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Continuous gametogenesis in the neotropical freshwater teleost, *Bryconops affinis* (Pisces:Characidae)

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Abstract. The gametogenesis of *Bryconops affinis* was studied by light, transmission and scanning electron microscopy. The spermatogenesis is semi-cystic and spermatids are released into the lumen of seminiferous tubules, where spermiogenesis is completed. Spermatozoa have an ovoid head, a rudimentary middle piece with a small number of mitochondria and long flagellum (primitive spermatozoa). The Sertoli and Leydig cells show secretory activity during spermatogenesis. By the end of this phenomenon, the Sertoli cells phagocytize the residual spermatozoa, while the Leydig cells show involuted characteristics. With regard to the oogenesis process, the oocyte development was divided into four stages based on the cytological characteristics of the oocyte and its surrounding layers. Ultrastructural analysis revealed that the zona pellucida is formed during the previtellogenic stage. Specializations associated to the outer layer of the zona pellucida may be related to the egg's adherence to the substrata. © 2001 Harcourt Publishers Ltd

Keywords: teleost, gametogenesis, *Bryconops affinis*, Sertoli cell, Leydig cell, adhesive egg

Introduction

The spermatogenic unit of fishes – the spermatocyst – consists of a germ cell or clones of isogenetic germ cells surrounded by cytoplasmic prolongations of one or more Sertoli cells that form the cyst wall (Loir et al., 1995; Pudney, 1995). In teleosts, two types of spermatogenesis are observed: a cystic type in which spermatogenesis is completed within the cysts, and a semi-cystic type, in which spermatogenesis occurs partly outside the cysts (Mattei et al., 1993).

Fish spermatozoa show more morphologic diversity than those of other vertebrates, and their ultrastructure provides parameters for phylogenetic analysis (Jamieson & Leung, 1991; Mattei, 1991). The Sertoli cells, in addition to their compartmentalization and secretion functions, also act as a barrier that inhibits communication between the germ cells and the vascular system (Grier, 1993; Loir et al., 1995). The interstitial or Leydig cells produce steroids and are present in variable numbers during the reproductive cycle (Arbuzova, 1995; Cauty & Loir, 1995; Kobayachi et al., 1998).

The basic pattern of oocyte growth is similar in teleosts (Tyler & Sumpter, 1996). Oocyte development into a mature egg is a complex process modulated by numerous environmental and endocrine factors (Coward & Bromage, 2000). The morphological characteristics of oocytes are important for an understanding of the dynamics of oogenesis, including oocyte final maturation and ovulation (Tyler & Sumpter, 1996).

Recently, Grier (2000) considered the 'follicular complex' to be the functional unit of the ovary and described it as being formed by two compartments separated by the basal membrane. The follicle, consisting of the oocyte and surrounding follicular cells, originates from the

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germ epithelium and the theca of undifferentiated cells located in the ovarian stroma.

The folliculogenesis process in teleosts varies among species. The zona pellucida reflects adaptations to different ecological conditions under which the egg develops (Stehr & Hawkes, 1979). Its inner layer protects the egg against mechanical injuries, and the outer layer against microorganisms. The surface ornaments are related to the egg's anchorage to different substrata (Kudo & Yazawa, 1997).

In this paper, histological and ultrastructural techniques were used to study the gametogenesis of *Bryconops affinis* (Characidae, Tetragonopterinae), a forager species previously thought to belong to the genus Creatochanes (Machado-Allison et al., 1993).

Methods

Animals

Specimens of *Bryconops affinis* (412 females and 223 males) were collected bimonthly in the period between March 1994 and January 1996 at the Três Marias reservoir (18–20°S, 44–46°W), located in the State of Minas Gerais, in Brazil's southeast region.

Light microscopy

Fragments of ovaries and testes were fixed in Bouin's fluid for 4–6 h, embedded in paraffin and glycol methacrylate (Technovit 7100), sectioned at 3–5 μm and stained with hematoxylin/eosin and toluidine blue-sodium borate.

The diameter of oocytes in different stages of development and the nucleus of spermatogenic cells were measured with a micrometric rule. Using five sections for each stage of the reproductive cycle, 100 oocytes and 100 nuclei of paraffin-embedded spermatogenic cells were measured. Only integer oocytes (with sections passing through the nucleus) and spermatogenesis cells with little retraction were selected for examination.

Transmission electron microscopy

Fragments of ovaries and testes of 10 specimens in reproductive activity were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.3 for 10 h at 4°C. The samples were post-fixed with 1% osmium tetroxide reduced with 1.5% potassium ferrocyanide for 2 hs, dehydrated and embedded in epon/araldite. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined on a Zeiss EM-10 microscope.

Scanning electron microscopy

The ovaries and testes of six specimens in reproductive activity, fixed as described previously, were submitted to triple post-fixation with osmium tetroxide for two hours in each step, intercalated with two mordants (tanic acid and thiosemicarbazid) for 20 min each. The samples were dehydrated, frozen in liquid nitrogen, fractured, dried at a critical point with CO_2, covered with gold and examined on a Zeiss DSM-950 microscope at 10–20 KV.

Results

Organization of the testes

The testes of *Bryconops affinis* were surrounded by a thin tunica albuginea (capsule) that projected to the interior of the organ, forming fiber septs that delimited the seminiferous tubules. During gonadal maturation, the germ cells contained in each cyst were all in a similar stage of development. The cysts were delimited by cytoplasmic process of Sertoli cells. Blood vessels, connective cells, collagenous fibrils, and Leydig cells were found inside the interstitial stroma. Leydig cells were abundant in all specimens examined.

Spermatogenesis and spermiogenesis

Inside the testes, the following spermatogenic cells were identified: spermatogonia, spermatocytes, spermatids, and spermatozoa. Spermatogonia, after consecutive divisions during spermatogenesis, underwent gradual reduction in the diameter of their nuclei, culminating with the formation of haploid cells, spermatids. After spermiogenesis, these spermatids originated spermatozoa which, due to chromatin condensation, showed a smaller nuclear diameter. The histological and ultrastructural characteristics of spermatogenic cells are shown on Table 1 and Figures 1–8. Hatching of the cysts occurred simultaneously with spermatid release into the lumen of the seminiferous tubules (Fig. 5), where spermiogenesis was completed (Figs 6–8).

Sertoli cells

These cells had a triangular or round nucleus with chromatin in a dispersed pattern or forming small clots. The cytoplasm showed rough and smooth endoplasmic reticulum cisterns (Figs 9–10). The Sertoli cells were connected by desmosomes and tight junctions (Fig. 11). After spermiation, residual spermatozoa were reabsorbed by the Sertoli cells (Fig. 12).

Interstitial or Leydig cells

At the ultrastructural level, Leydig cells in two distinct stages were found: cells in secretory activity, with a large, spherical, vesiculous nucleus, cytoplasm with abundant smooth endoplasmic reticulum and mitochondria with tubular crests (Fig. 13); and cells in involutive process, characterized by a retracted and irregular nucleus, expanded perinuclear cistern, and cytoplasm with myelin figures and numerous electron-lucent vesicles (Fig. 14).

Organization of the ovaries

The ovaries of *Bryconops affinis* were surrounded by serosa, under which the tunica albuginea could be seen,

Table 1 Ultrastructural and histological characteristics of spermatogenic cells of *B. affinis*

Stage	Morphology
Spermatogonia (Fig. 1)	Primary phase: vesiculous nucleus with $\varnothing = 7.54 \pm 0.92$ μm, finely granular chromatin and eccentric nucleoli. Poorly electron-dense cytoplasm, scattered endoplasmic reticulum, mitochondria in dispersed pattern or grouped by cementum. Secondary phase: nucleus with $\varnothing = 5.30 \pm 0.59$ μm, granular chromatin and eccentric or central nucleoli. Finely granular cytoplasm with few organelles.
Spermatocyte (Fig. 2)	Primary phase: spherical, centric nucleus with $\varnothing = 4.14 \pm 0.49$ μm. Poorly electron-dense chromatin and synaptonemal complexes. Finely granular cytoplasm with few organelles. Secondary phase: spherical nucleus with $\varnothing = 3.25 \pm 0.56$ μm and chromatin condensed into clots. Scarce, finely granular cytoplasm with few organelles.
Spermatids (Figs 3–6)	Nucleus with $\varnothing = 2.22 \pm 0.44$ μm and condensed chromatin. Initial phase: appearance of electron-lucent vesicles in cytoplasm. Intermediate phase: formation of implantation fossa. Advanced phase: flagellum begins to develop
Spermatozoa (Figs 7–8)	Head: nucleus with $\varnothing = 1.15 \pm 0.36$ μm and electron-dense chromatin. Middle piece: has few mitochondria. Flagellum: long, with axoneme pattern (9+2 arrangement).

Table 2 Ultrastructural and histological characteristics of ovarian follicles of *B. affinis* in different stages of development

Stage	Morphology
Young – O1 ($\varnothing = 72.77 \pm 13.31$ μm) (Fig. 16)	– large, vesiculous nucleus with many nucleoli. – cytoplasm strongly basophilic. – formation of microvilli from the oocyte. – pavimentous follicular cells with irregular nucleus contour and scattered cytoplasm. – basal membrane and theca poorly developed.
Previtellogenic – O2 ($\varnothing = 172.99 \pm 20.74$ μm) (Fig. 17)	– nucleus with peripheral nucleoli – basophilic, finely granular cytoplasm and with yolk nucleus – pavimentous folicullar cells – initial formation of zona pellucida – cytoplasmic processes of follicular cells associated with zona pellucida – fine basal membrane and theca
With cortical vesicles – O3 ($= 307.68 \pm 31.75$ μm) (Fig. 18)	– slightly basophilic and irregular nucleus – formation of cortical vesicles in peripheral ooplasm – fine zona pellucida with two layers in formation – electron dense globules associated with outer layer of zona pellucida – cubic follicular cells at light microscopy – elongations of follicular cells between globose material – connective theca with cells similar to fibroblasts
Vitellogenic – O4 ($\varnothing = 550.37 \pm 54.60$ μm) (Figs 19)	– irregular nucleus – acidophilic yolk globules fill up almost the entire ooplasm – cortical vesicles inside peripheral ooplasm – thick zona pellucida with compacted orifices-pores, showing thick electron-lucent inner layer and thin electron-dense outer layer – electron-dense globules, without orifices or pores, associated with outer layer of zona pellucida – funnel-shaped micropyle – prismatic follicular cells at light microscopy – thin theca

emitting septs to the interior of the organ and forming ovigerous lamellae containing oogonia and oocytes in different stages of development (Fig. 15).

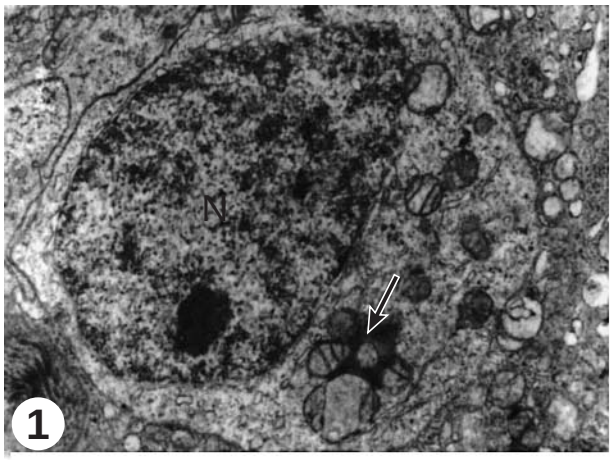
Oogenesis

This phenomenon was seen to initiate with the proliferation and differentiation of oogonias. These primordial germ cells, the smallest of the oogenic cells, were grouped in nests in the ovarian stroma and, after meiotic division and differentiation, they originated oocytes. These were classified into four groups, according to their stage of development, as determined by morphological changes in the nucleus, cytoplasm and follicular wall (Table 2).

Zona pellucida and follicular cells

Ultrastructural analysis showed that the zona pellucida was formed in the previtellogenic phase, after which globose specializations gradually attached to the outer layer of the zona pellucida (Figs 16–20).

The follicular cells had a vesiculous nucleus and a cytoplasm containing mitochondria, free ribosomes and rough endoplasmic reticulum. The cytoplasmic processes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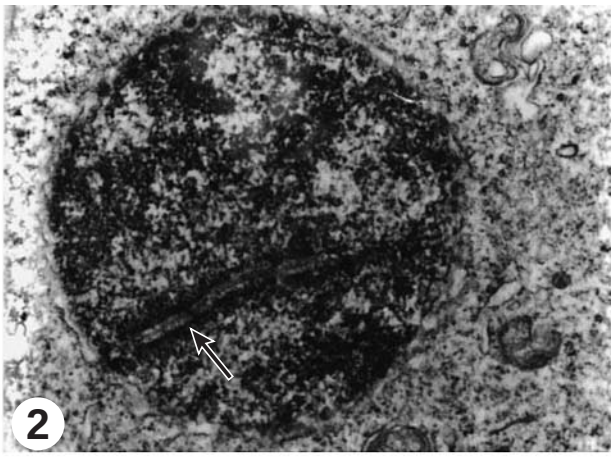
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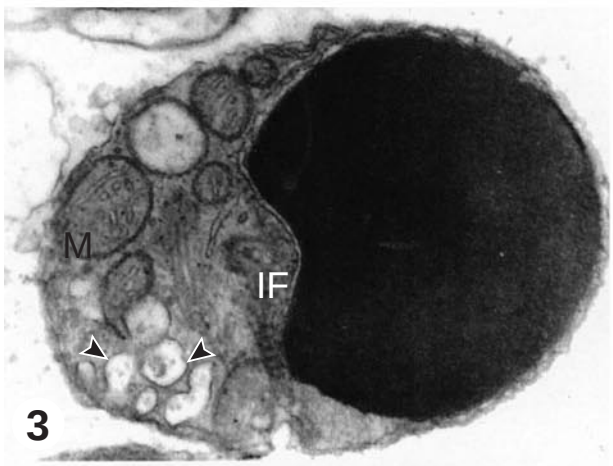
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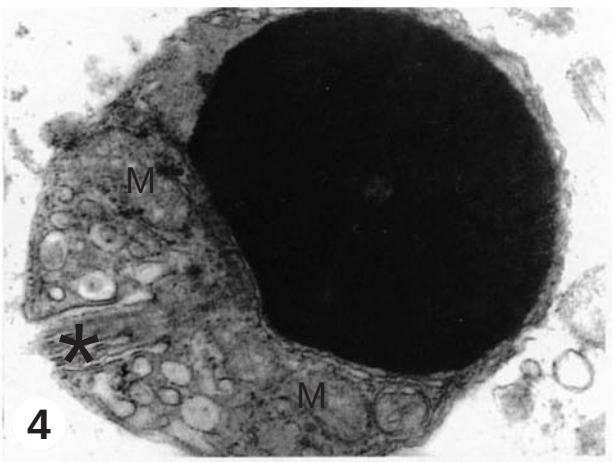
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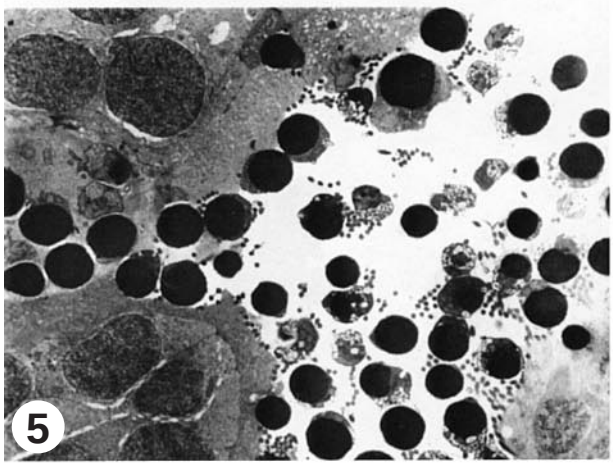
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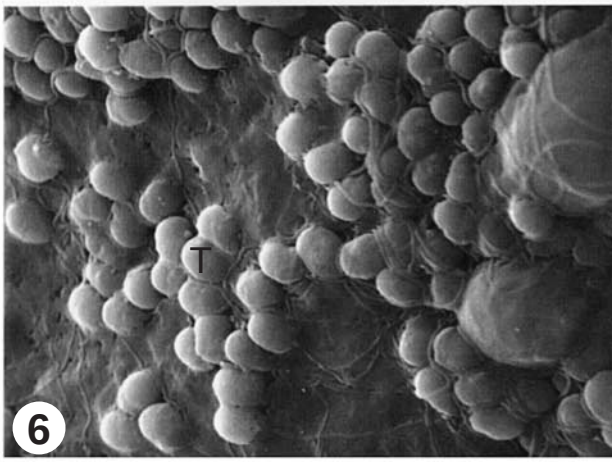
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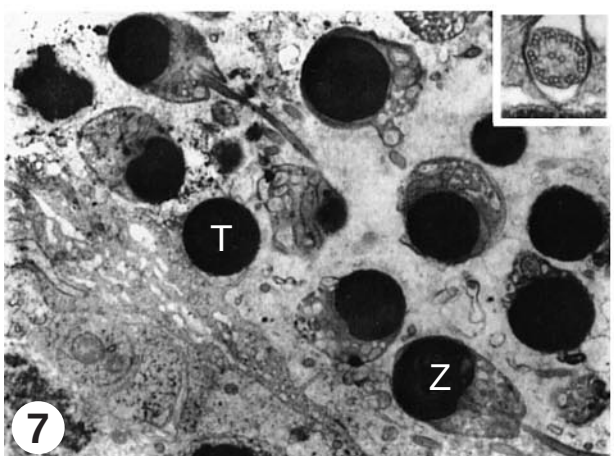
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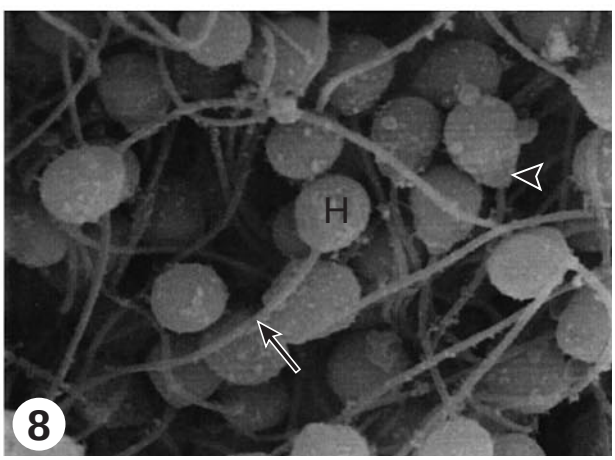
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Fig. 1 Primary spermatogonia: nucleus (N) with finely granular chromatin and evident nucleoli. Cytoplasm with mitochondria dispersed or grouped by cementum (arrow) – x10 600. **Fig. 2** Primary spermatocyte: nucleus with slightly compacted chromatin with characteristic synaptonemal complex (arrow) – x18 200. **Fig. 3** Spermatid in intermediate phase: nucleus with compacted, electron-dense chromatin. Cytoplasm with mitochondria (M), electron-lucent vesicles (arrow head) and formation of implantation fossa (IF) – x27 200. **Fig. 4** Spermatid in advanced phase: nucleus with electron-dense chromatin. Cytoplasm with mitochondria (M), electron-lucent vesicles, and initial formation of flagellum (*) – x29 300. **Fig. 5** Release of spermatids into the lumen of the seminiferous tubule – x2600. **Fig. 6** MEV – spermatids (T) associated to the wall of seminiferous tubules – x2000. **Fig. 7** Spermatids (T) and spermatozoa (Z) in the lumen of the seminiferous tubule. Spermatozoon consists of a round head joined to a middle piece and a long flagellum with a 9 + 2 (inset) microtubule – x6000 (inset – x33 600). **Fig. 8** MEV – Spermatozoa with ovoid head (C), short middle piece (arrow head) and long flagellum (arrow), in the lumen of the seminiferous tubule – x4500.

of these cells extended toward the oocyte, often reaching the zona pellucida pore canals.

Discussion

The reproductive activity in *Bryconops affinis* is continuous and the presence of spermatogenic and oogenic cells under their particular development stages was observed in all specimens examined bimonthly over a two-year period of study (Andrade, 1999). This pattern is also described for other teleosts, as a result of tropical water conditions (Lowe-McConnell, 1987).

In *B. affinis*, as in *Fundulus heteroclitus* (Selman & Wallace, 1986) and *Channa punctatus* (Srivastava & Singh, 1994), bursting of the cysts caused spermatids to be released into the lumen of the seminiferous tubules.

In the present study, primary spermatogonia showed groups of mitochondria bound together by cementum, resembling those observed by Clerot (1979), who reported that this cementum is made up of ribosomic RNA of nuclear origin.

The synaptonemal complexes are formed by pairing of the homologue chromosomes during the first meiotic division (Grier, 1975; Cruz-Landim & Cruz-Höfling, 1987), characterizing primary spermatocytes as observed in *Bryconops affinis*. In mammals, basic proteins of the synaptonemal complex combine with DNA and are involved in the chromosome structural organization that takes place in meiotic prophase (Offenberg et al., 1998; Yuan et al., 1998).

The stages of the spermatogenesis in *Bryconops affinis* were based on the modifications that occur in the nucleus and spermatid cytoplasm before the spermatozoon is formed. The distribution and organization of the cytoplasmic organelles and implantation fossa of spermatids of *Bryconops affinis* follow an identical pattern to that described by Thiaw et al. (1988). During spermatid differentiation, pinocytotic vesicles uptake exogenous substances that are important to the process of cytoplasm elimination (Thiaw et al., 1988). This extruded material is phagocytozed by Sertoli cells (Matos & Azevedo, 1989).

The spermatozoa of teleosts have no acrosomes and this characteristic may be related to the presence of a micropyle in the oocyte (Bromage & Roberts, 1995). However, vacuoles, which are vestiges of acrosomes, can be found in some teleosts (Billard, 1983). In *Bryconops affinis*, as in *Plagioscion squamosissimus* (Gusmão et al., 1999), no acrosomes or vestiges of these structures were observed. The spermatozoa of *Bryconops affinis* are simple, with a round head, rudimentary middle piece and poor in mitochondria, these being typical characteristics of primitive spermatozoa of the fishes with external fertilization (Billard, 1970; Jones & Butler, 1988; Silva & Godinho, 1991; Lahnsteiner et al., 1997).

In the species studied, the Sertoli cells were connected to each other through desmosomes and tight junctions. These junctions formed the morphological basis for the Sertoli cell barrier that inhibits communication between haploid germ cells and the vascular system (Silva & Godinho, 1989; Grier, 1993; Loir et al., 1995; Romagosa et al., 1999). According to Pudney (1995), in addition to supporting the cysts, the Sertoli cells also phagocytize residual spermatozoa. Secretory activity of Sertoli cells as observed in the present study was also described by Pudney (1995).

In the interstitial tissue of *Bryconops affinis*, interstitial or Leydig cells with a morphology that is typical of steroidogenic cells – abundant, smooth endoplasmic reticulum and mitochondria with tubular crests – were seen to occur. According to Payne et al. (1996), the products of the secretion of these cells are necessary for spermatogenesis and expression of secondary sexual characteristics.

In *Bryconops affinis*, gametogenesis is continuous, for that reason Leydig cells are observed throughout the whole reproductive cycle. Particularly those cells are more abundant at the initial maturation and gradually decrease in number during advanced maturation and spermiation (Andrade, 1999). In salmon *Oncorhynchus gorbuscha*, Leydig cells are more active before spermiation, with mitochondria, Golgi complexes and smooth endoplasmic reticulum being more abundant (Arbuzova, 1995). In the present study, Leydig cells in involutive process, characterized by irregular, smaller nuclei and fragmentation of the smooth endoplasmic reticulum into large vesicles, were also observed. Leydig cells in a similar involutive process were also reported in trout (Cauty & Loir, 1995).

Teleots may produce an indefinite number of oocytes during their life cycle (Tyler & Sumpter, 1996). Inside the ovary there is a permanent population of oogonias that can divide mitotically or meiotically to originate oocytes (Grier, 2000). Using a scanning electron microscopy, we found that, in females of *Bryconops affinis*, oogonias and young oocytes are present in the ovigerous lamella, near the tunica albuginea. During the maturation process, the

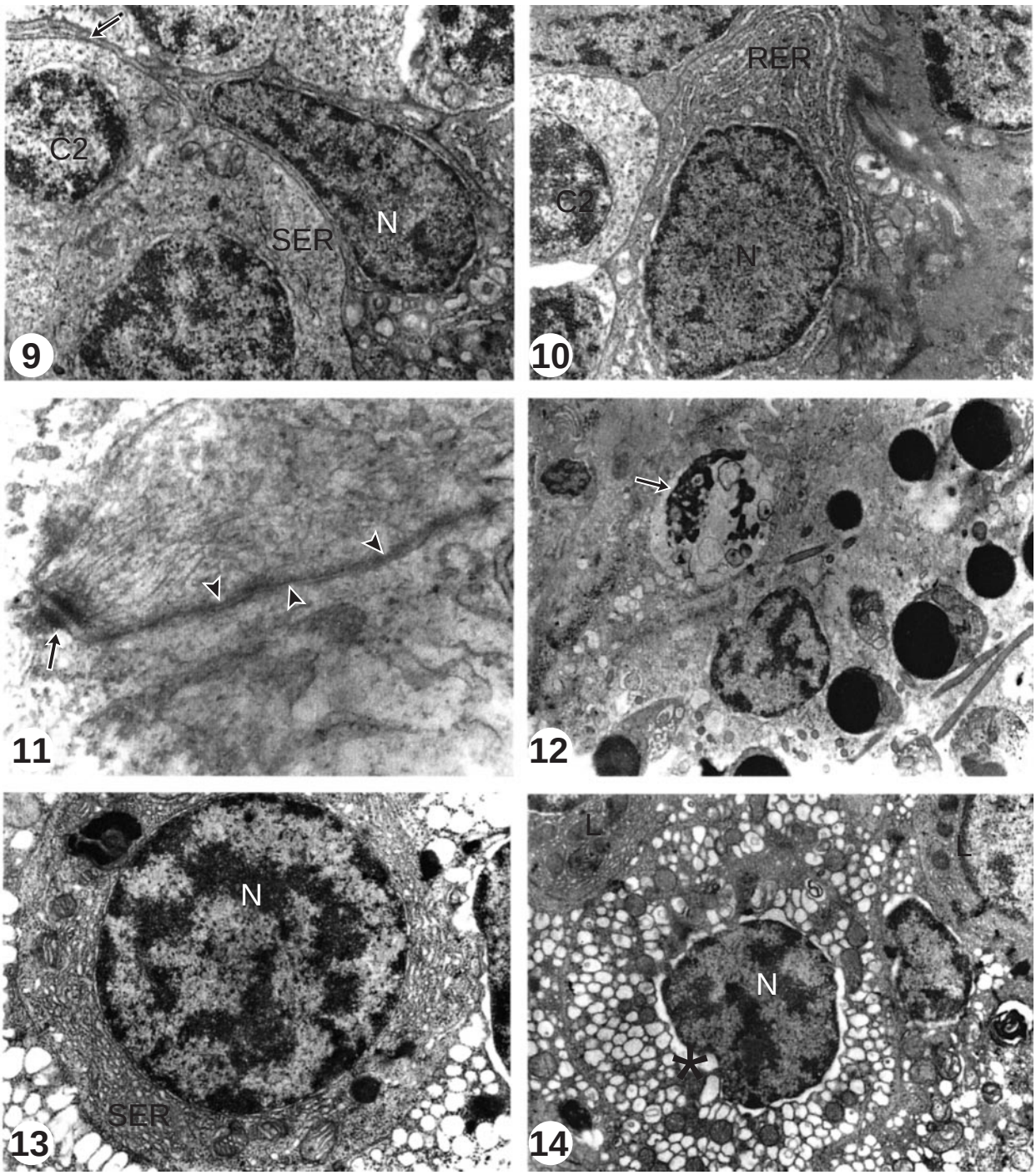
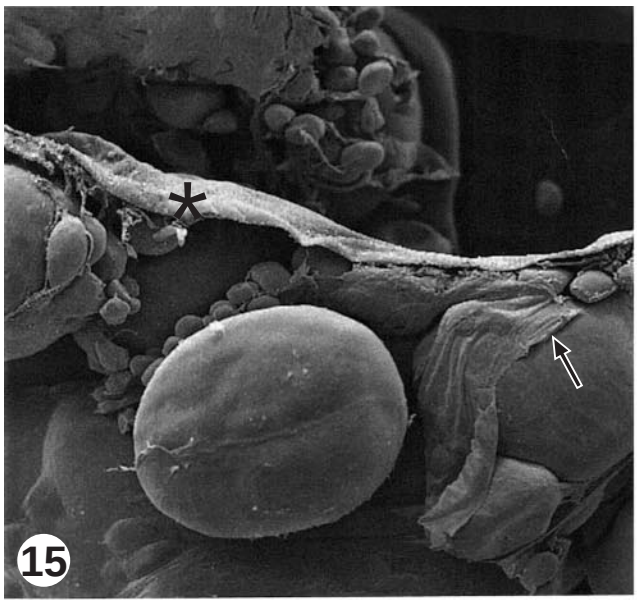


Fig. 9 Sertoli cells with oblong nucleus (N) and cytoplasm with smooth endoplasmic reticulum (SER). Cyst is delimited from the secondary spermatocyte (C2) by cytoplasmic process (arrow) – x9000. **Fig. 10** Sertoli cells in secretory activity: vesiculous nucleus (N) and abundant rough endoplasmic reticulum (RER). C2 = cysts of secondary spermatocyte – x8900. **Fig. 11** Desmosome (arrow) and tight junctions (arrow head) between neighbouring Sertoli cells – x32 100. **Fig. 12** Residual spermatozoon being reabsorbed by Sertoi cells (arrow) – x5500. **Fig. 13** Leydig cells in secretory activity: vesiculous nucleus (N), cytoplasm with smooth endoplasmic reticulum (SER) and mitochondria with tubular cristae – x12 600. **Fig. 14** Leydig cells in involutive process: retracted, irregular nucleus (N) with dilated perinuclear cisterns (*). Vacuolated cytoplasm. Leydig cells in secretory activity (L) – x7700.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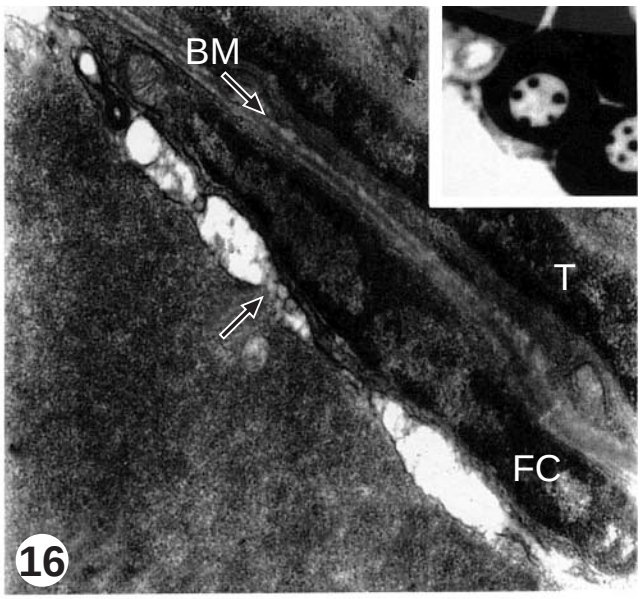
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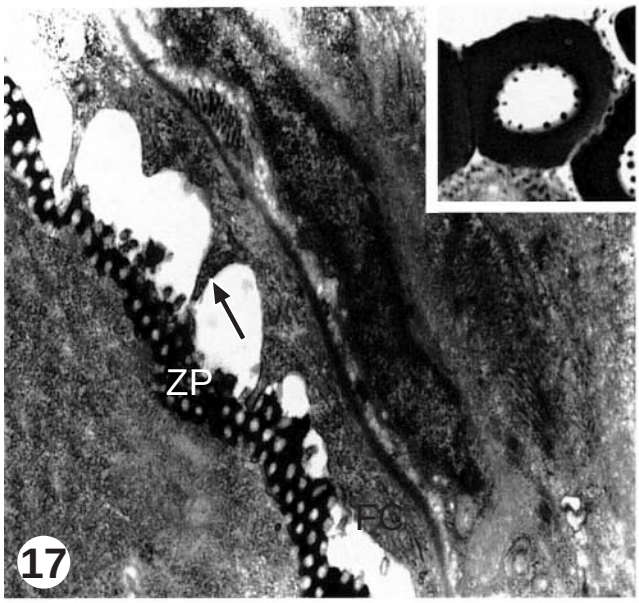
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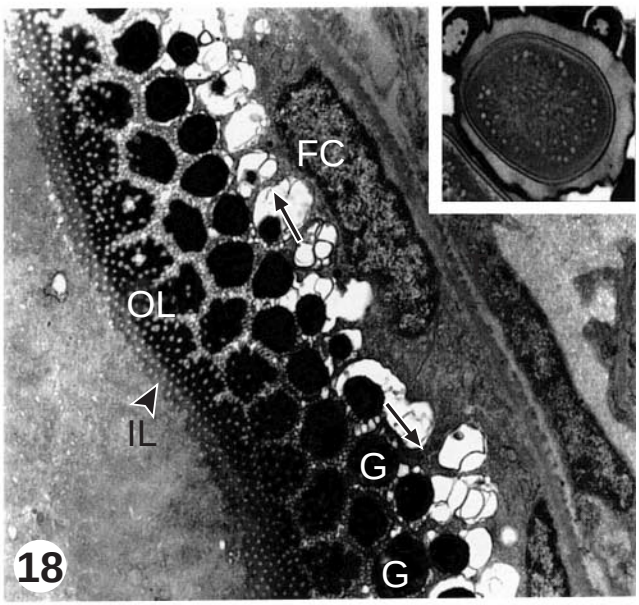
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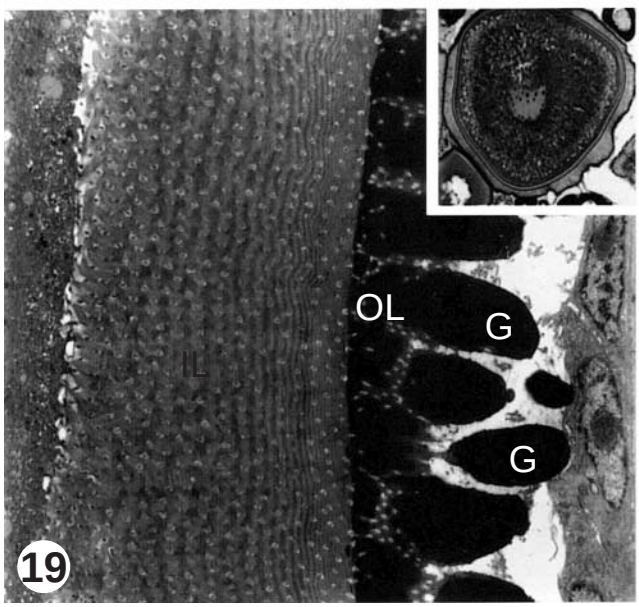
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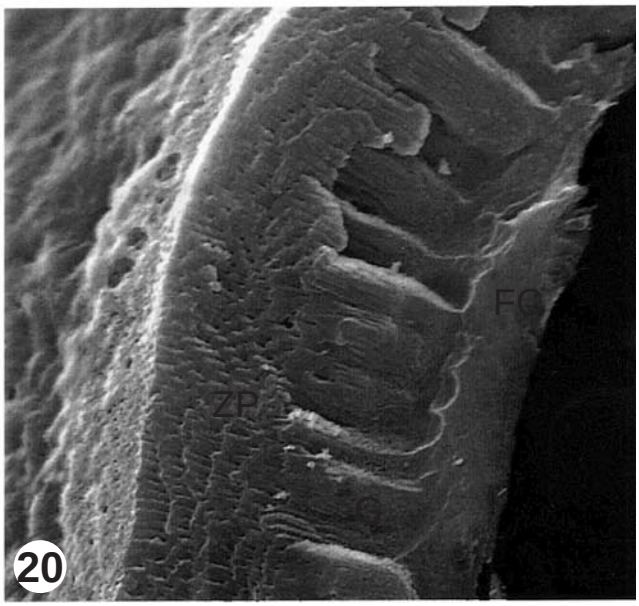
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Fig. 15 MEV – fractured ovary showing tunica albuginea (*) and ovigerous lamellae (arrow) with oocytes in different stages of development – x50. **Fig. 16** Young oocyte showing pavimentous follicular cell (FC) laid on basal membrane (BM) and theca (T). Small microvilli (arrow) emerge from the oocyte and follicular cells – x19400 (inset – x320). **Fig. 17** Formation of zona pellucida (ZP) on previtellogenic oocyte. Follicular cells emitting cytoplasmic processes (arrow) – x 13 800 (inset – x220). **Fig. 18** Zona pellucida differentiated into layers in an oocyte containing cortical vesicles. Outer layer (OL) with globose specializations (G) and inner layer beginning to be formed (IL). FC = follicular cells, T = theca, arrow = process of follicular cells – x5000 (inset – x70). **Fig. 19** Zona pellucida of vitellogenic oocyte with two layers perforated by pores: inner layer (IL) is less electron dense and thicker, the outer layer (OL) is more electron dense, thinner and with associated globose specializations (G) – x3100 (inset – x40). **Fig. 20** MEV – fractured follicular wall, with zona pellucida (ZP) with globose specializations (G), follicular cells (FC) – x1700.

oocytes of *Bryconops affinis*, as in other teleosts, start migrating toward to the centre of the ovary (Afonso-Dias & Hislop, 1996; Yoneda et al., 1998).

The zona pellucida of the oocytes of teleosts is a complex structure, generally consisting of two layers crossed by pores or canals containing oocyte microvilli and/or follicular cell processes (Guraya, 1996). In *Bryconops affinis*, the inner layer is thick and the outer layer is thin, electron-dense and with globose specializations. As in other teleosts, the zona pellucida of *Bryconops affinis* begins to be formed in the previtelogenic oocyte, with its outer layer being formed through electron-dense material deposition between microvilli of the oocyte and/or follicular cells (Anderson, 1967; Abraham et al., 1984; Rizzo & Bazzoli, 1991; Cruz-Höfling & Cruz-Landim, 1993).

In the present study, histologic analysis showed that follicular cells were first pavimentous, then cubic, and finally became prismatic at the end of oogenesis. Ultrastructural analysis revealed that electron-dense globules associated to the zona pellucida were responsible for the change in shape observed at light microscopy. The origin of the zona pellucida of teleosts is still a controversial issue, yet it is postulated that the oocyte, follicular cells and hepatic cells may play a role in the formation of this structure (Oppen-Berntsen et al., 1992) and that the specializations associated to it may originate from follicular cells (Guraya, 1996). Follicular cells and associated structures of *Bryconops affinis* showed positive reaction to Periodic-Acid-Schiff techniques and Alcian blue at pH 2.5, indicating the presence of mucosubstances which can be related to adhesive function (Bazzoli, 1992). Protuberances of several sizes and shapes, such as called here, globose specializations, attached to the zona pellucida, occur in different groups of teleosts, probably as a result of egg adherence to different substrata (Riehl & Patzner, 1998).

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