis structure

The testes of *C. dimerus* are elongated filiform paired organs, triangular or ovally shaped in cross-section. They are located ventral to the swimbladder and are connected to the dorsal body wall by a mesorchium. The efferent duct runs dorsally, parallel to the main artery and vein that drain to and from the organ (Fig. 1A,B). The wall of the testis is a serosa composed of a simple mesothelium and a thin sheath of underlying connective tissue. Two compartments can be distinguished within the testis: the germinal, composed of Sertoli cells and germ cells, and the interstitial, containing connective tissue (Fig. 1A).

The testes belong to the unrestricted lobular type. Spermatocysts containing clones of germ cells at different stages of spermatogenesis, from spermatogonia to spermatids, occur throughout the length of the lobules. The lobules end blindly at the periphery of the organ and are limited by a basement membrane that separates them from the interstitial tissue (Fig. 3A). Sperm is released during spermiation into the lobular lumen, which is continuous with the efferent duct (Fig. 1A,B).

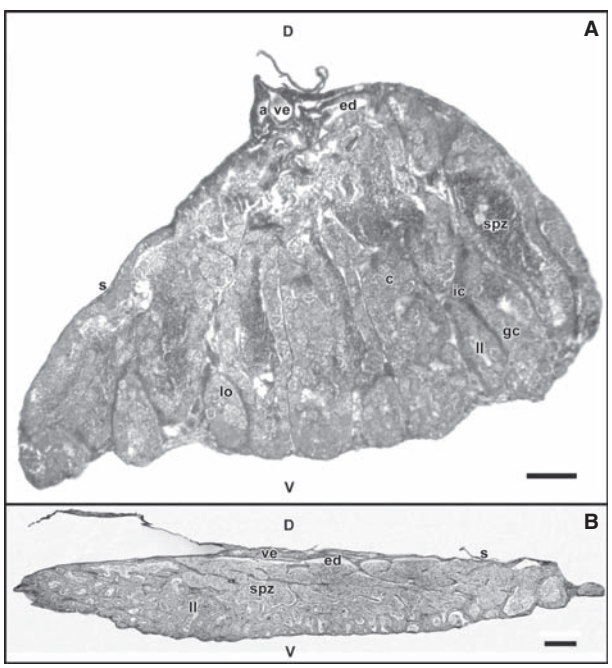

Fig. 1—Testis of *Cichlasoma dimerus* at mid-maturation. —**A**. Cross-section. —**B**. Sagittal section. Scale bars: (**A**) 100 μm, (**B**) 200 μm. a, artery; c, cyst; D, dorsal; ed, efferent duct; gc, germinal compartment; ic, interstitial compartment; lo, lobule; ll, lobular lumen; s, serose; spz, sperm; V, ventral; ve, vein.

Germinal compartment

The germinal compartment is formed by testicular lobules containing germ cells and Sertoli cells.

Type A spermatogonia (SPGA) are the largest germ cells in the testis. They are round to oval in shape, with a hyaline cytoplasm. They are characterized by a prominent spherical central nucleus containing a single nucleolus. Peripheral chromatin is frequently observed, in different degrees of condensation depending on the mitotic stage (Fig. 2A). TEM (Fig. 2B,C,E) reveals that SPGA are individually surrounded by Sertoli cell processes. Nuclei of SPGA exhibit relatively clear areas occupied by euchromatin and dense heterochromatin associated with the nuclear envelope. Smooth endoplasmic reticulum (SER), swirls of SER, abundant mitochondria with lamellar cristae, and cup-shaped mitochondria can be observed in the cytoplasm. Annulate lamellae usually appear as stacked cisternae with pore complexes similar to those of the nuclear envelope and may be disposed as concentric circles or even form amphitheater-like structures (Fig. 2D). Also present are clumps of electron-dense substance referred to as nuage that may become associated with mitochondria forming complexes (Fig. 2F).

Type B spermatogonia (SPGB) are grouped together within spermatocysts, bordered by Sertoli cell processes. SPGB are characterized by a round shape and a prominent spherical nucleus containing one nucleolus. Chromatin aggregation differs according to the cell cycle phase. SPGB are smaller in size than SPGA (Fig. 2A). Although difficult to discern, intercellular bridges between SPGB can sometimes be observed ultrastructurally. The cytoplasm contains annulate lamellae, mitochondria with lamellar cristae, a few cup-shaped mitochondria, scarce nuage, a Golgi apparatus, and SER. Swirls of SER are detected as well. A decrease in the number of mitochondria is evident when compared to SPGA (Fig. 3A,B).

Primary spermatocytes (SPCI) are similar in size and shape to SPGB. They possess a hyaline cytoplasm and a spherical nucleus that differs in appearance according to the stage of the meiotic prophase and contains one nucleolus (Fig. 2A). Under TEM (Fig. 3C), intercellular bridges can be observed. The cytoplasm contains scarce mitochondria with lamellar cristae, smaller in size than those of SPGB. Nuage, SER, and a Golgi apparatus are also observed, as well as annulate lamellae, which can be found in close association with the nuclear envelope (Fig. 3C inset). At pachytene, synaptonemal complexes are evident within the nucleus.

Secondary spermatocytes (SPCII) are rarely found in a testis section because this stage has a short duration. A decrease in size becomes evident when compared to the previous stage. The spherical nucleus is surrounded by a poorly discernable

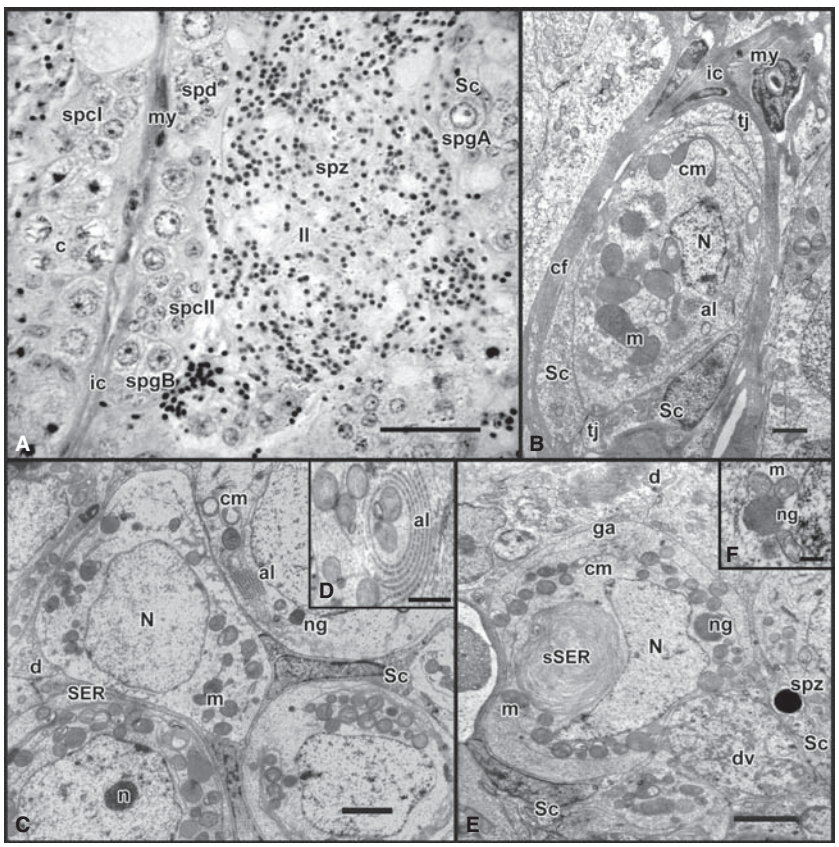

Fig. 2—Spermatogenesis in *Cichlasoma dimerus*. —**A**. Lobules under light microscopy. —**B, C, E**. SPGA under TEM. Note in Fig. E the presence of phagocytized sperm cells within Sertoli cells. —**D**. Detail of annulate lamellae. —**F**. Detail of a nuage–mitochondria complex. Scale bars: (**A**) 10 μm, (**B, C, E**) 2 μm, (**D, F**) 0.5 μm. al, annulate lamellae; c, cyst; cf, collagen fibers; cm, cup-shaped mitochondrion; dv, digestive vacuoles; ga, Golgi apparatus; ic, interstitial compartment; ll, lobular lumen; m, mitochondrion with lamellar cristae; my, myoid cell; N, nucleus; n, nucleolus; ng, nuage; Sc, Sertoli cell; SER, smooth endoplasmic reticulum; spcI, primary spermatocytes; spcII, secondary spermatocytes; spd, spermatid; spgA, spermatogonia A; spgB, spermatogonia B; spz, sperm; sSER, swirls of SER; tj, tight junction.

cytoplasm (Fig. 2A). Ultrastructurally (Fig. 3D), cytoplasmic limits are difficult to identify owing to the presence of numerous intercellular bridges joining adjacent cells, being only evident in the areas where intercellular spaces are distinguished. The nucleus has a well-defined regular outline. Mitochondria are small with lamellar cristae. Polyribosomes, SER, and multilamellate bodies are also observed. Centrioles can be observed close to the nucleus.

Spermatids (SPD). Early SPD have spherical nuclei with dense, irregular chromatin strands (Fig. 2A). During spermiogenesis, SPD undergo shape remodeling and size reduction. The intercellular spaces between cells within the spermatocyst become larger and the nuclei become smaller as the chromatin condenses. Under TEM (Fig. 3C,E), cytoplasmatic bridges are evident, and intercellular spaces are variable in size and shape. The nucleus contains one nucleolus. Centrioles, from which flagella will form, can be observed within the cytoplasm. The distribution and structure of other organelles are initially similar to those observed in SPCII.

Spermatozoa (SPZ). In this species, SPZ are biflagellate and belong to the anacrosomal type I aquasperm. Flagella remain separate from each other along their entire length (Fig. 4). Ultrastructurally, the cylindrical head is entirely occupied by the nucleus, which has highly condensed chromatin and a shallow nuclear fossa (Fig. 3F). The mid-piece contains four rings of nine spherical mitochondria with lamellar cristae (Fig. 3F,G,I). Densification occurs between contiguous outer membranes of adjacent mitochondria (Fig. 3G,I). The mitochondrial rings are separated from the two centrioles by a cytoplasmic canal (Fig. 3G). Each of them gives rise to a flagellum (Fig. 3F). In cross-sections, the typical 9 + 2 axoneme and two well-defined lateral fins are observed (Fig. 3H,I). The axoneme is a continuation of the basal body; the two central singlets originate shortly after the basal body. In the transition region between centriole and axoneme, nine well-developed satellite rays can be observed (Fig. 3G).

Sertoli cells (SC) are the only somatic cell type occurring within the germinal compartment. They adopt various shapes and have a triangular to elongated nucleus with an eccentric nucleolus (Fig. 2). Optically, the cytoplasm is difficult to identify, but under TEM it appears rather dense. More than a single SC envelopes SPGA (Fig. 2B). SC processes form the walls of spermatocysts. These cells are joined by desmosomes and tight junctions and are supported by the thin basement membrane that limits the testicular lobules, separating them from the interstitial compartment (Figs 2B,C,E and 3A,C). Numerous rounded mitochondria with lamellar cristae, free ribosomes, polyribosomes, rough endoplasmic reticulum, and a Golgi apparatus are observed (Figs 2B,C and 3A). SC phagocytize residual bodies and degenerating cells. Vacuoles containing cellular debris or whole sperm cells are frequently observed as large autophagic or digestive vacuoles within the

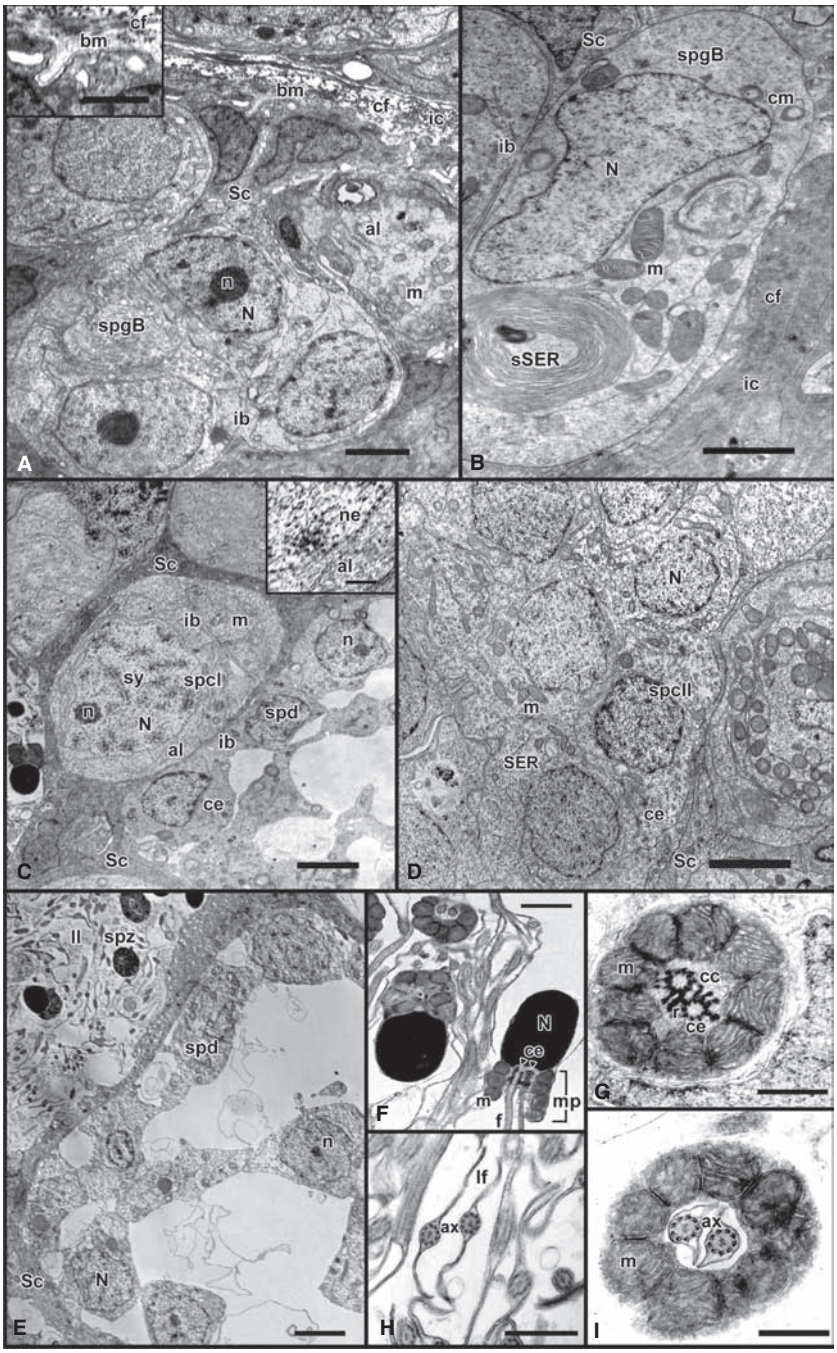

Fig. 3—Spermatogenesis in *Cichlasoma dimerus.* TEM. —**A, B**. SPGB. (Inset A) Detail of the basement membrane. —**C**. SPCI (at pachytene) and SPD. (Inset C) Detail of annulate lamellae in close association with the nuclear envelope.—**D**. SPCII. —**E**. SPD. —**F**. Longitudinal section of spermatozoon showing the two flagella. —**G, I**. Cross-section of the spermatozoan mid-piece showing the centrioles and axonemes, respectively. Note the densification between mitochondria. —**H**. Cross-section of flagella showing the lateral fins. Scale bars: (**A**–**E**) 2 μm, (**F**) 0.75 μm, (**G**–**I**) 0.5 μm. (Inset A, C) 1μm. al, annulate lamellae; ax, axoneme; bm, basement membrane; cc, cytoplasmic canal; ce, centriole; cf, collagen fibers; cm, cup-shaped mitochondrion; d, desmosome; f, flagellum; ib, intercellular bridge; ic, interstitial compartment; lf, lateral fins; ll, lobular lumen; m, mitochondrion with lamellar cristae; mp, mid-piece; N, nucleus; n, nucleolus; ne, nuclear envelope; r, radial fibrils; Sc, Sertoli cell; spcI, primary spermatocytes; spcII, secondary spermatocytes; spd, spermatid; spg B, spermatogonia B; spz, sperm; sSER, swirls of SER; sy, synaptonemal complex.

cytoplasm of SC (Fig. 2E). Spermiation is accomplished by rupture of the spermatocyst wall and release of spermatozoa into the lobular lumen (Fig. 7D). Following spermiation, a layer of SC persists lining the lobule walls (Fig. 9B,C,E).

Interstitial compartment

The interstitial compartment is located between lobules and is composed of Leydig cells, myoid cells, macrophages, collagen fibers, amyelinic nerves, and blood vessels (Figs 3B, 5 and 7C).

Leydig cells (Figs 5A and 7C) are oval cells which occur in small clusters in the space between adjoining lobules, near blood capillaries. The spherical nucleus contains compact chromatin bordering the nuclear envelope and a peripheral nucleolus. Mitochondria, free ribosomes, polyribosomes, lipid droplets, rough and smooth endoplasmic reticulum, and a Golgi apparatus are present in the cytoplasm.

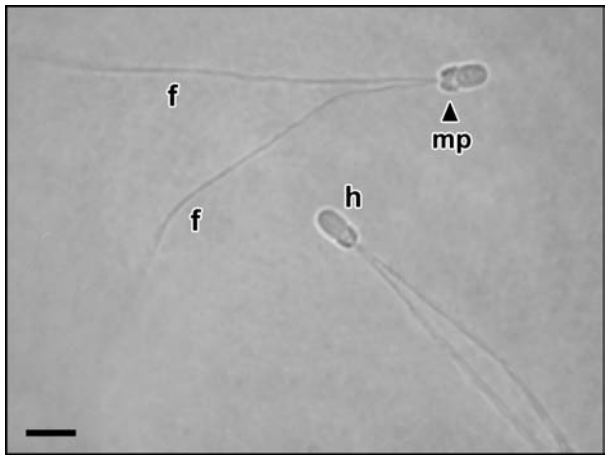

Fig. 4—Whole-mount spermatozoa of *Cichlasoma dimerus* under light microscopy. Note the presence of two long flagella. Scale bar: 2 μm. f, flagellum; h, head; mp, mid-piece.

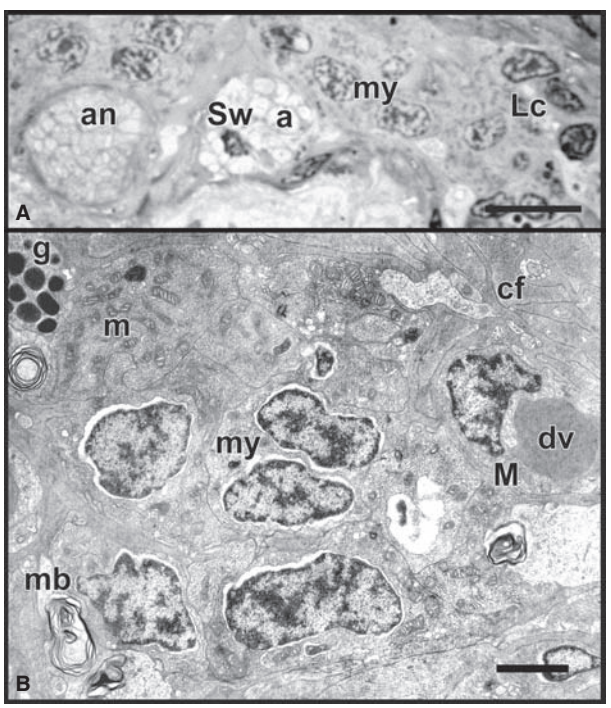

Fig. 5—Interstitial compartment of *Cichlasoma dimerus* testis. —**A**. Light microscopy. —**B**. TEM. Scale bars: (**A**) 10μm, (**B**) 2μm. a, axon; an, amyelinic nerve; ce, centriole; cf, collagen fibers; dv, digestive vacuole; g, granulocyte; Lc, Leydig cell; m, mitochondrion with lamellar cristae; M, macrophage; mb, multivesicular body; my, myoid cell; Sw, Schwann nucleus.

Myoid cells (Fig. 5) are the most abundant cell type found in the interstitial compartment. They reside in close proximity to collagen fibers, bordering the lobules. Ultrastructurally, the cytoplasm is commonly filled with microfilaments that run parallel to the long axis of the cell. The nucleus is variable in shape and contains clumps of heterochromatin. Mitochondria are the most abundant organelle. Centrioles and multivesicular bodies can also be observed. The remaining organelles are scarce.

Macrophages (Fig. 5B) are found in discrete groups or isolated in the testis interstitium, near Leydig cells, myoid cells, or blood vessels. They have an irregular outline because of the presence of cell processes. Under TEM, they show an irregular nucleus, with relatively condensed peripheral chromatin. Mitochondria and a Golgi apparatus are present, though frequently masked by the numerous digestive vacuoles.

Amyelinic nerves (Fig. 5A) are observed between myoid cells. In cross-section, each nerve is composed of many unmyelinated axons of variable diameter encompassed by a cellular sheath of Schwann cell projections.

Reproductive activity and changes in the germinal epithelium (GE)

The season of high reproductive activity in *C. dimerus* extended over 7 months, from September to March (spring and summer) (Fig. 6). Spawning was registered on average every 29.4 ± 16.2 days (mean ± SD) during this period, being 13 days the shortest interval between successive spawnings. Reproductive activity decreased from April to August, with occasional spawnings still occurring (Fig. 6). Changes in the GSI showed a correlation with the reproductive condition, beginning to increase in September and to decline in April (Fig. 6).

Changes in the germinal compartment allowed the recognition of five reproductive classes: regressed; early, mid-, and late maturation, and regression class. During the high reproductive season, each cycle went through the first four classes. The regressed class overlapped with the late maturation class, because while sperm was being released, spermatogonia were proliferating (Fig. 7D). The regression class occurred solely at the end of the final reproductive cycle and was evidenced in sexually inactive males during the low reproductive season.

The regressed class is a proliferative phase. Spermatogonia undergo rapid successive mitotic divisions. A characteristic continuous germinal epithelium, consisting of individual SPGA or clusters of SPGB surrounded by Sertoli cells processes forming spermatocysts, is observed from the distal end of the lobules to the dorsal duct (Fig. 7A,B). A positive reaction to nuclear PCNA antibodies in both germ and Sertoli cells reveals mitotic activity (Fig. 7D).

The lobular lumina are discontinuous in the germinal compartment of the regressed testis. In the absence of sperm, opposite sides of lobules are seen juxtaposed, frequently giving the appearance of solid cell cords (Fig. 7A).

The early maturation class (Fig. 8A,B) marks the beginning of meiotic division, defined by a continuous GE composed of juxtaposed SPGA and spermatocysts, predominantly containing SPCI at different stages of the first meiotic prophase. A

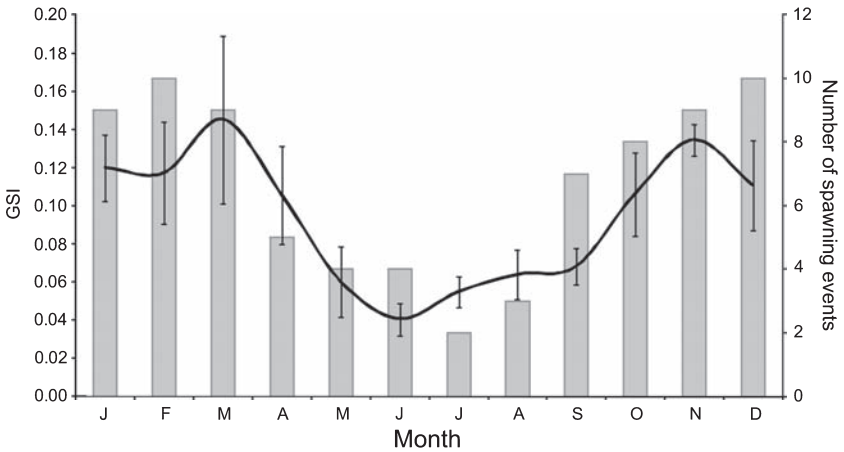

Fig. 6—Changes in the number of spawning events (bars) and GSI (line) in male *Cichlasoma dimerus* during the year. Error bars of GSI represent SD.

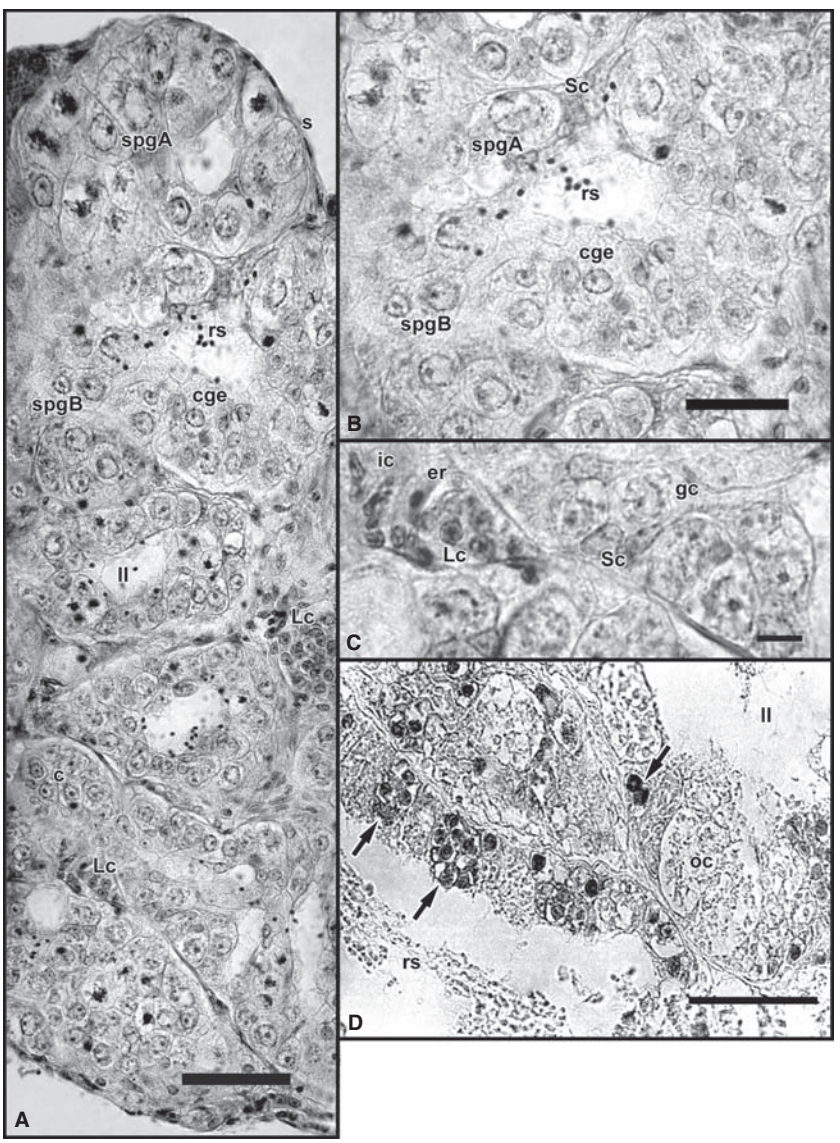

Fig. 7—Testis of *Cichlasoma dimerus* in regressed class under light microscopy. —**A**. Parasagittal section. —**B**. Detail of a lobule. —**C**. Cluster of Leydig cells in the interstitial compartment. —**D**. Positive reaction to nuclear PCNA (arrows). Note the open cyst releasing sperm, demonstrating overlapping with the late maturation class. Scale bars: (**A**) 30 μm, (**B**) 20 μm, (**C, D**) 10 μm. c, cyst, cge, continuous germinal epithelium; er, erythrocyte; gc, germinal compartment; ic, interstitial compartment; Lc, Leydig cell; ll, lobular lumen; oc, open cyst; rs, residual sperm; s, serose; Sc, Sertoli cell; spgA, spermatogonia A; spg B, spermatogonia B.

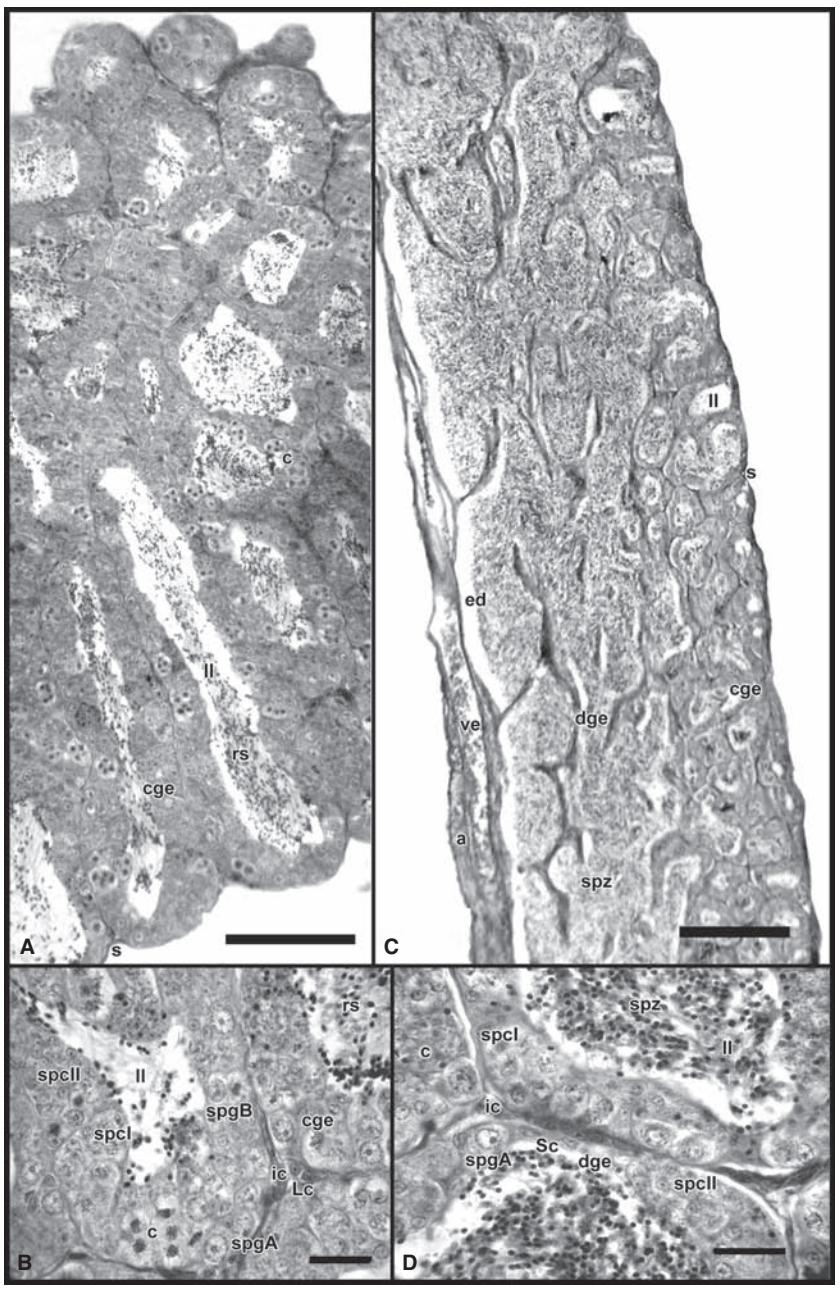

Fig. 8—Testis of *Cichlasoma dimerus* in early maturation (**A, B**) and mid-maturation (**C, D**) classes under light microscopy. —**A**. Parasagittal section. —**B**. Detail of a lobule. —**C**. Sagittal section. —**D**. Detail of a lobule. Scale bars: (**A**) 50 μm (**B, D**) 20 μm, (**C**) 100 μm. a, artery; c, cyst, cge, continuous germinal epithelium; dge, discontinuous germinal epithelium; ed, efferent duct; ic, interstitial compartment; Lc, Leydig cell; ll, lobular lumen; rs, residual sperm; s, serose; Sc, Sertoli cell; spcI, primary spermatocytes; spcII, secondary spermatocytes; spgA, spermatogonia A; spg B, spermatogonia B; spz, sperm; ve, vein.

few spermatocysts containing SPCII can also be observed. There is an increase in both height and width of the testes because of the elongation of lobules. In this class, lobules commonly have discontinuous lumina with residual sperm.

The mid-maturation class is characterized by the presence of a discontinuous GE near the efferent ducts and a continuous GE distally in the lobules (Fig. 8C). In the discontinuous GE, regions composed of Sertoli cells alone alternate with regions of Sertoli cells associated with germ cells, ranging from SPGA to SPZ (Fig. 8D). Mitotic division of spermatogonia continues. The testis lobules and efferent ducts become filled with sperm and their lumina converge. As mid-maturation progresses, sperm maturation followed by spermiation increases the amount of discontinuous GE toward the distal end of lobules (Fig. 8C). Near the testis duct, the function of lobules shifts from sperm production to sperm storage. There is a greater increase in lobule dorsal to ventral height, together with both branching of lobules at the periphery and formation of lateral anastomosis (Figs 1B and 8C).

The late maturation class is characterized by the presence of at least one lobule with discontinuous GE at the distal terminus (Fig. 9A). The morphology of each lobule does not

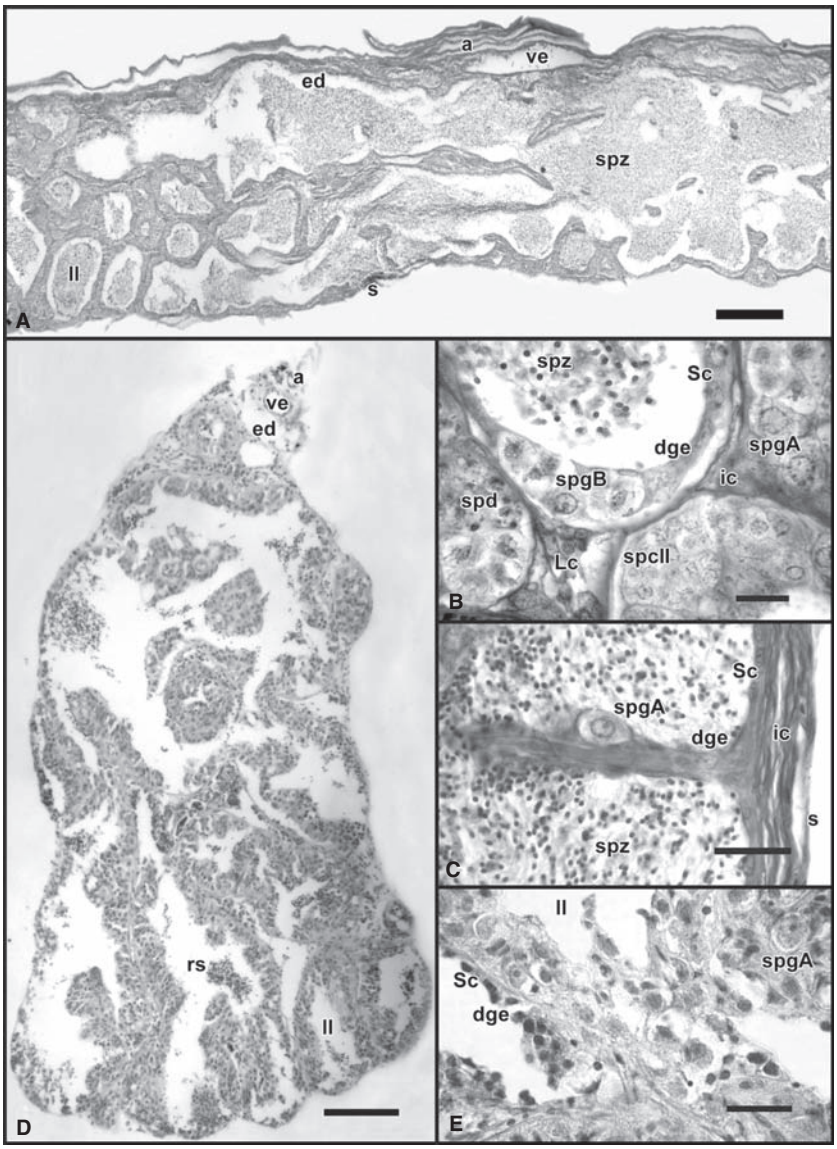

Fig. 9—Testis of *Cichlasoma dimerus* in late maturation (**A**–**C**) and regression (**D, E**) classes under light microscopy. —**A**. Sagittal section. —**B, C**. Detail of a lobule. —**D**. Cross-section. —**E**. Detail of a lobule. Scale bars: (**A, D**) 100 μm, (**B, C, E**) 20 μm. a, artery; dge, discontinuous germinal epithelium; ed, efferent duct; ic, interstitial compartment; Lc, Leydig cell; ll, lobular lumen; rs, residual sperm; s, serose; Sc, Sertoli cell; spcII, secondary spermatocytes; spd, spermatid; spgA, spermatogonia A; spgB, spermatogonia B; spz, sperm; ve, vein.

change in unison; as late maturation progresses, the discontinuous GE extends to all lobules (Fig. 9B,C). Sperm production remains mainly restricted to the peripheral portions of the testis. Testes become sperm storage organs; lateral anastomosis that first appeared between adjacent lobules during mid-maturation now become more evident and numerous. The efferent duct is filled with sperm (Fig. 9A) and has a simple squamous epithelium. During the late maturation class, testes reach their maximum size.

The regression class is only observed in sexually inactive males, which are predominant during the period of decreased reproductive activity. It is distinguished by the presence of scattered spermatocysts, some of them apparently degenerating. The discontinuous GE is composed mainly of Sertoli cells and dispersed SPGA that persist from the previous class. During regression, the lobular integrity of the testis is strongly altered. The presence of residual sperm and granulocytes is common (Fig. 9D,E). In this class, there is a decrease in GSI, which attains its minimal values (Fig. 6).

Within the interstitial compartment, collagen fibrils, myoid cells, and Leydig cells are easily distinguished throughout the year. Leydig cells are more easily observed from the proliferative phase to the early maturation class, coinciding with the absence of sperm within the testis (Figs 7A,C and 8B).

Discussion

As in all vertebrates, the testis in fish is composed of two main compartments, the germinal and the interstitial compartments. The basic functional unit of the spermatogenic

epithelium is a cyst, formed by a group of Sertoli cells surrounding and nursing one synchronously developing germ cell clone (Schulz *et al.* 2009). The cystic type of spermatogenesis is typical of fishes and amphibians (Callard 1991, 1996; Grier 1993; Mattei 1993).

Testicular structure in *C. dimerus* corresponds to an unrestricted lobular type (Grier 1981). The unrestricted spermatogonial distribution is characterized by the occurrence of spermatogonia all along the germinal compartment (Grier and Uribe Aranzábal 2009). In the present study, the characteristics of germ cell stages during spermatogenesis, from spermatogonia to spermatozoa, were studied in adult specimens of *C. dimerus*. Two types of spermatogonia could be described. SPGA were the largest cells, individually enveloped by Sertoli cells. SPGB occurred as two or more smaller cells per cyst, joined by cytoplasmic bridges (Billard 1984; Selman and Wallace 1986). Sertoli cells were hence in contact with a single syncytial germ cell clone throughout spermatogenesis, as proposed by Clérot (1971). Intercellular bridges were observed connecting germ cells, from SPGB to SPD, until spermiogenesis when the latter discard their excess cytoplasm as residual bodies. These bridges are thought to be responsible for the synchronous development of germ cells by allowing the exchange of molecules between cells (Gilbert 2003). SPCII were scarce and difficult to observe, probably due to their short lifespan because these cells undergo a rapid division to become SPD.

Annulate lamellae are normally found in rapidly dividing cells and germ cells (Bozzola and Russell 1992). In this study, they were observed in spermatogonia and SPCI as a cytoplasmic array of stacked membrane cisternae. Moreover, they were seen in close association with the nuclear membrane, either integrating into or being released from it. Annulate lamellae contain densely packed pore complexes which are similar in structure to nuclear pore complexes. The function of annulate lamellae is believed to be the storage of nuclear pores and nuclear membrane (Stafstrom and Staehelin 1984). It is disputed whether, during cell division, pieces of the fragmented nuclear envelope may become excluded from the re-forming nuclear membrane and thus give rise to annulate lamellae in the cytoplasm.

Another characteristic organelle in germ cells is the nuage, which appear as large amounts of electron-dense material. It is assumed that this dense material is composed of long half-life ribonucleoproteins and mRNA (Knaut *et al.* 2000; Houwing *et al.* 2007). In *C. dimerus*, nuage and their aggregates with mitochondria occurred mainly from spermatogonia to SPD.

Mitochondria with lamellar cristae were observed in all germ cell stages. Spermatogonia, in particular, also presented large amounts of cup-shaped mitochondria.

Spermatozoa in this species correspond to the anacrosomal type I aquasperm (Mattei 1970, 1991; Jamieson 1991). In addition, light microscopy and TEM revealed that they are biflagellate. Biflagellate sperm have only been observed in few other teleost species, including the apogonid *Paranocheilus* sp., the zoarcid *Zoarces elongates,* and the gobiesocid *Lepadogaster lepadogaster*, among perciforms (Jamieson 2009). The existence of two flagella is even more exceptional among cichlids. To our knowledge, the only cichlid with biflagellate sperm described so far is *Satanoperca jurupari*, a self-fertilizing hermaphrodite with introsperm-type spermatozoa (Matos *et al.* 2002). Spermatozoa in *C. dimerus* showed lateral fins on both flagella, a trait observed in the sperm tail of several species, including *S. jurupari* (Matos *et al.* 2002) and other perciforms such as *Boleophthalmus pectinirostris* (Gobiidae) (Chung 2008). Lateral fins are present in most externally fertilizing sperm except for Ostariophysi (Characiform, Cypriniform, and Siluriform clades) (Burns *et al.* 2009). The mitochondrial collar is formed by four rings of nine round mitochondria, rendering, in general, a total number of 36.

In this study, none of the three characteristics that seem to be exclusively observed in Cichlidae sperm – compact filamentous clusters of chromatin, a slightly eccentric nuclear fossa, and about 10 mitochondria within the mid-piece (Quagio-Grassiotto *et al.* 2003) – were evidenced. In *C. dimerus*, sperm presents homogeneously condensed chromatin, a mainly central nuclear fossa and a number of mitochondria that considerably exceeds 10. The spermatozoan ultrastructure of more cichlid species must be analyzed to evaluate the phylogenetic significance of these characters.

Spermatocysts are bordered by Sertoli cell processes joined laterally by desmosomes and tight junctions. These processes completely envelop germ cells throughout spermatogenesis, isolating them from contact with both the basement membrane, clearly observed under TEM, and the lobular lumen. The definition of testicular germinal epithelium is based on Sertoli cells fulfilling the criteria used to define an epithelium (Grier and Lo Nostro 2000). The Sertolian component of the germinal epithelium persists as a monolayer after the release of sperm into the lobular lumen, whereas the germinal component becomes scattered constituting the 'discontinuous germinal epithelium' (Grier and Taylor 1998).

In *C. dimerus*, phagocytized spermatozoa were observed in the cytoplasm of Sertoli cells. This function constitutes a mechanism to eliminate residual sperm. In agreement with this function of Sertoli cells, it comes as no surprise that melano-macrophage centres were not observed. Similar findings were made in testes of cobia, *R. canadum* (Brown-Peterson *et al.* 2002), confirming that sperm phagocytosis by macrophages is far less common that phagocytosis by Sertoli cells (Scott and Sumpter 1989). In other species such as common snook, *C. undecimalis* (Grier and Taylor 1998) and swamp eel, *Synbranchus marmoratus* (Ravaglia and Maggese 1995), residual spermatozoa appeared to be phagocytized by melano-macrophage centers in postreproductive males.

As is the norm for teleosts, Leydig cells were found as interstitial components in the testis of *C. dimerus*, in close association with blood vessels and located in the spaces between lobules. Blood vessels contained formed elements

(erythrocytes, thrombocytes, and four types of leukocytes: lymphocytes, monocytes, heterophils, and eosinophils), whose morphological features were previously described for this species by Rey Vázquez and Guerrero (2007). In addition to Leydig cells, Polder (1971) described another cellular type composing the interstitium in this species (referred to as *A. portalegrensis* in the scientific literature at that time), the 'lipoid cells'. They differed from Leydig cells by the absence of sudanophil droplets in Sudan black–stained gelatine sections and did not show any correlation with reproductive behavior (Polder 1971). Although in this study, lipoid cells were not identified under optical microscopy, by the light of current knowledge, they are probably previous functional stages of fully differentiated and actively secreting Leydig cells. Lipid content of Leydig cells undergoes pronounced seasonal changes in most teleost species, because it serves as the main source of gonadal steroids synthesis (Chung 2008). However, lipoid droplets were not observed in Leydig cells of *Thalassoma duperrey* (Hourigan *et al.* 1991), *Esox lucius* (Grier *et al.* 1989), *Esox niger* (Grier *et al.* 1989), and *Oryzias latipes* (Gresik *et al.* 1973).

Based on the criteria of the changes occurring in the germinal epithelium, between continuous and discontinuous epithelia, and the stages of germ cells present, Grier and Taylor (1998) and Grier (2002) proposed five reproductive classes during the annual reproductive cycle for Perciformes: regressed; early, mid-, and late maturation, and regression classes. In this study, all five reproductive classes could be identified and described for *C. dimerus*. Because this species is a multiple spawner, they did not occur as one lineal sequence throughout the year, as would be expected in a single spawner. The season of higher reproductive activity extended over 7 months, from September to March, during which spawning took place on average every 29.4 ± 16.2 days as the result of a completed cycle. In these successive cycles not all reproductive classes were present, with the regression class being absent. The regressed class overlapped with the late maturation class, because while sperm was being released, spermatogonia were simultaneously proliferating. This simultaneity was better evidenced by immunohistochemical detection of PCNA during the reproductive cycle. Therefore, the regressed class in *C. dimerus* could only be clearly defined at the beginning of the reproductive period, mainly at the end of August, whereas the regression class occurred in sexually inactive males during the low reproductive period (April–August). Similar dynamics were observed in common snook, where proliferation of spermatogonia in preparation for the following spawning event begins during the late maturation class (Taylor *et al.* 1998). From our understanding, the classification proposed by Grier and Taylor (1998) is the most appropriate according to the changes in histological characteristics of gonads and their functional correlation. Based on this classification and in order to avoid nomenclature conflicts, Grier and Uribe Aranzábal (2009) recently proposed that the term 'maturation' should be replaced by 'GE development'. In addition, the regressed class has been usually referred to as 'resting'. However, Brown-Peterson *et al.* (2002) suggested that this term should not be used and, accordingly, Grier and Uribe Aranzábal (2009) stated that 'resting stage' does not apply to fish gonads because gonads of teleosts never 'rest'. To solve this discrepancy, Brown-Peterson *et al.* (2007) proposed discarding the use of the terms 'regressed' and 'resting' in favor of 'regenerating'. In our opinion, as spermatogonia undergo rapid successive mitotic divisions during the regressed class, 'proliferative class' would result in a more appropriate and less confusing term to describe the events that occur at this period.

Associated with the changes in the breeding activity throughout the year, the GSI began to increase in September at the beginning of spring and declined in April with the onset of autumn and a descent of temperature. During the summer months, the variability in GSI values was greater than in winter. This was because of successive cycles taking place during the reproductive season, meaning that fish could be sacrificed while undergoing different reproductive conditions. In contrast, during winter, when reproduction reached its minimum, most fish exhibited regression testes and variability in GSI was correspondingly smaller.

In the natural environment, *C. dimerus* probably displays a marked breeding seasonality, with no reproduction occurring during the autumn–winter season. Under the laboratory conditions used in the present study, this seasonality was attenuated and spawning took place throughout the whole year. However, the reproductive activity was lower during the months of low temperature and testes of most males showed a regression condition, which was rarely observed during the high reproductive season.

Reproduction in *C. dimerus* is regulated by both temperature and photoperiod conditions (Fiszbein *et al.* 2010) as occurs in other teleosts (Bromage *et al.* 2001; Cinquetti and Dramis 2003; Alvarenga *et al.* 2006; Batlouni *et al.* 2006; Singh *et al.* 2010), which would explain the changes in GSI and spawning frequency recorded during this study.

Acknowledgements

This study was performed with financial support from the University of Buenos Aires, contract grant number UBACYT × 457 and CONICET, contract grant number PIP 2302.

References

Alvarenga, E. R., Bazzoli, N., Santos, G. B. and Rizzo, E. 2006. Reproductive biology and feeding of Curimatella lepidura (Eigenmann & Eigenmann) (Pisces, Curimatidae) in Juramento reservoir, Minas Gerais, Brazil. – *Revista Brasileira de Zoologia* **23**: 314–322.

Batlouni, S. R., Romagosa, E. and Borella, M. I. 2006. The reproductive cycle of male catfish *Pseudoplatystoma fasciatum* (Teleostei, Pimelodidae) revealed by changes of the germinal epithelium. An approach addressed to aquaculture. – *Animal Reproduction Science* **96**: 116–132.

Billard, R. 1984. Ultrastructural changes in the spermatogonia and spermatocytes of *Poecilia reticulata* during spermatogenesis. – *Cell and Tissue Research* **237**: 219–226.

Bozzola, J. J. and Russell, L. D. 1992. *Electron Microscopy. Principles and Techniques for Biologist*, pp. 542. Jones and Bartlett Publishers, London.

Bromage, N., Porter, M. and Randall, C. 2001. The environmental regulation of maturation in farmed finfish with special references to the role of photoperiod and melatonin. – *Aquaculture* **197**: 63–69.

Brown-Peterson, N. 2003. The reproductive biology of spotted seatrout. In: Bortone, S. (Ed.): *Biology of the Spotted Seatrout*, pp. 99–134. CRC Press, Boca Raton, Florida.

Brown-Peterson, N. J., Grier, H. J. and Overstreet, R. M. 2002. Annual changes in germinal epithelium determine male reproductive classes of the cobia. – *Journal of Fish Biology* **60**: 178–202.

Brown-Peterson, N., Lowerre-Barbieri, S., Macewicz, B., Saborido-Rey, F., Tomkiewicz, J. and Wyanski, D. 2007. An improved and simplified terminology for reproductive classification in fishes. Available at: http://hdl.handle.net/10261/11844 (accessed on 11 July 2007).

Burns, J. R., Quagio-Grassiotto, I. and Jamieson, B. G. M. 2009. Ultrastructure of spermatozoa: Ostariophysi. In: Jamieson, B. G. M. (Ed.): *Reproductive Biology and Phylogeny of Fishes (Agnathans and Bony Fishes): Phylogeny Reproductive System Viviparity Spermatozoa*, Vol. 8A, pp. 287–387. Science Publishers, Enfield, NH, USA.

Callard, G. V. 1991. Spermatogenesis. In: Pang, P. and Schreibman, M. (Eds): *Vertebrate Endocrinology: Fundamentals and Biomedical Implications*, pp. 303–341. Academic Press, New York.

Callard, G. V. 1996. Endocrinology of Leydig cells in nonmammalian vertebrates. In: Payne, A. H., Hardy, M. P. and Russell, L. D. (Eds): *The Leydig Cell*, pp. 308–331. Cache River Press, Vienna, IL, USA.

Chung, E. Y. 2008. Ultrastructure of germ cells, the Leydig cells, and Sertoli cells during spermatogenesis in *Boleophthalmus pectinirostris* (Teleostei, Perciformes, Gobiidae). – *Tissue and Cell* **40**: 195–205.

Cinquetti, R. and Dramis, L. 2003. Histological, histochemical and enzyme histochemical and ultrastructural investigations of the testis of *Padogobius martensi* (Pisces,Gobidae) between breeding seasons. – *Journal of Fish Biology* **63**: 1402.

Clérot, J. C. 1971. Les ponts intercellulaires du testicule de gardon: organisation syncitiale et synchronie de la différenciation des cellules germinales. – *Journal of Ultrastructural Research* **37**: 690–703.

Fiszbein, A., Cánepa, M., Rey Vázquez, G., Maggese, C. and Pandolfi, M. 2010. Photoperiodic modulation of reproductive physiology and behaviour in the cichlidfish *Cichlasoma dimerus*. – *Physiology & Behavior* **99**: 425–432.

Gilbert, S. 2003. *Developmental Biology*, pp. 1–838. Sinauer Associates Inc., USA.

Godinho, H. P. 2007. Reproductive strategies of fishes applied to aquaculture: bases for development of production technologies. – *Revista Brasileira de Reprodução Animal* **31**: 351–360.

Gresik, E. W., Quirk, J. G. and Hamilton, J. B. 1973. A fine structural and histochemical study of the Leydig cells in the testis of the teleost, *Oryzias latipes* (Cypriniforme). – *General and Comparative Endocrinology* **20**: 86–98.

Grier, H. J. 1981. Cellular organization of the testis and spermatogenesis in fishes. – *American Zoologist* **21**: 345–357.

Grier, H. J. 1993. Comparative organization of Sertoli cells including the Sertoli cell barrier. In: Russell, L. D. and Griswold, M. D. (Eds): *The Sertoli Cell*, pp. 703–739. Cache River Press, Clearwater.

Grier, H. J. 2002. The germinal epithelium: its dual role in establishing male reproductive classes and understanding the basis for indeterminate egg production in female fishes. In: Creswell, L. R. (Ed.): *Proceedings of the Fifty-third Annual Gulf and Caribbean Fisheries Institute, Biloxi, Mississippi November 2000*, pp. 537–552. Alabama Sea Grant Consortium, Fort Pierce, FL.

Grier, H. and Lo Nostro, F. 2000. The germinal epithelium in fish gonads: the unifying concept. In: Norberg, B., Kjesbu, O. S., Taranger, G. L., Anderson, E. and Stefansson, S. O. (Eds): *Proceedings of the Sixth International Symposium on the Reproductive Physiology of Fish*, pp. 233–236. The University of Bergen, Bergen, Norway.

Grier, H. J. and Taylor, R. G. 1998. Testicular maturation and regression in common snook. – *Journal of Fish Biology* **53**: 521–542.

Grier, J. H. and Uribe Aranzábal, M. C. 2009. The Testis and Spermatogenesis in Teleosts. In: Jamieson, B. G. M. (Ed.): *Reproductive Biology and Phylogeny of Fishes (Agnathans and Bony Fishes). Phylogeny, Reproductive System, Viviparity, Spermatozoa*, pp. 119–142. Science Publishers, Enfield.

Grier, H., van der Hurk, R. and Billard, R. 1989. Cytological identification of cell types in the testis of *Esox lucius* and *E. niger*. – *Cell and Tissue Research* **257**: 491–496.

Hourigan, T. F., Nahamura, M., Nagahama, Y., Yamauchi, K. and Grau, E. G. 1991. Histology, ultrastructure and *in vitro* steroidogenesis of the testes of two male phenotypes of the protogynous fish *Thalassoma duperrey* (Labridae). – *General and Comparative Endocrinology* **83**: 193–217.

Houwing, S., Kamminga, L. M., Berezikov, E., Cronembold, D., Girard, A., van den Elst, H., *et al.* 2007. A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in Zebrafish. – *Cell* **129**: 69–82.

Jamieson, B. G. M. 1991. *Fish Evolution and Systematics: Evidence from Spermatozoa*, pp. 319. Cambridge University Press, Cambridge.

Jamieson, B. G. M. 2009. Ultrastructure of Spermatozoa: Acanthopterygii continued: Percomorpha. In: Jamieson, B. G. M. (Ed.): *Reproductive Biology and Phylogeny of fishes (Agnathans and Bony Fishes), Phylogeny, Reproductive System, Viviparity, Spermatozoa*, pp 503–684. Science Publishers, Enfield.

Knaut, H., Pelegri, F., Bohmann, K., Schwarz, H. and Nüssslein-Volhard, C. 2000. Zebrafish vasa RNA but not its protein is a component of the germ plasm and segregates asymmetrically before germline specification. – *Journal of Cell Biology* **149**: 875–888.

Kullander, S. O. 1983. *A Revision of the South American cichlid Genus Cichlasoma (Teleostei: Cichlidae)*, pp. 296. Swedish Museum of Natural History, Stockholm.

Lo Nostro, F., Grier, H., Andreone, L. and Guerrero, G. 2003. Involvement of the gonadal germinal epithelium during sex reversal and seasonal testicular cycling in the protogynous swamp eel, *Synbranchus marmoratus* Bloch, 1795 (Teleostei, Synbranchidae). – *Journal of Morphology* **258**: 107–126.

Lo Nostro, F., Antoneli, F., Quagio-Grassiotto, I. and Guerrero, G. 2004. Testicular interstitial cells, and steroidogenic detection in the protogynous fish, *Synbranchus marmoratus* (Teleostei, Synbranchidae). – *Tissue and Cell* **36**: 221–231.

Matos, E., Santos, M. N. S. and Azevedo, C. 2002. Biflagellate spermatozoon structure of the hermaphrodite fish *Satanoperca jurupari* (Heckel, 1840) (Teleostei,Cichlidae) from the Amazon River. – *Brazilian Journal of Biology* **62**: 847–852.

Mattei, X. 1970. Spermiogené se comparé des poisson. In: Baccetti, B. (Ed.): *Comparative Spermatology*, pp. 57–72. Academic Press, New York.

Mattei, X. 1991. Spermatozoon ultrastructure and its systematic implications in fishes. – *Canadian Journal of Zoology* **69**: 3038–3055.

Mattei, X. 1993. Peculiarities in the organization of testis of *Ophidion* sp. (Pisces: Teleostei). Evidence for two types of spermatogenesis in teleost fish. – *Journal of Fish Biology* **43**: 931–937.

Nagahama, Y. 1983. The functional morphology of teleost gonads. In: Hoar, W. S. and Randall, D. J. (Eds): *Fish Physiology, Vol. 9. Part A*, pp. 433. Part B, 477pp. Academic Press, New York.

Nakatani, K., Agostinho, A. A., Baumgartner, G., Bialetzki, A., Sanches, P. V., Makrakis, M. C. and Pavanelli, C. S. 2001. *Ovos e Larvas de Peixes de Água Doce: Desenvolvimento e Manual de Identificação*, pp. 378. Eduem, Maringá.

Nelson, J. S. 2006. *Fishes of the World*, 4th edn. pp. 601. John Wiley and Sons, New York.

Nóbrega, R. H., Batlouni, S. R. and França, L. R. 2009. An overview of functional and stereological evaluation of spermatogenesis and germ cell transplantation in fish. – *Fish Physiology and Biochemistry* **35**: 197–206.

Ortego, L. S., Hawkins, W. E., Walter, W. W., Krol, R. M. and Benson, W. H. 1994. Detection of proliferating cell nuclear antigen in tissues of the three small fish species. – *Biotechnic and Histochemistry* **69**: 6.

Pandolfi, M., Cánepa, M. M., Meijide, F. J., Alonso, F., Rey Vázquez, G., Maggese, M. C. and Vissio, P. G. 2009. Studies on the reproductive and developmental biology of *Cichlasoma dimerus* (Percifomes, Cichlidae). – *Biocell* **33**: 1–18.

Parenti, L. and Grier, H. 2004. Evolution and phylogeny of gonad morphology in bony fishes. – *Integrative and Comparative Biology* **44**: 333–348.

Polder, J. J. K. 1971. On gonads and reproductive behaviour in the cichlid fish *Aequidens portalegrensis* (Hensel). – *Netherlands Journal of Zoology* **21**: 265–365.

Pudney, J. 1995. Spermatogenesis in nonmammalian vertebrates. – *Microscopy Research and Technique* **32**: 459–497.

Pudney, J. 1996. Comparative cytology of the Leydig cell. In: Payne, A. H., Hardy, M. P. and Russell, L. D. (Eds): *The Leydig Cell*, pp. 98–142. Cache River, Vienna, Ill.

Quagio-Grassiotto, I., Antoneli, F. N. and Oliveira, C. 2003. Spermiogenesis and sperm ultrastructure in *Cichla intermedia* with some considerations about Labroidei spermatozoa (Teleostei, Perciformes, Cichlidae). – *Tissue and Cell* **35**: 441–446.

Ravaglia, M. A. and Maggese, M. C. 1995. Melano-macrophage centers in the gonads of the swamp eel, *Synbranchus marmoratus* Bloch (Pisces, Synbranchidae): histological and histochemical characterization. – *Journal of Fish Diseases* **18**: 117–125.

Rey Vázquez, G. and Guerrero, G. A. 2007. Characterization of blood cells and hematological parameters in *Cichlasoma dimerus* (Teleostei, Perciformes). – *Tissue and Cell* **39**: 151–160.

Schulz, R. W., de Franca, L. R., Lareyre, J. J., LeGac, F., Chiarini-García, H., Nóbrega, R. H. and Miura, T. 2009. Spermatogenesis in fish. – *General and Comparative Endocrinology* **165**: 390–411.

Scott, A. P. and Sumpter, J. P. 1989. Seasonal variations in testicular germ cell stages and in plasma concentrations of sex steroids in male rainbow trout (*Salmo gairdneri*) maturing at 2 years old. – *General and Comparative Endocrinology* **73**: 46–58.

Selman, K. and Wallace, R. A. 1986. Gametogenesis in *Fundulus heteroclitus*. – *American Zoologist* **26**: 173–192.

Singh, R., Chaturvedi, S. K. and Abhinav 2010. Effect of photoperiod and temperature on testicular regression in *Channa punctatus*. – *Journal Environmental Biology* **31**: 307–310.

Stafstrom, J. P. and Staehelin, L. A. 1984. Are annulate lamellae in the Drosophila embryo the result of overproduction of nuclear pore components?. – *The Journal of Cell Biology* **98**: 699–708.

Taylor, R. G., Grier, H. J. and Whittington, J. A. 1998. Spawning rhythms of common snook in Florida. – *Journal of Fish Biology* **53**: 502–520.