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Postnatal development and histofunctional differentiation of the oviduct in the broad-snouted caiman (*Caiman latirostris*)

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ABSTRACT

Caiman latirostris is a South American crocodilian species characterized as a sentinel of the presence of endocrine-disrupting compounds (EDCs). Evaluating developmental events in hormone-dependent organs, such as the oviduct, is crucial to understand physiological postnatal development, to identify putative periods of exposure sensitive to EDCs, and/or to identify biomarkers useful to evaluate the effects of EDC exposure. In this study, we describe the histomorphological features of *C. latirostris* oviducts by establishing the ontogeny of changes at cellular, tissue and molecular levels from the neonatal to the pre-pubertal juvenile stages. Since the histological diagnosis of the adenogenic oviduct lies on a group of features, here we defined a histofunctional score system and a cut-off value to distinguish between preadenogenic and adenogenic oviducts. Our results showed that the maturation of the *C. latirostris* oviduct is completed postnatally and characterized by changes that mimic the pattern of histological modifications described for the mammalian uterus. Ontogenic changes in the oviductal epithelium parallel changes at subepithelial level, and include collagen remodeling and characteristic spatial-temporal patterns of α-actin and desmin. The expression pattern of estrogen receptor alpha and progesterone receptor evidenced that, even at early postnatal developmental stages, the oviduct of *C. latirostris* is a target organ of endogenous and environmental hormones. Besides, oviductal adenogenesis seems to be an estrogen-dependent process. Results presented here provide not only insights into the histophysiological aspect of caiman female reproductive ducts but also new tools to better characterize caimans as sentinels of endocrine disruption.

1. Introduction

The reptilian female reproductive system consists of a pair of ovaries and oviducts, where the term 'oviducts' designates the structures derived from the Müllerian ducts in the embryonic period (Fox, 1977; Wake, 1985). It is well established that reptilian oviductal functions are influenced by ovarian sex steroids (Reviewed by Gist, 2011). In oviparous species, such as *Caiman latirostris*, a South American crocodilian species characterized as a sentinel of endocrine disruption (Stoker et al., 2003, 2008, 2011; Rey et al., 2006, 2009; Poletta et al., 2009; Hayes et al., 2011; Durando et al., 2013, 2016), a prominent function of the oviducts is to provide the egg white proteins and eggshell to ovulated eggs. Recently, we have assessed the relationship between the burden of organochlorine compounds, which behave as endocrine-disrupting compounds (EDCs), in *C. latirostris* eggs and eggshell features. Our results suggested a direct effect of exposure to organochlorine compounds on mother oviductal functions evidenced by decreased eggshell porosity (Stoker et al., 2013). Evaluating oviductal developmental events is crucial to understand normal postnatal development and to identify periods that are sensitive to exposure to environmental EDCs.

Early studies in *Alligator mississippiensis* indicate that, as that of other vertebrates (Arango and Donahoe, 2010), the crocodilian female reproductive tract completes its development after birth

Abbreviations: ANOVA, analysis of variance; BM, basement membrane; *C. latirostris*, *Caiman latirostris*; DAB, diaminobenzidine; EDCs, endocrine-disrupting compounds; ER, estrogen receptor; ERα, estrogen receptor alpha; FRT, female reproductive tract; GAM, gonadal-adrenal-mesonephros; IOD, integrated optical density; ip, intraperitoneal; OCCs, organochlorine compounds; PAS, periodic acid-Schiff; PCNA, proliferating cell nuclear antigen; PR, progesterone receptor; Pre-A, preadenogenic; WNT, wingless-type MMTV integration site family; α-SMA, smooth muscle alpha actin.

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(Forbes, 1940). Besides, the oviduct of *A. mississippiensis* adults has seasonal variation (Palmer and Guillette, 1992; Bagwill et al., 2009); however, little is known about the early postnatal development of the oviduct and adenogenesis in crocodiles and reptiles in general.

In mammals, the process of postnatal differentiation of the uterus is divided into two periods: organogenetic and functional (Kurita et al., 2001). Organogenetic differentiation is the process by which the identity of the Müllerian epithelium is determined, and appears to be irreversible once adulthood is reached. The morphogenetic events that occur during this postnatal stage of development are considered common for mammals and are carried out independently of estrogens (Bartol et al., 1993; Couse and Korach, 1999; Gray et al., 2001). However, the presence of steroid hormones or substances with hormonal activity at these critical stages can affect the temporal pattern of changes and/or normal function of the uterus (Varayoud et al., 2008; Bosquiazzo et al., 2010, 2013; Vigezzi et al., 2015). In mice, functional differentiation is a process that occurs after puberty and is regulated by estrogen and progesterone (Kurita et al., 2001). Gray et al. (2001) grouped the changes that occur during the organogenetic differentiation of the mammalian uterus into three main events: organization and stratification of the endometrial stroma, differentiation and growth of the myometrium, and coordinated development of endometrial glands. The period during which the morphogenetic event of adenogenesis happens is highly variable among different species of vertebrates (Gray et al., 2001).

The aim of the present study is to describe the histomorphological features and identify biomarkers of histofunctional differentiation of the oviduct of *C. latirostris*, by establishing the ontogeny of changes at cellular, tissue, and molecular levels from the neonatal to the pre-pubertal juvenile stage.

2. Material and methods

2.1. Animals

All laboratory and field work was conducted according to the published guidelines for the use of live amphibians and reptiles in field and laboratory research (American Society of Ichthyologists and Herpetologists, 2004) and in full compliance with the Institutional Committee of Bioethics in Animal Care and Use of the Universidad Nacional del Litoral, Santa Fe, Argentina.

C. latirostris eggs were collected shortly after oviposition from nests randomly selected from the Chaco Province in Argentina. The collection sites were characterized by low anthropogenic intervention, and were located upstream of urbanized, industrial and farming areas, thus minimizing exposure to sewage or agriculture and/or feedlot run-off putative sources of EDCs. Eggs were transported to the laboratory and incubated at 30 °C (female-producing temperature), as previously described (Stoker et al., 2003, 2008). Upon hatching, neonates were individually identified, weighed, measured and housed in controlled conditions. Housing facilities have been previously described in detail (Zayas et al., 2011; Durando et al., 2016).

Four developmental stages were chosen for the study: neonatal (10 days), early postnatal (40 days), late postnatal (90 days) and pre-pubertal juvenile (12–31 months) (Edwards et al., 2004). Biometric characteristics of each stage are summarized in Table 1. Caimans were euthanized with sodium pentobarbital (ip). At the neonatal and early postnatal stages, the oviduct is a thin structure that runs attached to the Gonadal–Adrenal–Mesonephros (GAM) complexes; thus, the GAM complexes and oviducts were dissected and immediately fixed for histological studies. In advanced developmental stages (late postnatal and juvenile), the oviduct was dissected from the GAM complexes, sectioned into three segments (caudal, middle and rostral) and processed separately. The oviduct regions (infundibulum, uterine tube, isthmus, uterus and vagina) were identified following the criteria established for *A. mississippiensis* (Girling, 2002). The uterine tube was the region of choice for this study. The uterine tube is a structurally and functionally relevant region of the reptilian oviduct (Gist, 2011).

Table 1
Female biometric characteristics.

	Neonatal (n = 10)	Early postnatal (n = 15)	Late postnatal (n = 14)	Pre-pubertal juvenile (n = 7)
Age	10 days	40 days	90 days	12–31 months
Body Mass (g)	45.6 ± 4.2	57.3 ± 1.8	162.8 ± 14.9	1623.6 ± 518.9
Snout-Vent Length (cm)	10.5 ± 0.7	12.5 ± 0.2	15.8 ± 4.2	37.2 ± 2.9
Total Length (cm)	21.9 ± 2.0	26.0 ± 0.4	30.3 ± 5.1	79.6 ± 7.9

Results are expressed as (X ± SD).

2.2. Sample processing

After dissection, GAM complexes or oviducts were fixed by immersion in 4% phosphate-buffered formalin (pH 7.4) for 6 h at room temperature. Fixed tissues were dehydrated, cleared in xylene (Biopack, Buenos Aires, Argentina), and embedded in paraffin (Biopack). Serial transverse sections (5 μm) were stained as described below. All the evaluations were performed in at least three sections separated 150 μm from each other.

2.3. Oviduct histoarchitecture and morphometric analysis

For regular histological examination and morphometric evaluation, samples were stained with trichromic Picrosirius solution and counterstained with Harris hematoxylin (Biopur, Rosario, Argentina). To visualize secretion products rich in glycoproteins, samples were stained with periodic acid-Schiff (PAS) (Biopur) (Junqueira and Junqueira, 1983) and the PAS-positive spatial distribution pattern was established. To analyze levels of organization of collagen fibers in the subepithelial compartment, the technique of choice was trichromic Picrosirius solution counterstained with Harris hematoxylin for polarized light microscopy (Montes and Junqueira, 1991; Rodríguez et al., 2003).

2.3.1. Epithelial height

The epithelial height of the oviduct, which was used as a biomarker of estrogen action, was evaluated using an image analysis system. Images were recorded by a SPOT color video camera (Diagnostic Instruments Inc., USA) attached to an Olympus BH2 microscope (Olympus Optical, Tokyo, Japan). Images were analyzed using the Image Pro-Plus 4.1.0.1 system (Media Cybernetics, Silver Spring, MD, USA). The basal and apical edges of the epithelium were manually delimited and the mean epithelial height was calculated. Calibration with reference rulers was performed at the beginning of each measurement.

2.3.2. Scoring system to distinguish between preadenogenic and adenogenic oviducts

After identifying a core group of histomorphological features and changes in the spatial staining pattern that precedes the presence of glands, we proposed a system for quantitative evaluation, based on the concept that the histological diagnosis of the adenogenic oviduct lies on a constellation of features rather than on any individual feature. Each stained oviduct section was evaluated to identify particular features and the number of times that each of these features was observed in each oviduct sample was recorded. The overall mean