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Tissue and Cell 39 (2007) 89–98

Tissue&Cell

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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.

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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0040-8166/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tice.2007.01.004

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 ± 1) °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 dehydratation in increasing concentrations of alcohol series, the embryos, fry, and adult gonads were embedded in paraplast. Semiserial sections of 7 μm 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.