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Anatomical, histological and immunohistochemical study of testicular development in *Columba livia* (Aves: Columbiformes)

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ABSTRACT

In this work, testicular ontogeny is analyzed at the anatomical, histological and immunohistochemical levels; the latter through the detection of GnRHR and PCNA in the testicles of embryos, neonates and juveniles of *Columba livia*. We analyzed 150 embryos, 25 neonates and 5 juveniles by means of observations under a stereoscopic magnifying glass and scanning electron microscope (SEM). The histological analysis was performed using hematoxylin-eosin staining techniques and the PAS reaction. For the immunohistochemical analysis, the expression of GnRHR and PCNA in embryos corresponding to stages 41, 43 and in neonates of 2, 5, 7 and 75 days post-hatch was revealed in testicular histological preparations. That gonadal outline is evident in stage 18. In stage 29, the testes are constituted of a medulla in which the PGCs are surrounded by the Sertoli cells, constituting the seminiferous tubules. From stage 37 a greater organization of the tubules is visualized and at the time of hatching the testicle is constituted of the closed seminiferous tubules, formed of the PGCs and Sertoli cells. The Leydig cells are evident outside the tubules. In the juvenile stages, the differentiation of germline cells and the organization of small vessels that irrigate the developing testicle begin to be visible. In the analyzed stages, the immunodetection of the GnRHR receptor and PCNA revealed specific marking in the plasma membrane and in the perinuclear zone for GnRHR and in the nucleus of the germline cells in juvenile testicles for PCNA. These results can be used as a basis for further study of endocrine regulation events during testicular ontogeny in avian species.

1. Introduction

The study of gonadal development has been approached in model species such as *Gallus gallus domesticus* and *Coturnix coturnix* (González Morán, 2007, González Morán et al., 2008; Chang et al., 2012; Rong et al., 2013; Intarapat and Stern, 2014). These species have a morphogenetic pattern of the precocious-2 type, are born covered with feathers, with eyes open, locomotive organs well developed, and able to feed on their own (Starck and Richlefs et al., 1998).

The gonads develop primary sexual cords on the genital crest toward the end of the 18HH stage (Olea et al., 2016), but there is no difference between males and females, so it is a bipotential gonadal state. After the stage of bipotentiality or undifferentiation, the sexual cords differ in the seminiferous tubules of the testicles and in the medullary cords of the ovary. Both the left and right gonads continue to develop in the male, while in the female the left gonad develops in the ovary and the right one regresses (Smith et al., 2010).

In the case of males, the seminiferous tubules are constituted of PGCs, supporting cells and Sertoli cells, supported on a basal lamina and surrounded by a layer of peritubular myoid cells. These tubules are derived from the primary cords of the embryonic testis. The Leydig cells are the steroidogenic cells present in the interstitium next to the blood vessels. The somatic cells of the underlying mesonephros can also contribute to the early gonads (Gonzalez Moran and Soria Castro, 2010a,b; Chang et al., 2012).

In the males, during this process, the Sertoli cells and the PGC_S occupy the primary sexual cords, which subsequently anastomose with each other, forming the networks seen in the histological sections (Romanoff, 1960; Morrish and Sinclair, 2002; Gonzalez Moran and Soria Castro, 2010a,b).

Studies on morphology and development of the tests are mainly performed on mammals and some birds. To date, the morphological development of the testis has been well studied in some species of birds, such as poultry and quails (Gonzalez Moran, 1997; Gonzalez Moran and

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Soria Castro, 2010a; Chang et al., 2012). Studies with the main focus on early embryological events and throughout testicular ontogeny have not been reported, except for *Struthio camelus* by Hassanzadeh et al. (2014). They have analyzed in detail the morphological development of the testis, including the development of sexual cords, testicular capsules and epithelial ducts, as well as the evaluation of morphometric changes in the testicle and its tubular compartments.

During testicular gonadogenesis for the detection of proliferating cells by immunohistochemical assay, the expression of PCNA, a protein involved in cell proliferation, was analyzed. It was developed for tissues of different groups of vertebrates (Korfsmeier, 2002). This protein is widely used in bioassays to detect tumor cells, but PCNA is also expressed during cell proliferation in vertebrates and invertebrates (Ortego et al., 1994). PCNA has been found as a useful marker for the detection of mitotically active cells in reproductive organs of zebrafish (Korfsmeier, 2002) and birds (Gerzilov et al., 2016).

Many hormones that are classified as neuropeptides are synthesized in the vertebrate gonads in addition to the brain. Receptors for these hormones are also expressed in gonadal tissue. There is strong evidence to suggest the presence of gonadotropin-releasing hormone (GnRH) and its receptor (GnRH-R) in the gonads of several vertebrate species (Carou et al., 2017). The occurrence of GnRH and its receptor in the gonads has been demonstrated in mammals (Bahk et al., 1995; Dong et al., 1996 and Oikawa et al., 1990), birds (Sun et al., 2001), and, to the extent that the species of the genus is a species, reptiles (Ikemoto and Park, 2007) and amphibians (Di Matteo et al., 1988; Di Mateo et al., 1996; Lin and Peter, 1996; Pati and Habibi, 1998; Von Schalburg et al., 1999; Bogerd et al., 2002). It is believed that GnRH may play an autocrine and paracrine role in the regulation of gonadal development and function, including testicular steroidogenesis in rats and frogs (Hsueh et al., 1983; D'Antonio et al., 1992), spermatogenesis in fish (Andreu Vieyra and Habibi, 2001;Andreu Vieyra et al., 2005) and oocyte meiosis and follicular steroidogenesis in fish (Pati and Habibi, 2000).

As previously mentioned, only testicular development events for *G. gallus domesticus*, *C. coturnix*, *C. japonica* and *Struthio camelus* have been described, and remain unknown for other taxa. *Columba livia* is a cosmopolitan species with large populations in urban areas. They have a relatively short incubation period (17 days) and their embryos are easy to manipulate and present a pattern of semialtricial-2 development. Because of these characteristics, this species is a good model for addressing different types of studies related to developmental biology.

The objective of this work is to describe and characterize testicular development in *Columba livia*, at anatomical, histological and immunohistochemical levels, from the detection of the GnRH receptor, its relationship with the cells of the germline and with the expression of PCNA. The work aims to expand knowledge about gonadal development in birds and provide useful data to make comparisons with other species already studied.

2. Materials and methods

2.1. Obtaining study material

To obtain the study material, 10 nests of *Columba livia* were selected at the university campus of the Facultad de Ciencias Exactas y Naturales y Agrimensura de la Universidad Nacional del Nordeste, Corrientes, Argentina (*Faculty of Exact and Natural Sciences and Surveying at the National University of the Northeast*). Nests were checked daily between November and May 2011–2012 and 2013–2014, between 7 a.m. and 9 p.m. to obtain recently laid eggs, as laying occurs mostly in the early hours of the day. 2 eggs were collected per day, per nest, from the 10 nests, and were taken to the laboratory where they were incubated in a culture oven at 35–37 °C with 40–45% moisture.

In order to analyze specimens at different stages of development, the embryos, neonates, and juveniles were euthanized. In the case of embryos the following protocol was followed: every 6–10 h for the early (E.) stages (E. 10–22) and every 12–24 h for the middle and late stages (E. 30 to 43). For the identification of the stages, the table of heterochronic events proposed for the species under study was followed (Olea and Sandoval, 2012). For the neonates, euthanasia of 2, 10, 15 and 25 days post-hatching (dph) and juveniles of 75 dph. The slaughter of the specimens was carried out following the standard method established in the Guide for Animal Euthanasia proposed by the IACUC (The Institutional Animal Care and Use Committee) and CICUAL-Med-UNNE 0004-CICUAL/17.

Embryos in E. 10–30 were fixed and preserved whole in 10% buffered formalin. The samples of stages superior to 30, neonates and juveniles were dissected in order to extract the genital system, which was fixed in a solution of Bouin for 48 h and subsequently preserved in 10% formaldehyde.

180 animals were analyzed using 60 embryos for the early stage (Stages 15–30), 60 for the middle stage (Stages 31–40), 30 for the late stage (Stages 41–45), 25 neonates and 5 juveniles, which were later incorporated into the scientific collection of the *Compared Anatomy of Chordates* subject of the Biology Department of the *Facultad de Ciencias Exactas y Naturales y Agrimensura de la Universidad Nacional del Nordeste*.

2.2. Anatomical studies

The gonadal structures were characterized at each stage of their development, and the key stages in which the gonad differentiation was produced were determined. To this end, the analysis was divided into the following steps:

2.2.1. Morphological analysis of the gonads

Embryo dissection and analysis of the topography and morphology of gonadal structures at different stages of development were performed based on the observation of a stereoscopic microscope and a scanning electron microscope (SEM). The preparation of the specimens for SEM was carried out following the standardized dehydration protocol in solutions of increasing concentration of acetone (12.5, 25, 50, 75 and 100%), dried at a critical point and metalized with gold-palladium. The observations were made using a JEOL JSM-5800 LV microscope belonging to the Scanning Electron Microscopy Service of the *Secretaría General de Ciencia y Técnica de la Universidad Nacional del Nordeste (UNNE)*.

2.2.2. Histological analysis of the gonads

The gonads were fixed in Bouin's solution and later preserved in 10% buffered formalin. Histological preparations were carried out following the conventional techniques of dehydration, inclusion in paraffin and colorations. Dehydration was performed in increasing concentrations of ethyl alcohol (70, 80 and 96%) and butyl alcohol (100%) for 25–45 min depending on sample size. Inclusion in butyl paraffin (50–50%) for 24 h and pure paraffin overnight was performed. After this, the blocks were made and the samples were prepared to obtain cross-sections or longitudinal sections of 5–7 microns. These were obtained with a Spencer manual rotary microtome. Samples were stained with Hematoxylin-Eosin. The preparations were observed and photographed with a Trinocular Microscope (Leica, inc. Model DME) and supported using image capturing software Leica LASZ.

2.3. Immunohistochemical studies to determine GnRHR

To establish the presence of the GnRH receptor in germline cells in differentiation in the neonatal and juvenile testis of *C. livia*, expression of GnRHR was revealed using an anti-GnRHR antibody PABX (1 mg / ml), clone GNRH03 made in mice by the company Thermo Scientific Cat. # MS-1139-P1 ABX (Carou et al., 2017), in a working dilution 1: 800, incubated for 60 min at 37 °C; and the development kit according to the indirect protocol of "L-streptoavidin biotin" - HRP (DAKO