

4-2007

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Arezo, M.J., S. D'Alessandro, N. Papa, R. de Sá and N. Berois. 2007. Sex differentiation pattern in the annual fish *Austrolebias charrua* (Cyprinodontiformes: Rivulidae). *Tissue and Cell* 39(2):89-98.
<https://doi.org/10.1016/j.tice.2007.01.004>

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Sex differentiation pattern in the annual fish *Austrolebias charrua* (Cyprinodontiformes: Rivulidae)

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Received 30 December 2006; received in revised form 25 January 2007; accepted 26 January 2007

Available online 30 March 2007

Abstract

Sex differentiation process, determination of sexual strategy, and gametogenesis of the annual fish *Austrolebias charrua* are established. Evidence of histological sex differentiation in an antero-posterior gradient was observed in pre-hatching stages. Sexual strategy corresponds to the “differentiated gonochoric” pattern. Histological analyses of adult gonads showed an asynchronous spawning mode for females and continuous spawning for males. Mature oocytes presented fluid yolk. Testis organization corresponded to a restricted spermatogonial model. Herein, we report the ultrastructural organization of the vitelline envelope and the main features of the sperm of *A. charrua*. Taking together these results also contribute to phylogenetic studies and provide base line data to propose *A. charrua* as a biomonitor of contamination in a protected area. © 2007 Elsevier Ltd. All rights reserved.

Keywords: Gonadal development; Annual fishes; *Austrolebias*

1. Introduction

Successful reproduction of an individual depends on events that begin early in its life. Sex determination and differentiation are fundamental components of the genetic information passed on from generation to generation. Teleosts are an attractive group of organisms for the study of the evolution of these events because members of this class exhibit a broad range of sexual strategies ranging from hermaphroditism to gonochorism and from environmental to genetic sex determination (Devlin and Nagahama, 2002).

Although zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) have been the most used teleost models in developmental biology studies, species of annual fishes are excellent organisms for comparative analyses as they show unique reproductive and developmental characteristics. Annual fishes are exposed to an extremely variable environment. They inhabit temporary ponds that undergo drying during summer resulting in the death of the entire adult population.

The developing embryos remain buried in the bottom mud and hatch, after the pond is flooded, in the next rainy season (Wourms, 1964, 1967). In contrast with other teleosts, annual fishes exhibit a unique developmental pattern (Myers, 1952). Epiboly is temporally and spatially separated from organogenesis and embryos undergo one or more reversible arrests (diapauses) at three different stages (Wourms, 1972a,b,c). These developmental adaptations are closely related to their life cycle.

Teleosts are becoming increasingly important indicators of environmental health. Considerable information exists suggesting that pollutants may cause serious impacts on fish reproduction: sex differentiation, gonad morphology, rates of gametogenesis and sex phenotypes (reviewed by Devlin and Nagahama, 2002; Arukwe and Goksøyr, 2003). Moreover, *Cynopoecilus melanotaenia*, an annual fish species, was suggested as a sensitive model organism to assess the impact of environmental pollution (Arenzon et al., 2003).

Critical to the understanding of sex-determination processes are studies examining the origin and development of cells involved in the formation of the gonad (Devlin and Nagahama, 2002). There is any evidence about sex deter-

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Fig. 1. Map of Uruguay showing *Austrolebias charrua* collecting area (black zone).

mination and differentiation mechanisms in annual fishes. In this study, as a first comprehensive approach, we determine primary sex differentiation (both at morphological and temporal scales) from early embryos to adults of the annual fish *Austrolebias charrua* (Costa and Cheffe, 2001). In addition, we present the adult gonad organization and the main characteristics of oogenesis and spermatogenesis.

2. Materials and methods

Adult females and males of *A. charrua* were collected during the rainy season (May to August) in temporary ponds from Departamento de Rocha, Uruguay (Fig. 1). They were kept in the laboratory in 30 l aquaria, filled with continuously aerated and dechlorinated tap water (pH 7–7.5), and exposed to natural light. Water was partially changed every 5 days. Water temperature was $(19 \pm 1)^\circ\text{C}$. Specimens were fed once a day with live *Tubifex* sp. Spawning occurred daily from fish pairs or groups of one male and two females isolated in aquaria that had containers with peat moss on the bottom. Early embryos were collected from the peat moss, raised, and developmental stages were classified according to Arezo et al. (2005).

2.1. Hatching

Containers with peat moss were maintained in the aquaria for 1 month; subsequently, they were dried in darkness for at

least 2 months. For hatching, each peat moss container was placed in a 10 l aquarium and covered with dechlorinated water. After 6–12 h hatched fry were observed. They were fed with freshly hatched *Artemia* sp. nauplii for the first 15 days and then with *Daphnia* sp.

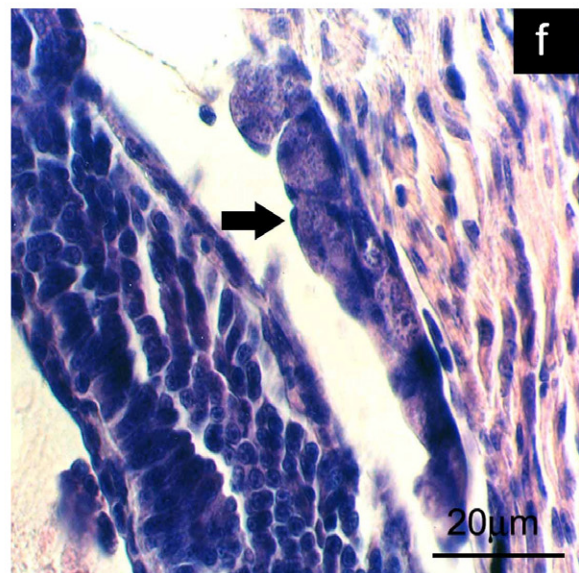
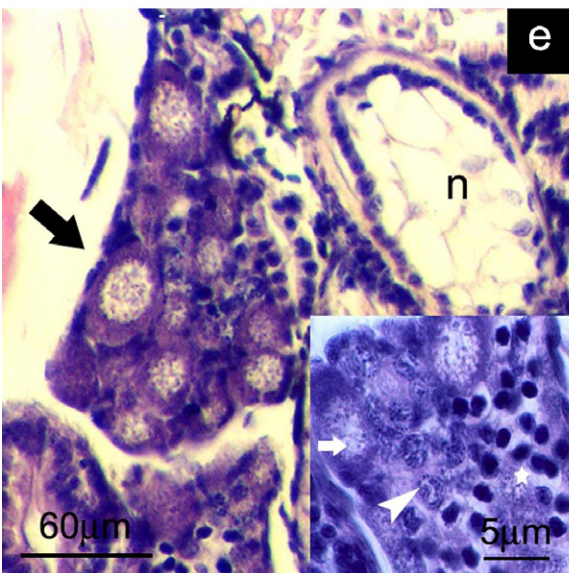
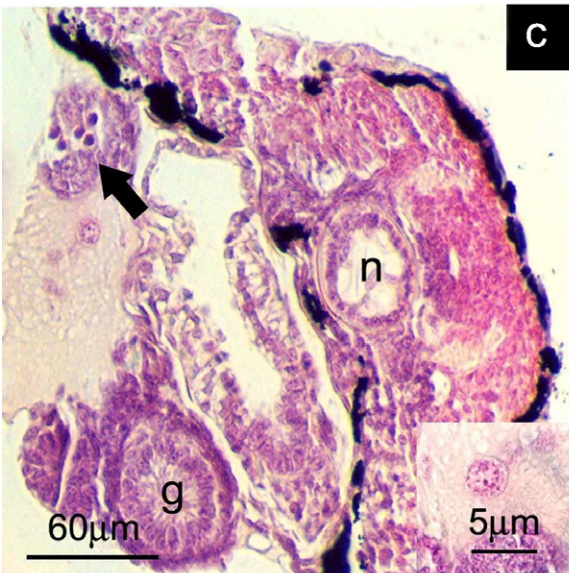
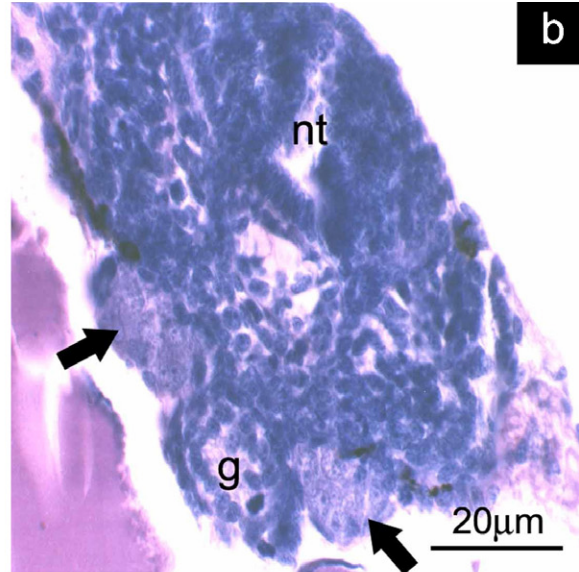
2.2. Histological studies

Embryos were placed in Stockard solution (formalin, glacial acetic acid, glycerine, distilled water, 5:4:6:85; Costello et al., 1957) for 48 h, then dechorionated using fine tweezers and fixed in Bouin's solution for 30 min. Fry were killed in a 10 min exposure to a solution of 1% 2-phenoxyethanol (Sigma) and directly fixed in Bouin's solution for 2 h. Ovaries and testes were obtained from adult fish from May to December (10 females and 10 males each month), killed by immersion in freshwater containing 5% of 2-phenoxyethanol (Sigma) until death. The gonads were removed and fixed in Bouin's solution for 3 h. After washing and dehydration in increasing concentrations of alcohol series, the embryos, fry, and adult gonads were embedded in paraplast. Semiserial sections of $7\ \mu\text{m}$ thickness were stained with hematoxylin and eosin and mounted in Entellan (synthetic medium) (Ganter and Jolles, 1970). Sections were examined and photographed using an Olympus-Vanox light microscope. Measurements were carried out directly under the microscope using an ocular micrometer (E. Leitz 1/100 mm). Micrographs were taken using Ilford PANF-50 ASA film.

2.3. Ultrastructural studies

Ovulated oocytes and testes for scanning electron microscope analysis (SEM), were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at room temperature, dehydrated in acetone series, dried at the CO_2 critical point and coated with gold using a Pelco 90000 sputter-coater. Samples were examined with a JEOL JSM 25 S II scanning electron microscope. For transmission electron microscope studies (TEM), pieces of fresh ovaries were fixed overnight at 4°C in a solution of 4% paraformaldehyde and 2, 5% glutaraldehyde in 0.1 M phosphate buffer, pH 7. Tissues were then washed six times in 0.1 M phosphate buffer and post-fixed in a 1% solution of osmium tetroxide prepared in phosphate buffer, pH 7.4, for 1 h and 30 min. After six washes in phosphate buffer, dehydration was accomplished in an increasing acetone series with a final wash in 100% acetone. The samples were included in araldite (Durcupan ACM, Fluka). Gold and silver sections obtained from a RMC MT-X ultramicrotome were stained with uranyl acetate followed

Fig. 2. Gonadal differentiation: embryonic stages (hematoxylin and eosin). (a) Longitudinal section of 3 weeks post-fertilization embryo: arrow: gonadal primordia area; y, yolk. (b) Transverse section of gonadal primordia in the same stage: arrows, gonadal primordia; g, gut; nt, neural tube. (c) Thirty days post-fertilization embryo showing gonadal type I (arrow). Germ cells undergoing mitosis are recognized by basophilic nuclei with condensed chromatin: g, gut; n, notochord; square, high magnification of a germ cell arriving to the developing gonad. (d) Thirty days post-fertilization embryo showing gonadal type II (arrow): y, yolk; g, gut. (e) Pre-hatching embryo: developing ovary (arrow); n, notochord; square, high magnification of the gonad; star, oogonia population; arrowhead, chromatin nucleolar stage oocytes; arrow, perinucleolar stage oocytes. (f) Pre-hatching embryo: presumptive male; arrow: undifferentiated gonad.



by lead citrate standard conditions. Observations were made with a JEOL JEM-1010 transmission electron microscope.

Stages of embryonic development were determined in relation to post-fertilization time and morphological features. Stages of oocyte maturation and testicular organization were defined according to Wallace and Selman (1981) and Grier (1981), respectively.

3. Results

3.1. Gonadal differentiation

About 3 weeks post-fertilization, *A. charrua* embryos ($n=36$) occupied more than a half the distance around the yolk, the eyes were slightly pigmented, few blood cells were circulating, and otic vesicles contained no otholiths. Paired gonad primordia were observed dorso-laterally to the gut and ventrally to the notochord in the mid region of the embryo's antero-posterior axis (Fig. 2a and b). They consisted of clusters of germ cells with no signs of differentiation that were surrounded by flattened somatic cells (undifferentiated stage) (Fig. 2b). Germ cells were recognized based on their morphological features: round shape, large cell size ($12 \pm 2, 5 \mu\text{m}$), high nucleus/cytoplasm ratio, darkly stained nucleoli, and hyaline cytoplasm (Fig. 2b and c).

Thirty days post-fertilization, embryos ($n=43$) were fully extended around the perimeter of the yolk, the eyes were heavily pigmented, blood circulation was apparent and contained many blood cells, otic vesicles possessed two otholiths, and few melanophores were scattered over the body. At this stage, two kinds of gonadal tissue were observed in different individuals. Gonadal tissue type I consisted of two different populations of germ cells. One type of cell exhibited morphological characteristics resembling undifferentiated germ cells as described in the previous stage whereas the other type (cells showing basophilic nuclei with condensed chromatin) was undergoing mitosis (Fig. 2c). Gonadal tissue type II showed fewer cells and all of them had characteristics similar to those observed in the undifferentiated stage (Fig. 2d).

In pre-hatching embryos (about 38 days post-fertilization, $n=42$), the tip of the tail touched the margin of the eyes and the skin was darkly pigmented. Some individuals had a true developing ovary in which oogonia and pre-vitellogenic oocytes were observed. Oogonia showed large euchromatic nuclei and scarce cytoplasm (some of them undergoing mitosis). Early meiotic oocytes presented nuclei with thin chromatin strands while chromatin nucleolar-stage oocytes were identified by having scarce cytoplasm and eccentric basophilic nucleus. Perinucleolar-stage oocytes were

characterized by the occurrence of peripheral nucleoli and basophilic cytoplasm (Fig. 2e). Other individuals presented germ cells in a morphological undifferentiated state; these were classified as presumptive males (Fig. 2f).

At hatching (at least 98 days post-fertilization, $n=40$) and 7 days post-hatching ($n=10$), the ovaries had increased in size, exhibited the same cell populations as the previous stage (Fig. 3a), and showed an antero-posterior gradient of sex differentiation (data not shown). The presumptive testes remained undifferentiated (Fig. 3b). In fry of 30 days post-hatching ($n=15$), the ovaries mostly consisted of perinucleolar stage oocytes (Fig. 3c). In other individuals of the same age, true developing testes containing meiotic spermatocyte cysts were found (Fig. 3d).

In juveniles (45 days post-hatching, $n=10$), some individuals had ovaries showing the pre-vitellogenic oocyte types described above and putative cortical-alveolar stage oocytes. These oocytes are bigger than the perinucleolar stage oocytes and presented putative cortical alveoli. The ovaries also showed vitellogenic oocytes (Figs. 3e and 4a). Other individuals had testes in which all the cellular stages of spermatogenesis were seen, including free spermatozoa (Fig. 3f). Vitellogenic oocytes and spermatogenic stages are described below.

3.2. Morphology of the adult gonads

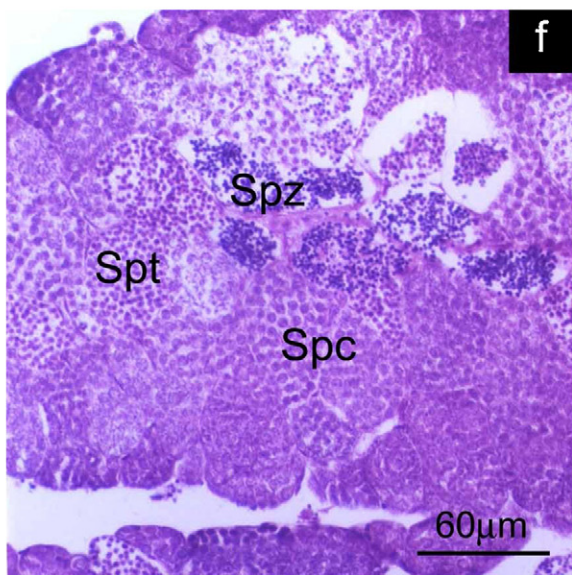
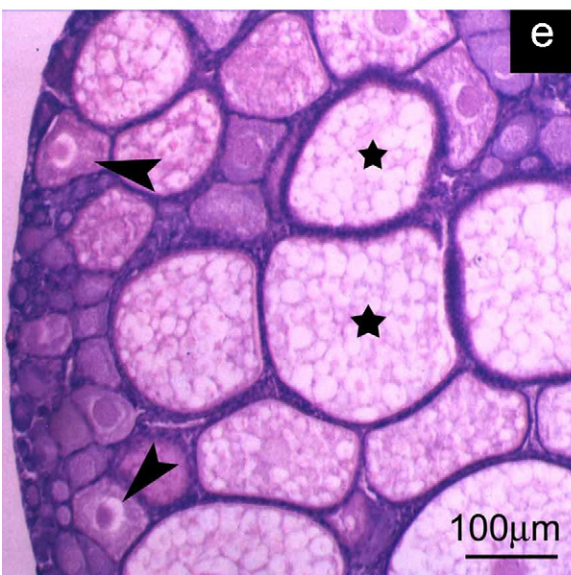
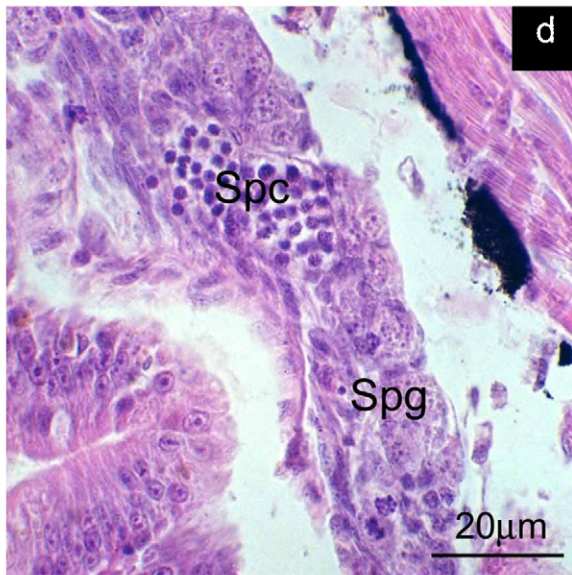
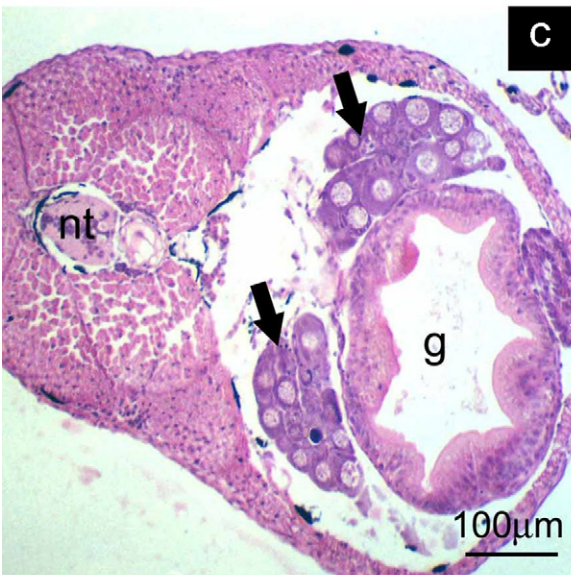
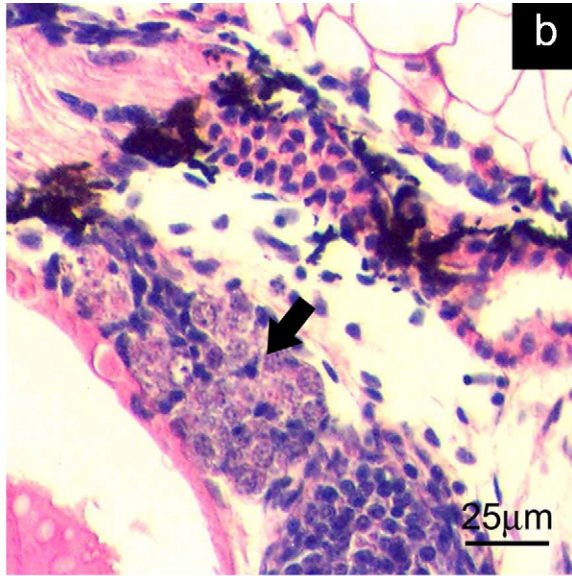
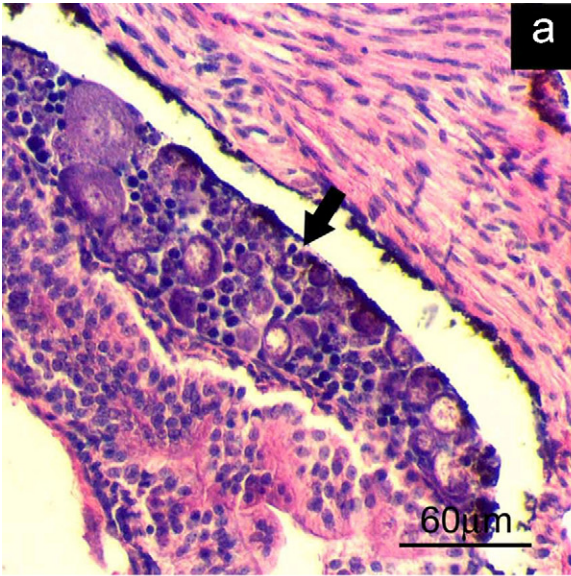
3.2.1. Female

The female reproductive system of *A. charrua* consisted of two elongated ovaries (7–12 mm long) covered by a pigmented visceral peritoneum and attached to the dorsal body wall by a mesovarium. The ovaries were located ventrally to the swim bladder and dorsally to the gut. The ovarian wall consisted of smooth muscle fibres and connective tissue. The ovarian parenchyma consisted of different kinds of developing follicles that almost filled the entire organ. Among the follicles, particularly central in the gonad, we found ducts of different diameter that exhibited a stratified and folded epithelium. Neither an ovarian cavity nor ovigerous lamellae were observed.

Female germ cells were classified into four groups: oogonia, pre-vitellogenic, vitellogenic, and fully grown oocytes according to their size, nuclear and cytoplasmic morphology, and presence and structure of the vitelline envelope. All stages were observed from sexual maturation (May) to senescence (December).

The oogonia were the smallest germinal cells and they were grouped in clusters (data not shown). The pre-vitellogenic oocytes generally were found in the periphery of the ovary. They were associated with follicle cells in the

Fig. 3. Gonadal differentiation: post-hatching stages (hematoxylin and eosin). (a) Hatching day fry: arrow, ovary. (b) Hatching day fry: arrow, presumptive testis. (c) Thirty days post-hatching fry: arrows, ovaries; g, gut; nt, neural tube. (d) Thirty days post-hatching fry: Spg, spermatogonia; Spc, spermatocyte cysts. (e) Forty-five days post-hatching juvenile: arrowheads, putative cortical-alveolar stage oocytes; star, vitellogenic oocytes. (f) Forty-five days post-hatching juvenile: Spc, spermatocytes; Spt, spermatids; Spz, spermatozoa.



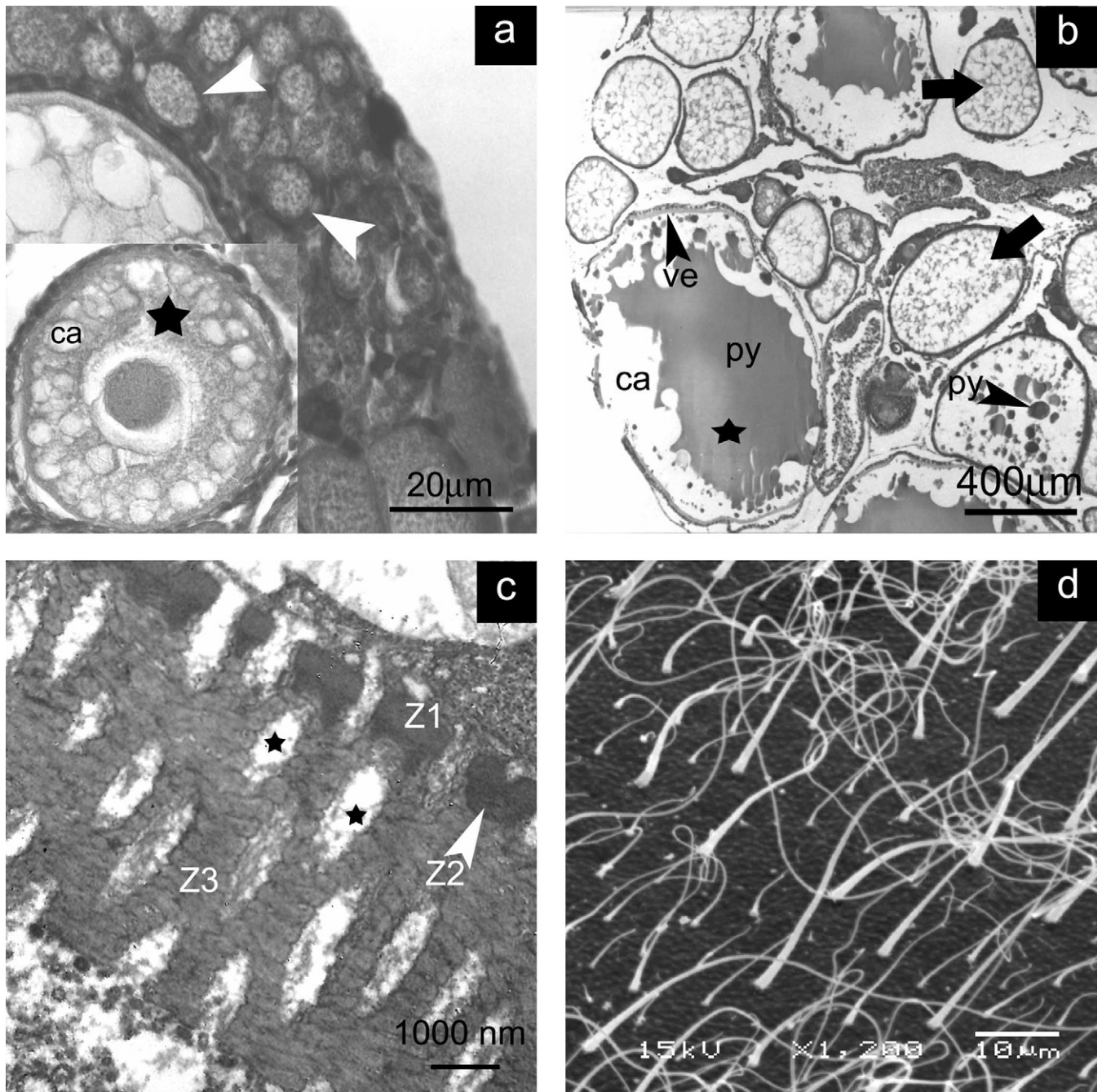
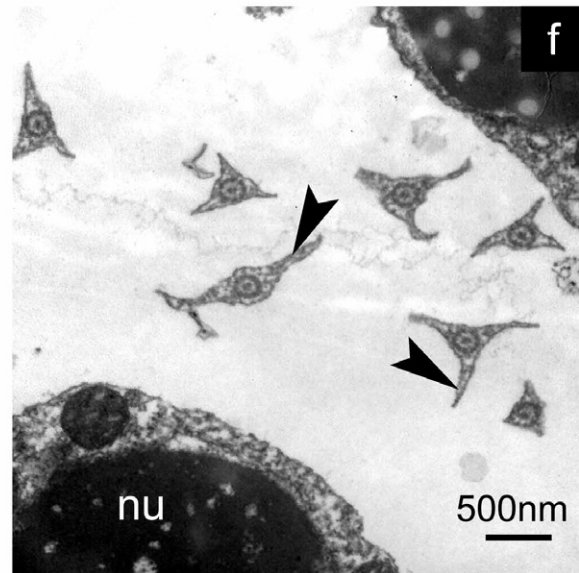
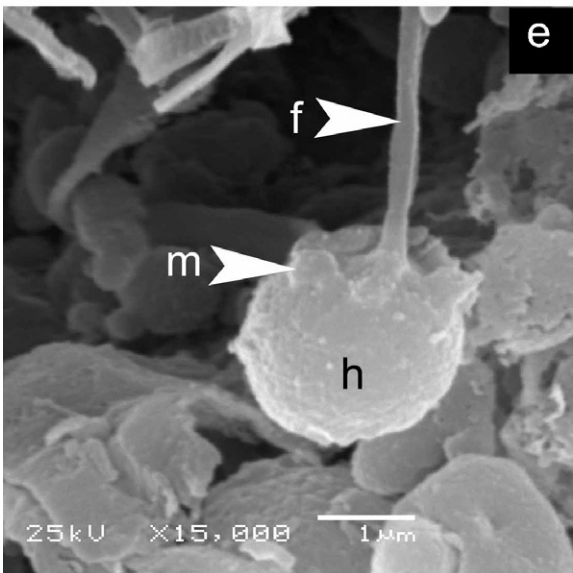
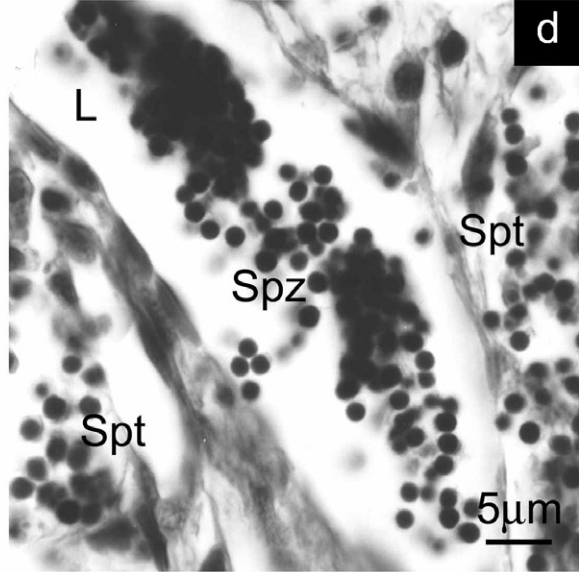
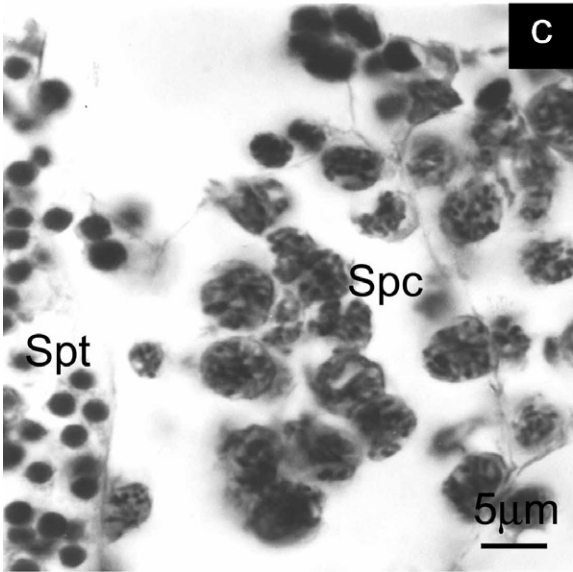
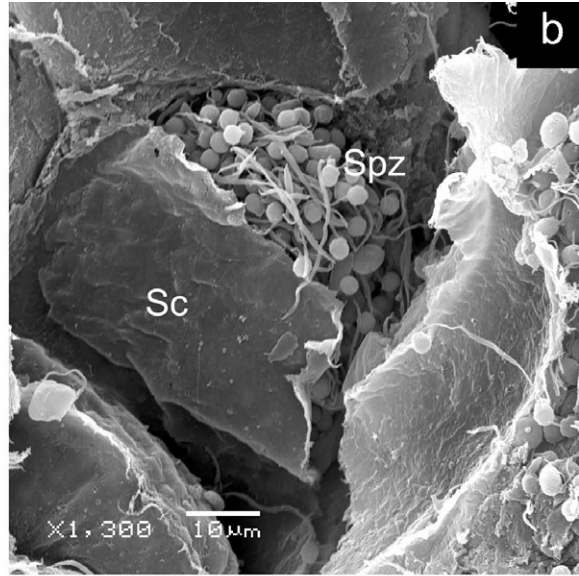
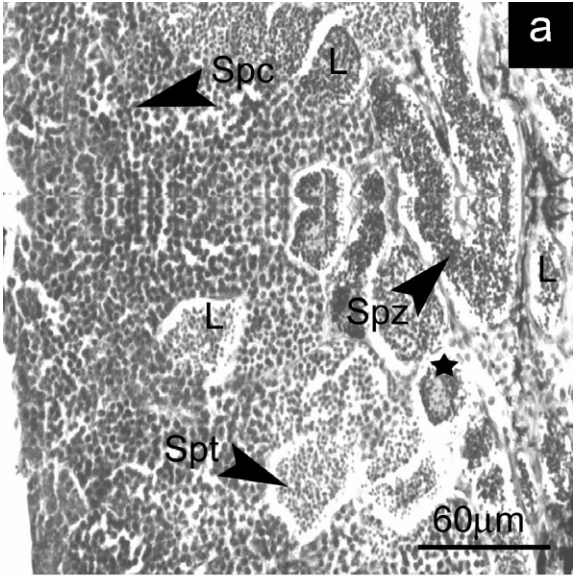


Fig. 4. Morphology of the adult gonads: ovaries. (a) Pre-vitellogenic oocytes: arrowheads, perinucleolar stage oocytes; star, putative cortical alveolar stage oocytes; ca, putative cortical alveoli (hematoxylin and eosin). (b) Vitellogenic oocytes (arrows) and fully grown oocyte (star); ve, vitelline envelope; py, protein yolk; ca, putative cortical alveoli (hematoxylin and eosin). (c) Ultrastructure of vitelline envelope: Z₁, zona radiata externa; Z₂, middle layer; Z₃, zona radiata interna; stars, channels (TEM). (d) Vitelline envelope surface showing the two kinds of filaments (SEM).

Fig. 5. Morphology of the adult gonads: testes. (a) Transverse section of testicular lobules showing cysts. Each cyst contained germ cells in the same stage of spermatogenesis. At left, testis periphery; at right, testis central zone; L, lobule; Spc, spermatocytes; Spt, spermatids; Spz, free spermatozoa; star, packed spermatozoa (hematoxylin and eosin). (b) Packed spermatozoa within a cyst: Spz, spermatozoa; Sc, Sertoli cell (SEM). (c) High magnification of a spermatocyte cyst (Spc): Spt, spermatids (hematoxylin and eosin). (d) High magnification of spermatozoa (Spz) and spermatids (Spt) cysts: L, lobule (hematoxylin and eosin). (e) Sperm surface: h, spherical head; m, mitochondria; f, flagellum (SEM). (f) Sperm tail lateral fins (arrowheads): nu, nucleus (TEM).



ovarian follicle and had a high nucleus/cytoplasm ratio. At the end of this stage, the oocytes were characterized by the presence of putative cortical alveoli beneath the cell membrane (Fig. 4a). Vitellogenic oocytes undergo successive stages during oogenesis. First, the cytoplasm had big vacuoles that appeared empty with the staining techniques used. The nucleus still showed several nucleoli in the periphery (data not shown). Subsequently, a fine granular acidophilic material began to accumulate among the vacuoles and progressively fused to form the yolk. Between the cell membrane of the oocyte and the follicle cells, the vitelline envelope was observed. Finally, fully grown oocytes were the largest cells and showed a large amount of acidophilic yolk fluid filling the cytoplasm. The putative cortical alveoli were visible without colour with the staining techniques used and the nucleus appeared displaced to one pole of the oocyte (data not shown). At this stage, there were no morphological changes related with the process of hydration (Fig. 4b).

The vitelline envelope showed radial striation and its thickness gradually increased to $6.0\ \mu\text{m}$ (± 1.0) for fully grown oocytes. TEM analyses of ovulated oocytes showed a vitelline envelope consisting of three layers: a zona radiata externa, formed by a homogeneous electron dense layer (Z_1), a middle electron-denser layer (Z_2), and a heterogeneous electron-dense zona radiata interna (Z_3). The latter was the widest and the last to appear during the deposition process. The entire vitelline envelope was crossed by channels (Fig. 4c). SEM analyses revealed a rough outer surface (sticky *in vivo*) that was ornamented by dense hair-like filaments. Based on their thickness and regular organization two kinds of filaments were recognized. Thick and thin filaments had an overall cone-shaped morphology (Fig. 4d).

3.2.2. Male

The male reproductive system consisted of a pair of elongated testes, triangular in transverse section, located between the swim bladder and the gut. They were covered by a darkly pigmented visceral peritoneum and both were connected with the genital papilla by a common deferent duct. The parenchyma was organized in lobules limited externally by a basal membrane. Within the lobules, spermatogenesis occurred in cysts. Among lobules, a vascular interstitial connective tissue and Leydig-like cells were observed. Lobules extended from the periphery to the central region of the gonad where they opened in a network of deferent ducts (Fig. 5a).

Within the lobule, each cyst consisted of clusters of germ cells in the same stage of spermatogenesis surrounded by Sertoli cells (Fig. 5b). Five spermatogenic stages were identified according to germ cell size and nuclear and cytoplasm characteristics: spermatogonia, spermatocytes I and II, spermatids, and sperms. All spermatogenic cysts were observed from sexual maturation (May) to senescence (December).

Spermatogonia were the largest cells and they were round cells with fine granular chromatin. They were located at the blind boundaries of the lobules at the periphery of the testis (data not shown). The morphology of the nuclei of spermatocytes

decreased in size as the cell proceeds through meiosis. Spermatids showed a small amount of acidophilic cytoplasm and a rounded nucleus with increasing chromatin condensation during spermiogenesis. Light microscopy analyses showed sperm packed in cysts and also free in the lumen of the deferent duct (Fig. 5a, c and d). In SEM and TEM analyses, the sperm exhibited a spherical head ($3.8 \pm 0.5\ \mu\text{m}$ diameter), a midpiece containing round mitochondria arranged in a single layer, and a flagellar tail. The tail had two, three, or four short lateral fins along almost the entire length (Figs. 5e and f).

4. Discussion

Gonadal differentiation, determination of sexual strategy, and gametogenesis have not previously been reported for annual fishes. These fish exhibit differences in timing of developmental stages and hatching (Wourms, 1972a; Lesseps et al., 1975; Van Haarlem, 1983). In addition, the possibility to undergo diapauses of variable length (Wourms, 1972c) increases their developmental asynchrony. Considering this developmental variability *A. charrua* embryos were classified using time post-fertilization in association with morphological features.

4.1. Sex differentiation

Previous studies showed that presence of gonadal structures (i.e., ovarian cavity and efferent duct formation), difference in germ cell number, and female precocious meiosis are valid criteria to determine gonadal sex at an early phase in development. However, which is the most reliable criterion varies depending on the studied species. A safe generalization is that, for most teleosts, germ cells in putative ovaries outnumbered those in putative testes (reviewed by Nakamura et al., 1998; Strüssman and Nakamura, 2002). Using the difference in germ cell number criterion, we observed the first evidence of histological sex differentiation in embryos at 30 days post-fertilization in *A. charrua*.

Sex differentiation has been shown to occur at different post-hatching times beginning earlier in females than in males in most gonochoric teleosts examined to date (reviewed by Nakamura et al., 1998; Strüssman and Nakamura, 2002). However, in *O. latipes* (Beloniformes, the sister Order to Cyprinodontiformes) and *Gambusia affinis* (Cyprinodontiformes) germ cells proliferate at a higher rate in presumptive ovaries during pre-hatching stages (Hamaguchi, 1982; Koya et al., 2003). This characteristic was also found in *A. charrua*.

Different sexual strategies are found among teleost fishes, ranging from species where testes and ovaries are in separate individuals (gonochorism) to species containing functional male and female tissues in the same individual (synchronous hermaphroditism) (reviewed by Devlin and Nagahama, 2002). Even among gonochorists, Yamamoto (1969) identified two patterns of gonadal development. In the

“differentiated” type, the early gonads directly develop into a testis or ovary whereas in the “undifferentiated” type the early gonads first pass through an ovary-like stage. By definition, transient occurrence of intersex gonads is not present in the differentiated gonochorists; sporadic intersexes would appear only in “undifferentiated” gonochorists (Yamamoto, 1969; Strüssman and Nakamura, 2002). Our histological analyses of gonadal differentiation from 3 weeks post-fertilization embryos to adults showed neither 100% ovary-like stage nor intersex gonads in the 341 individuals examined. Therefore, we conclude that *A. charrua* sexual strategy corresponds to a “differentiated gonochoric” pattern.

4.2. Adult gonads

Morphology of the gonads has traditionally been used to identify annual reproductive cycles, onset of reproductive maturity, spawning rhythms, and various other aspects of the reproductive biology. Histological analysis is considered an accurate method to determine the reproductive pattern in teleosts (Parenti and Grier, 2004).

A. charrua, spawn daily from the time they reach sexual maturity until senescence. Oocytes and spermatogenic cysts in all different stages of development were observed during the breeding months. These observations are in agreement with an asynchronous spawning pattern for females (reviewed by Wallace and Selman (1981)) and a continuous spawning for males.

Another aspect related to spawning is how ovulated oocytes are released to the environment. The different diameter ducts found in *A. charrua* accord with the description of an ovarian duct network that opens into a hollow structure, namely “oocyte chamber”, for this and other *Austrolebias* species. Since this “oocyte chamber” contained ovulated oocytes it was suggested that it could function as an oocyte storage compartment (Pereiro, personal communication). This structure seems to be similar to the “ovisac” described in the non-annual cyprinodontid *Fundulus heteroclitus* (Brummett et al., 1982).

The histological organization of the testes of *A. charrua* corresponds to the restricted lobular pattern because spermatogonia are confined to the distal end of the lobules (Grier, 1981). Fishes belonging to the Series Atherinomorpha (which include Cyprinodontiformes) uniquely present this type of testis whereas the unrestricted pattern (spermatogonia occurring along the testicular lobules) is present in most teleosts.

The main cellular aspects of gametogenesis in *A. charrua* can be summarised as: first, during vitellogenesis, the yolk appears as small globules that continuously fuse. This process of deposition results in a fluid mass that occupies almost entirely the mature oocyte. This supports the unique fluid yolk character previously identified for Atherinomorpha (Parenti and Grier, 2004). These authors attribute phylogenetic value to this condition that, along with the restricted spermatogonial pattern, appears to be unique derived characters for Atherinomorpha. Second, the eggs of *A. charrua* are laid in the

bottom mud (i.e., demersal condition). The lack of hydration and the ultrastructure of the outer surface of the vitelline envelope containing sticky and adhesive hair-like filaments accord to their demersal condition. The description of the ultrastructure of the vitelline envelope for *A. charrua* shows a trilaminar organization similar to the envelopes previously described for other ovipary fish species (Guraya, 1986; Berois et al., 2007). However, this vitelline envelope exhibit morphological differences at the inner zone (Z_3) when compared with other closely related species of *Austrolebias* (Casanova et al., unpublished). Finally, the main characteristics of *A. charrua* sperm were described. As in other teleostean fishes with external fertilization, this species presents the typical uniflagellate anacrosomal aquasperm (Jamieson, 1991). Current studies in sperm morphology comparing related species of *Austrolebias* suggest differences at ultrastructural level. These differential traits could be proposed as a useful tool for phylogenetic studies (Casanova et al. in preparation).

Considering that *A. charrua* inhabits the “Bañados del Este”, a Uruguayan area declared a Biosphere Reserve Area (UNESCO, 1976) and that the present study establishes baseline data regarding sex differentiation under laboratory conditions, this species may represent a useful taxon to monitor the effects of contamination in a protected area.

Acknowledgments

The authors wish to thank MSc. Gabriela Casanova and Br. Alvaro Olivera for their technical support (TEM). We are grateful for the suggestions and improvements made by the reviewers of the Tissue & Cell. The financial support was provided by Facultad de Ciencias and PEDECIBA (Programa de Desarrollo de Ciencias Básicas), Universidad de la República.

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