

Histological and immunohistochemical study of the ovaries and oviducts of the juvenile female of *Caiman latirostris* (Crocodylia: Alligatoridae)

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ABSTRACT. The purpose of this study was to examine the ovaries and the oviduct of juvenile females of the broad-snouted caiman, *Caiman latirostris* (Daudin, 1802), using light microscopy, histochemical and immunocytochemical techniques. The ovarian cortex was observed to contain groups of germinative cells and abundant previtellogenic follicles at different stages of development. In previtellogenic follicles, the oocyte was surrounded by a single cuboidal layer of granulosa cells, supported by the theca layer. The theca was formed by a concentric layer containing collagenous fibers, predominantly type I, and several smooth muscle fibers. The inner perivitelline layer appeared as a narrow basophilic region between the oocyte and the granulosa. The ovarian medullary region of loose connective tissue contained blood and lymphatic vessels, as well as numerous lacunae, being covered by a simple squamous epithelium, supported by a thin layer of connective tissue. The oviduct of *C. latirostris* showed five histologically distinct regions: infundibulum, magnum, isthmus, uterus and vagina. The infundibulum was composed of an irregularly folded mucosa covered by a simple columnar epithelium with the presence of ciliated cells, weakly reactive to PAS staining. In the magnum, the mucosa was highly folded, and pseudostratified columnar epithelium contains mucous cells, which reacted positively to PAS staining. The isthmus was lined by an epithelium of ciliated and non-ciliated secretory cells, but no gland was visualized in mucosa. In the uterus, the folded mucosa was composed of a simple epithelium of high cylindrical cells and the lamina propria, which was predominantly formed by strongly birefringent fibers, but yellow-green weakly birefringent fibers were also present. The epithelium of the vagina contained intensely ciliated and non-ciliated cells, both of which were positive to PAS staining and no gland was observed in this region. Although the ovary and the oviduct showed some morphological particularities, they are similar to other crocodylians.

KEY WORDS. Female reproductive system; morphology; reptiles.

Crocodylians are reptiles that belong to the order Crocodylia, which is divided into two families: Alligatoridae and Crocodylidae. Five species can be found in Brazil: *Caiman latirostris* (Daudin, 1802), broad-snouted caiman, *Melanosuchus niger* (Spix, 1825) (black caiman), *Paleosuchus palpebrosus* (Cuvier, 1807) (dwarf caiman), *Caiman crocodilus crocodilos* (Linnaeus, 1758) (common caiman) and *Caiman crocodilus yacare* (Daudin, 1802) (yacaré caiman), all belonging to the family Alligatoridae (IUCN 2015).

Broad snouted caimans are widely distributed in South American aquatic ecosystems (YANOSKY 1990, VERDADE 1995). Caimans spend a large portion of their lives in the water. They are long-lived animals and are at the top of the food chain. As in other countries, the broad-snouted caiman was placed in

the endangered species list of Brazil, since populations are low due to habitat pollution and predatory hunting for commercial purposes (BERNARDES et al. 1990, GROOMBRIDGE 1987). *Caiman latirostris* is still included in listed on CITES Appendix I (except the population of Argentina) that include all species threatened with extinction, which are or may be affected by trade (IUCN 2015). Because of its economic potential (for leather and meat) there has been great interest in recent years in its commercial breeding (AVEIRO 2012).

In the oviduct of reptiles, five regions are recognized: infundibulum, magnum, isthmus, uterus and vagina (GIRLING 2002). Crocodylians are unusual reptiles in that there are two separate regions, one for formation of the eggshell membranes and the other for the calcareous layer (PALMER & GUILLETTE 1992).

In this respect, these animals are similar to birds, in which a soft shell membrane is formed in the isthmus and the calcareous shell is formed in the uterus (AL-KINDI et al. 2006).

Various articles have described the oogenesis process and histology of the ovaries and oviducts of reptiles, such as of the species *Mabuya brachypoda* (Taylor, 1956) (HERNANDEZ-FRANYUTTI et al. 2005), *Sceloporus grammicus* (Weigmann, 1828) (LOZANO et al. 2014), sea turtles of the species *Caretta caretta* (Linnaeus, 1758), *Dermochelys coriacea* (Vandelli, 1761) and *Chelonia mydas* (Linnaeus, 1758), (CERIANIA & WYNEKEN 2008), the freshwater turtles *Chelydra serpentina* (Linnaeus, 1758) (AL-KINDI et al. 2006) and *Phrynops geoffroanus* (Schweigger, 1812) (FIRMIANO et al. 2012) and the snake *Naja kaouthia* (Lesson, 1831) (TUMKIRATWONG et al. 2012). In crocodylians, the reproductive system has been described of *Alligator mississippiensis* (Daudin, 1802) (JOSS 1989, GUILLETTE et al. 1994, 1995, 1997, URIBE & GUILLETTE 2000), *Caiman crocodilus* (Linnaeus, 1758) (CALDERÓN et al. 2004) and *Caiman yacare* (COUTINHO et al. 2005). In *C. latirostris*, VERDADE (1995) described the reproductive biology of males and females and STOKER et al. (2003, 2008) reported alterations in the follicular dynamics and steroid levels caused by exposure to endocrine disruptor chemicals (EDCs).

The reproductive cycle of crocodylians is more complex and evolved than in other reptiles. In general, caimans, alligators and the smaller crocodile species reach sexual maturity while still relatively small in relation to their full adult size, while the large crocodile species only become sexually mature when they reach relatively larger sizes (MAGNUSSON et al. 1989). Female broad-snouted caimans take seven to eight years to reach reproductive maturity and males take 10 years. In *C. yacare*, the vitellogenic capacity can start earlier, at around 5 years of age, but mature ovaries with advanced vitellogenic follicles have only been observed in 7-year old females (COUTINHO et al. 2005).

Knowing the reproductive biology of an animal is a key factor to achieve success in its long-term rearing. The objective of this study was to characterize the structure of the ovaries and oviducts of the juvenile female *Caiman latirostris* through histochemical and immunohistochemical techniques. This new information increases our understanding of the morphological changes during puberty, thereby enhancing the breeding of this commercially valuable reptile.

MATERIAL AND METHODS

Eight two-year old juvenile females of *Caiman latirostris*, from Bonsucesso Farm (Nossa Senhora do Amparo District of Barra Mansa City, Rio de Janeiro State, Brazil), were obtained after slaughter at a slaughterhouse located near the city of Itaguaí. The breeder is registered in accordance with the Brazilian environmental agency (IBAMA) and is authorized by the local government (Rio de Janeiro State Environmental Agency, FEEMA) and regional board of veterinary medicine. The animals were subjected to hypothermia prior to being slaughtered after exsanguination.

For the light microscopy study, we removed from each animal three ovary samples and one sample from each region of the oviduct (infundibulum, magnum, isthmus, uterus and vagina). The fragments were collected and fixed by immersion in Bouin's liquid for six hours. The samples were then processed according to the histological technique for paraffin embedding. Five-micrometer-thick serial slices were stained with hematoxylin-eosin and Gomori's trichrome (LILLIE & FULLMER 1976) to analyze the general morphology and the arrangement of collagenous fibers. The Weigert's resorcin-fuchsin staining was used to visualize the elastic fiber system (LILLIE & FULLMER 1976). Staining with 1% Alcian blue (AB) 8GX at pH 2.5 (KIERNAN 1990) was employed to detect sulfated and carboxylated acidic glycoconjugates. The periodic acid and Schiff reagent (PAS) staining was employed to detect neutral glycoproteins. Moreover, the picosirius-polarization method (JUNQUEIRA et al. 1979) was utilized to observe the arrangement of collagenous fibers.

For immunohistochemistry, paraffin sections were de-waxed, dehydrated and washed in phosphate buffered saline (PBS), followed by incubation with methanol containing 0-3% H₂O₂ for 15 minutes to block any endogenous peroxidase. The sections were then incubated with a 1:100 dilution of bovine serum albumin (B4287; Sigma) in phosphate buffered saline (PBS) in a humid chamber for 30 minutes at room temperature, followed by incubation overnight with anti-smooth muscle α actin (Cat. no. 08-0106, Zymed, California, USA) diluted to 1:200 at 4°C. Sections were then incubated with biotinylated secondary antibody (PK 7200, Vector Laboratories, Inc., U.K.) for 30 minutes, then with ABC, diluted to 1:200, for 30 minutes (both from PK 6200, Vector Lab. Inc.). Subsequently, the peroxidase label was revealed by reaction with DAB at room temperature before dehydration and mounted using Enthelan (Merck).

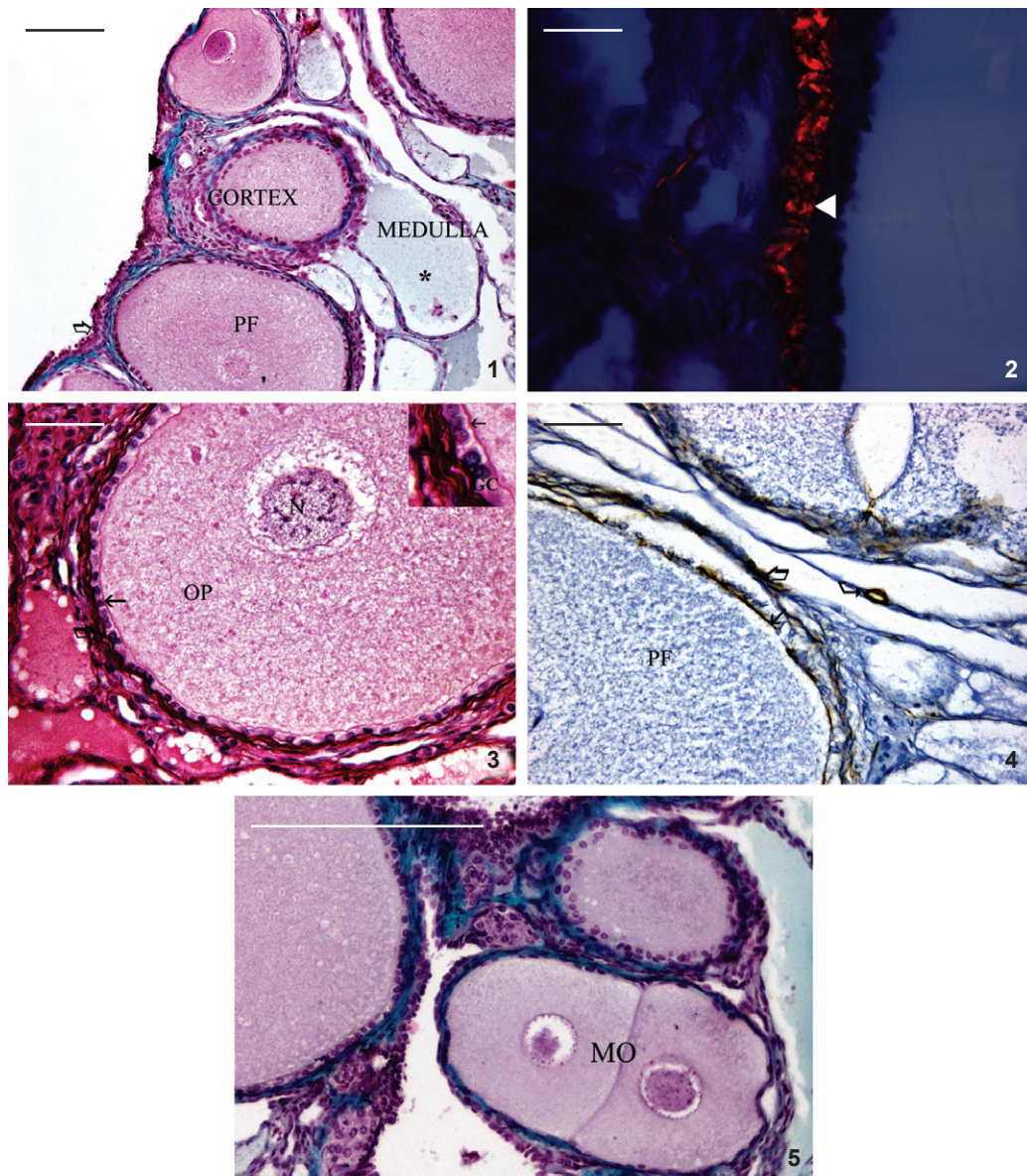
Oocytes and follicles were classified according to previously established criteria for *A. mississippiensis* and *C. crocodilus* (URIBE & GUILLETTE 2000, CALDERÓN et al. 2004). In the stages I and II (or early previtellogenesis) oocyte growth is initiated and in stage III follicles are in advanced stages of previtellogenesis. To quantify the diameter of the oocytes of the ovarian follicles three sections of each animal were digitized. Selected images used for measurements and quantification were obtained using a light microscope (Leica DM500) coupled to a Digital Camera Leica ICC50 HD. Image analysis was carried out using the Image J system (National Institute of Health, EUA).

RESULTS

The ovaries of juvenile females of the *C. latirostris* are characterized by an irregularly shaped, elongated structure, and present a cortical and a medullar region (Fig. 1). The cortical region is covered by an epithelium that varies from cuboidal simple to squamous. Below the epithelium a thin tunica albuginea is formed by dense unordered connective tissue, revealed

by Gomori's trichrome (Fig. 1). Staining with picosirius red revealed red strongly birefringent fibers, indicating the predominance of type I collagen (Fig. 2). In the tunica albuginea, thin smooth muscle fibers were also observed, detected by the immunohistochemical method for α -smooth muscle actin (SMA).

The ovarian cortex was observed to contain groups of germinative cells and abundant previtellogenic follicles at different developmental stages, along with sparse follicles in the initial stage of development (stage I). These contained an oocyte surrounded by squamous follicular cells.



Figures 1-5. Photomicrographs of a cross section of the ovary of *Caiman latirostris*. (1) Cortical region with previtellogenic follicles (PF) and a medullar region with lacunae covered by a simple squamous epithelium (*). The ovarian surface epithelium (\Rightarrow), tunica albuginea (\blacktriangleright). Gomori's trichrome. (2) Tunica albuginea formed by strongly birefringent fibers (\blacktriangleright), indicating the predominance of type I collagen. Picosirius red. (3) Previtellogenic follicles (stage III). Oocyte nucleus (N) and ooplasm (OP). The oocyte is surrounded by a single cuboidal layer of granulosa cells (GC), supported by a theca layer (\Leftarrow). Note the presence the inner perivitelline layer (\Leftarrow). Picosirius red. (4) Smooth muscle cells in blood vessel walls (\rightarrow) and in theca layer (\curvearrowright). Note positive reaction in the inner perivitelline layer (\Leftarrow). (5) Previtellogenic follicles containing multiple oocytes (MO). Gomori's trichrome. Scale bars: 1 = 100 μ m, 2 = 20 μ m, 3-4 = 50 μ m, 5 = 200 μ m.

Follicles in stage II had diameters ranging from 0.12 to 0.15 mm, with large nuclei, and were completely surrounded by squamous to cuboidal follicular cells. Follicles in advanced stages of previtellogenesis (stage III) measured between 0.33 and 0.43 mm in diameter and contained oocytes immersed in abundant follicular liquid. The nucleus of the oocyte is oval, eccentric or central, and contains lampbrush chromosomes and nucleoli. The ooplasm contains small uniformly distributed vacuoles. The oocyte is surrounded by a single cuboidal layer of granulosa cells, supported by a theca layer (Fig. 3). The theca is formed by a concentric layer of collagenous fibers, predominantly type I (Fig. 3), and several smooth muscle fibers (Fig. 4). With increased follicular size, the inner perivitelline layer (Fig. 3), which is equivalent to the zona radiata, appears like a narrow basophilic band established in the border region between oocyte and granulosa cells. This region was positive for SMA immunohistochemical method (Fig. 4). Previtellogenic follicles containing multiple oocytes were observed (Fig. 5). Vitellogenic follicles, atresic follicles and corpus luteum were not identified.

The ovarian medullary region is composed of loose connective tissue with blood and lymphatic vessels, as well as numerous lacunae covered by a simple squamous epithelium, supported on a thin layer of connective tissue, hence classified as a chordolacunar type ovary. The lacunae contain an acidophilic substance (Fig. 1). No elastic system fibers were observed in the ovarian stroma.

The oviduct of the *C. latirostris* juvenile females presented the same five histologically distinct regions as other reptiles: infundibulum, magnum, isthmus, uterus and vagina, all of them formed by three concentric layers: mucosa, muscular and serosa (Fig. 6).

The infundibulum is composed of an irregularly folded mucosa covered by a simple columnar epithelium with the presence of ciliated cells, weakly reactive to PAS staining. The lamina propria presents small simple tubular glands without the presence of acid or neutral mucins (AB and PAS negative, respectively) and dense unmodeled connective tissue. The muscular layer is formed by two layers of smooth muscles.

In the magnum, the mucosa is highly folded. The epithelium lining is pseudostratified columnar and contains cells with characteristics of mucosa cells, which react positively to PAS staining, demonstrating the presence of neutral glycoproteins (Fig. 7). Intraepithelial glands were present. The lamina propria has varied small glands (tubulo-alveolar, branched tubular and branched acinar) that are PAS negative. The lamina propria contains smooth muscles. The muscle layer is formed by smooth muscle tissue with a circular internal and longitudinal external arrangement and the serosa was clearly visible, with the presence of a mesothelium formed by a simple squamous epithelium (Fig. 8).

The isthmus is an intermediate area between the magnum and uterus. It presents an epithelium composed of cili-

ated and non-ciliated secretory cells, but contains no glands in its mucosa. The other layers of the isthmus have similar form to those of the other regions.

In the uterus, the epithelium is formed by high cylindrical cells with basal nuclei and the mucosa is folded (Fig. 9). The lamina propria is predominantly formed by strongly birefringent fibers stained red by the picrosirius method, but yellow weakly birefringent fibers are also present. This region has small glands in the lamina propria (Fig. 10). The muscles are formed by two layers, one internal circular and the other external longitudinal, which is thicker.

The most caudal region of the oviduct, which leads out to the common urogenital or cloacal opening, is called the vagina. The epithelium contains intensely ciliated cells as well as non-ciliated cells, both of which are positive to PAS staining. No glands were observed in this region. The muscles are formed of two smooth muscle layers.

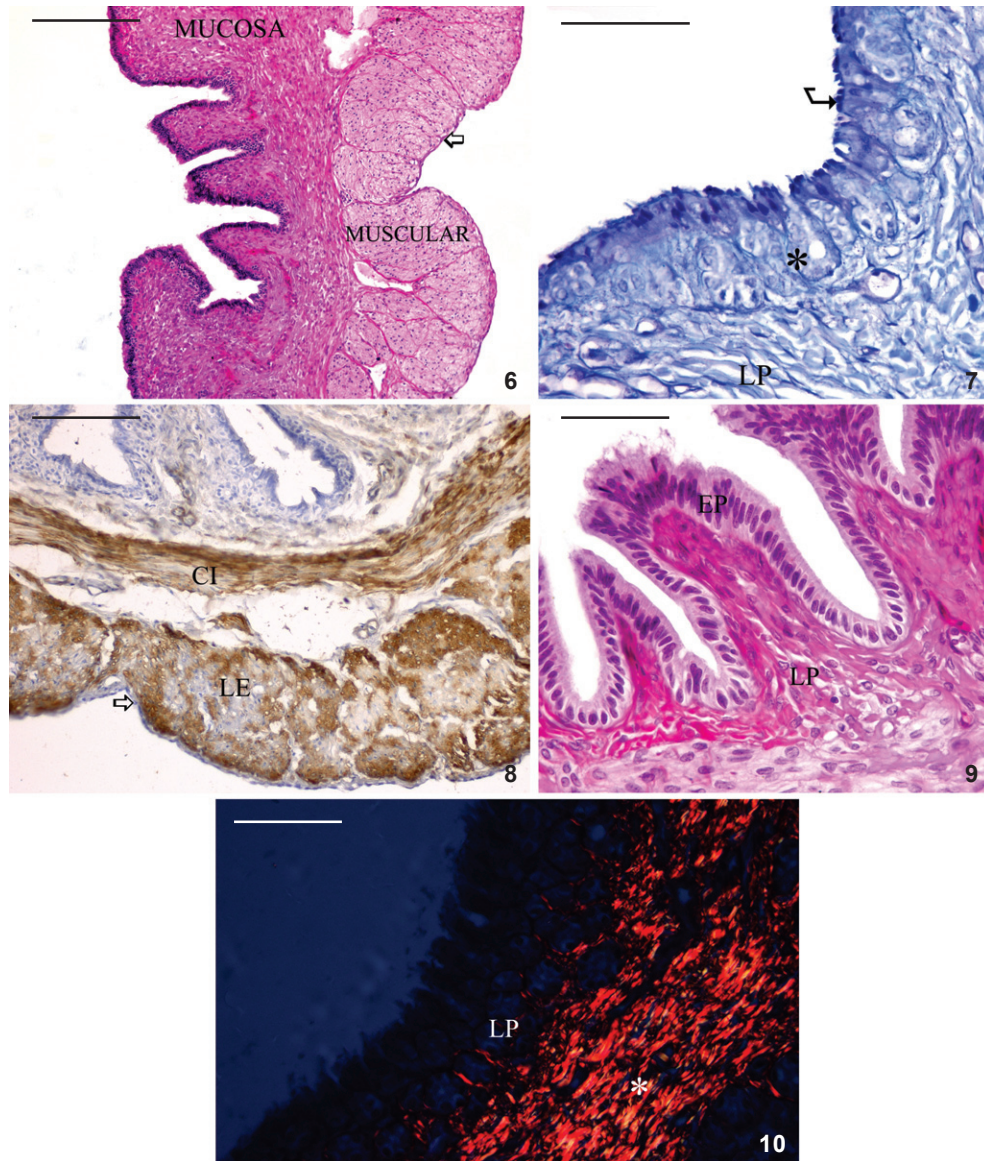
DISCUSSION

The gonadal histoarchitecture of the non-reproductive *C. latirostris* females examined was similar to that of other crocodylians, such as *C. crocodilus* (CALDERÓN et al. 2004) and *A. mississippiensis* (URIBE & GUILLETTE 2000). The chordolacunar type ovary has also been described in birds and testudines (CALLEBAUT 1988, CERIANI & WYNEKEN 2008). This type of ovary is characterized by the presence of various medullary lacunae that support the developing follicles, with the necessary expansive properties during follicular development. These may also participate in ovulation (CALLEBAUT 1988).

The epithelium of the ovaries examined is cuboidal to squamous and contains dense connective tissue and small bundles of smooth muscle, constituting the thin ovarian wall or tunica albuginea. CALDERÓN et al. (2004) using ultra-thin sections identified fibers of elastin lamina underline the epithelium of *C. crocodiles*. However, these fibers were not observed in our study using the staining with Weigert's resorcinfuchsin.

The germinative cells were found in the cortical ovary of *C. latirostris*. These cells were also identified in *C. crocodilus* (CALDERÓN et al. 2004) and the neonatal ovary of *C. latirostris* (STOKER et al. 2008). It is widely accepted that after birth the primordial germ cells of mammals do not proliferate. Thus, it is the ovarian reserve of primordial follicles that comprises the source of female gametes for life (VAN VOORHIS 1999). In many reptiles, it is known that oogonia proliferation occurs in the gonad during embryonic development and continues in adulthood (LESSMAN 1999). However, mitotic figures were frequently seen in germ cells in neonatal and 3-monthold *C. latirostris* (STOKER et al. 2008).

Previtellogenesis is a stage of development that involves emergence of a variety of follicles, ranging from primordial follicles that have recently migrated out of the germinal bed to large follicles immediately prior to vitellogenesis (VIEIRA et al.



Figures 6-10. Photomicrographs of a cross section of the oviduct of *Caiman latirostris*. (6) The infundibulum with three concentric layers: mucosa, muscular and serosa (↷). Hematoxylin and eosin. (7) Magnum lining by pseudostratified columnar epithelium. Note mucosa cells, which react positively to PAS staining (arrow) and intraepithelial glands (*). Lamina propria (LP). AB pH 2.5. (8) Smooth muscle cells in magnum. Muscle layer formed by smooth muscle tissue with a circular internal (CI) and longitudinal external (LE) arrangement and the serosa (arrow). (9) Uterus with folded mucosa and epithelium formed by high cylindrical cells. The lamina propria (LP). Hematoxylin and eosin. (10) Uterus. The lamina propria is predominantly formed by strongly birefringent fibers (*) stained red by the picrosirius method. Note small glands in the lamina propria (LP). Scale bars: 6 = 200 μ m, 7, 9, 10 = 50 μ m, 8 = 100 μ m.

2010). The previtellogenic follicles of *C. latirostris* are abundant and contain a nucleus, ooplasm and a layer of follicular or granulosa cells. Three types of follicular cells (pyriform cells) were observed in the snake *N. kaouthia* (TUMKIRATWONG et al. 2012). In *C. latirostris*, the granulosa is a single layer, like in birds (GURAYA

1989), *C. crocodilus* (CALDERÓN et al. 2004) and *A. mississippiensis* (LANCE 1989, URIBE & GUILLETTE 2000). These granulosa cells are apparently involved in the early steps in oocyte development. As the time of ovulation approaches, granulosa cells and some thecal cells accumulate cholesterol-positive lipids, and follow-

ing ovulation, proliferate and luteinize to form the corpora luteum (NORRIS 2007, TUMKIRATIWONG et al. 2012). The follicular diameters in the previtellogenic *C. latirostris* specimens (0.12 to 0.43mm) were similar to those observed in *C. crocodilus* (0.15 to 0.39 mm) (CALDERÓN et al. 2004).

Distinct immunostaining for α -smooth muscle actin was regularly found in the follicular walls of the previtellogenic follicles of *C. latirostris*. The presence of smooth muscle fibers outlining the theca of vitellogenic follicles, in the chordae that suspend the follicles, and below the ovarian epithelium was observed in *C. crocodilus* (CALDERÓN et al. 2004). JONES (1987), studying scaled reptiles, VAN NASSAUW et al. (1991), analyzing testudines, and CALDERÓN et al. (2004), studying alligators, all suggested the participation of these structures during follicular development and ovulation processes, through contractile mechanisms.

In the corpora luteum, follicular atresia and formation of the corpora atretica follicles are common elements in reptilian ovaries, as in other vertebrates (NORRIS 2007). However, as in juvenile females of *C. crocodilus* (CALDERÓN et al. 2004), these structures were not detected in *C. latirostris*. This absence is due to the fact that these structures are reported only in reproductive females.

The reptilian oviduct is an organ whose multiple functions reflect the variety of reproductive patterns exhibited by diverse group of animals (GIRLING 2002). In reptiles, the term 'oviducts' designates the structures derived from the Müllerian ducts in the embryonic period (WAKE 1985). The oviduct in reptiles is divided into five anatomical regions: infundibulum, magnum, isthmus, uterus and vagina (FOX 1977, GIRLING 2002). However, the different regions are not recognized in all reptilian species, and additional regions may also be included, like in the oviparous lizards *Crotaphytus collaris* (Say, 1823) and *Eumeces obsoletus* (Baird & Girard, 1852) (GUILLETTE et al. 1989), the viviparous lizard *Hoplodactylus maculatus* (Gray, 1845) (GIRLING et al. 1997), the snakes *Diadophis punctatus* (Linnaeus, 1766) (PERKINS & PALMER 1996) and *Seminatrix pygaea* (Cope, 1871) (SEVER et al. 2000), in gecko *Hemidactylus mabouia* (Moreau De Jonnés, 1818) (NOGUEIRA et al. 2011).

The oviduct of *C. latirostris* shows the same histological layers found in other species of reptiles, like the turtles *P. geoffroanus* (FIRMIANO et al. 2012) and *C. serpentina* (AL-KINDI et al. 2006) and lizards *C. collaris* and *E. obsoletus* (GUILLETTE et al. 1989). From the outer surface to the lumen, these are as follows: serosa, muscle and mucosa.

In the infundibulum of *C. latirostris*, the mucosa contains folds that gradually increase in height toward the magnum, as in the gecko *Hemidactylus turcicus* (Linnaeus, 1758) (GIRLING et al. 2000) and in snake *D. punctatus* (PERKINS & PALMER 1996). The infundibulum contains a simple ciliated columnar epithelium, in contrast to that found in *P. geoffroanus* (FIRMIANO et al. 2012), which reacts negatively to PAS. In *C. latirostris* the epithelial cells react positively, indicating neutral glycoconjugates. Like

in *C. serpentina* (AL-KINDI et al. 2006), in *C. latirostris* we only observed a few scattered endometrial glands in the lamina propria.

The magnum of turtles and crocodylians is responsible for the production of albumen: egg white proteins that surround the egg prior to oviposition in turtles and crocodylians, but not in squamates (GIRLING 2002). The magnum of *C. latirostris* is highly pleated and the mucosa has high longitudinal elevations, as described in *C. serpentina* (AL-KINDI et al. 2006). The pseudostratified columnar epithelium of the tube of *C. latirostris* responds positively to PAS staining, indicating the production of neutral glycoconjugates, a result also found in *D. punctatus* (PERKINS & PALMER 1996), *H. turcicus*, *Saltuarius wyberba* (Couper, Schneider & Covacevich, 1997) and *H. maculatus* (GIRLING et al. 1998), and *H. mabouia* (GIRLING et al. 2000). This secretion may be involved in protecting and lubricating epithelial surfaces, thereby facilitating the passage of oocytes through the oviduct (SEVER et al. 2000, GIRLING et al. 2000). In female *C. latirostris* specimens, we observed only small endometrial glands in the lamina propria. However, the endometrial glands are numerous and occupy most of the lamina propria in *C. serpentina* (AL-KINDI et al. 2006) and *P. geoffroanus* (FIRMIANO et al. 2012). These differences can be explained by the fact that the females examined in this study had not yet reached reproductive age.

The isthmus is narrow and connects the magnum with the uterus. The isthmus often appears to share similarities with both its neighboring regions (GIRLING 2002). Few glands are found in the mucosa of *C. serpentina* (AL-KINDI et al. 2006). However, in *C. latirostris* no glands were found.

The uterine mucosa of *C. latirostris* is pleated, as observed in *C. serpentina* (AL-KINDI et al. 2006). The luminal epithelium in *C. latirostris* is similar to that of oviparous squamates, composed by a columnar epithelium with both ciliated and nonciliated cells (PALMER et al. 1993, PERKINS & PALMER 1996, GIRLING et al. 1998). The lamina propria of *C. latirostris* predominantly contains collagenous fibers and small glands. In *C. serpentina* (AL-KINDI et al. 2006), the endometrial glands dominate the lamina propria. A thick muscular wall with an outer longitudinal and inner circular layer was observed, like in the turtle *P. geoffroanus* (FIRMIANO et al. 2012).

The mucosa epithelium of the vagina is composed of ciliated and non-ciliated cells. SEVER et al. (2000) suggested that ciliated cells may function in sperm transport or the movement of mucus and debris from the oviduct. On the other hand, non-ciliated cells, which stain positively for carbohydrate substances, contain numerous secretory granules (GIRLING et al. 1997, 1998). In the common gecko, *H. maculatus*, goblet cells were also present, but in *C. latirostris* this cell type was not observed. In the vagina of *P. geoffroanus* (FIRMIANO et al. 2012), glands or gland-like crypts between the mucosal folds can be present in the anterior vagina and are associated with sperm storage, but in *C. latirostris* these glands were not observed.

The morphology of the ovaries of juvenile females of *C. latirostris* is similar to that of other species of crocodylians, for instance *C. crocodilus* and *A. mississippiensis*, which includes follicular histological features and a well-developed chordolacunar system. However, despite numerous studies on the oviducts of reptiles, more studies in crocodylians are needed to clarify their morphology as well as their reproductive cycles.

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