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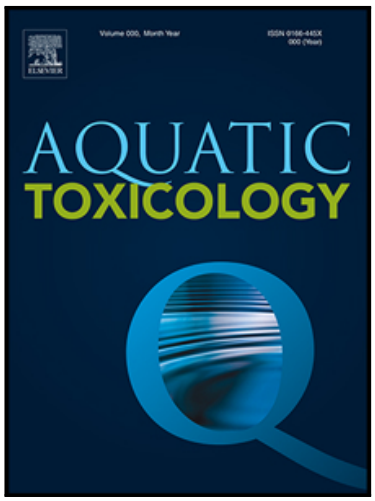

Exposure to Xenoestrogens Alters the Expression of Key Morphoregulatory Proteins of Oviduct Adenogenesis in the Broad-Snouted Caiman (Caiman latirostris)

Y.E. Tavalieri , R. Alarcón , M.V. Tschopp , G. Canesini , E.H. Luque , M. Muñoz-de-Toro , G.H. Galoppo

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Exposure to Xenoestrogens Alters the Expression of Key Morphoregulatory Proteins of Oviduct Adenogenesis in the Broad-Snouted Caiman (*Caiman latirostris*)

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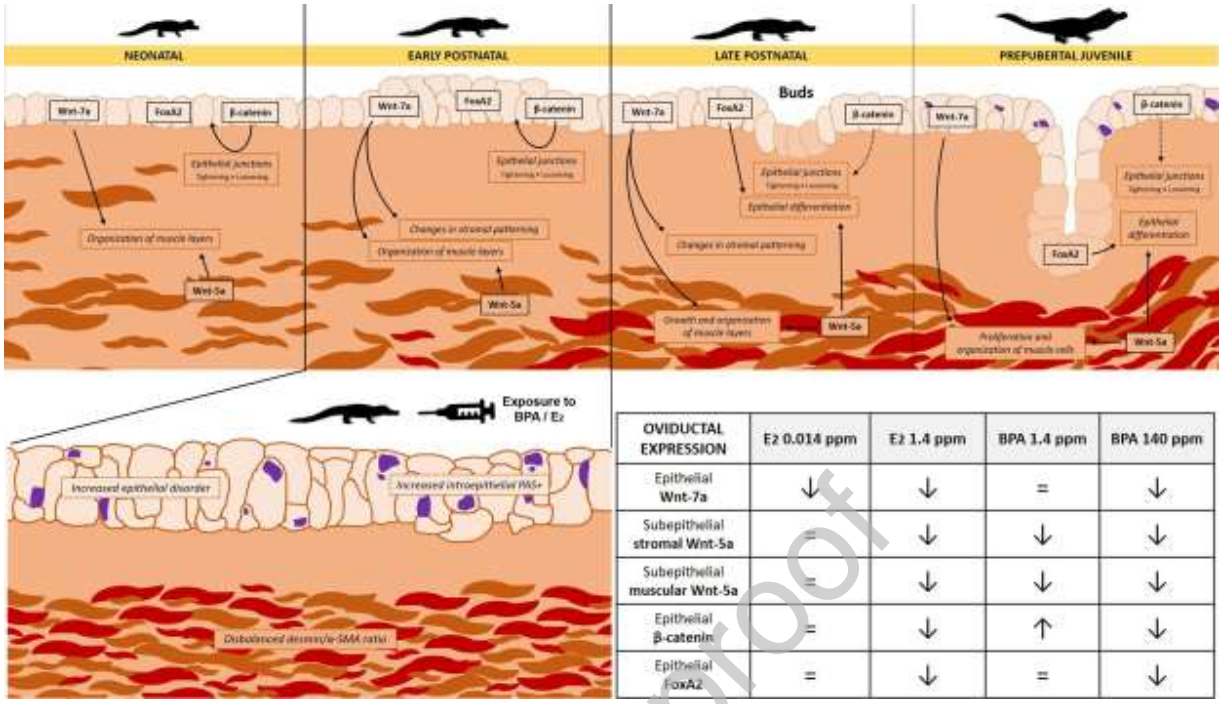
Highlights

- Wnt proteins regulate the postnatal differentiation of *Caiman latirostris* oviduct.
- FoxA2 and β-catenin play a role in the oviduct morphogenesis of *C. latirostris*.
- E_2 and BPA alter the expression of regulatory proteins of oviduct differentiation.
- E_2 and BPA modify patterns of oviduct muscle organization and gland morphogenesis.
- Effects of BPA exposure alert about plastic contamination in aquatic environments.

Graphical Abstract



OVIDUCTAL EXPRESSION	E2 0.014 ppm	E2 1.4 ppm	BPA 1.4 ppm	BPA 140 ppm
Epithelial Wnt-7a	↓	↓	=	↓
Subepithelial stromal Wnt-5a	=	↓	↓	↓
Subepithelial muscular Wnt-5a	=	↓	↓	↓
Epithelial β-catenin	=	↓	↑	↓
Epithelial FoxA2	=	↓	=	↓

Abstract

Endocrine disrupting compounds (EDCs) are contaminants ubiquitously found in the environment, which pose a potential threat to aquatic and wetland ecosystems. *Caiman latirostris*, a crocodilian species that inhabits South American wetlands, is highly sensitive to EDC exposure. Previously, we reported that early postnatal exposure to EDCs such as Bisphenol A (BPA) and 17β-Estradiol (E_2) alters *C. latirostris* oviduct differentiation. The aim of this work was to elucidate the molecular mechanisms behind this alteration. To accomplish this, we established the ontogenic changes in histological features and the expression of Wnt-7a, Wnt-5a, β-catenin, FoxA2, desmin, and alpha smooth muscle actin (α-SMA) in the oviduct of *C. latirostris*. Then, we evaluated the effects of BPA and E_2 exposure on these histological features and protein expressions. Our results showed that during the postnatal differentiation of the oviduct the presence of histological features related to adenogenesis is associated with the levels of expression of FoxA2, β-catenin, Wnt-5a and Wnt-7a. Early postnatal exposure to BPA and E_2

decreased the presence of histological features related to adenogenesis and altered the levels of expression of FoxA2, β-catenin, Wnt-5a and Wnt-7a, as well as the desmin/α-SMA ratio. These findings suggest that altered levels of Wnt-7a, Wnt-5a, β-catenin and FoxA2 could play a role in the BPA and E_2–induced alteration in oviduct differentiation in *C. latirostris*. Thus, impaired adenogenesis and, probably, impaired reproduction in wildlife naturally exposed to BPA and other estrogenic agonists cannot be completely ruled out.

Keywords

Crocodilian; Plastic pollution; Endocrine disruptor; Bisphenol A; Estrogenic agonists; Wnt signaling pathway.

Abbreviations

ANOVA, analysis of variance; BPA, Bisphenol A; DAB, 3,3´diaminobenzidine tetrahydrochloride hydrate; EDCs, endocrine-disrupting compounds; E_2, 17β-Estradiol; FoxA2, Forkhead box protein A2; FRT, female reproductive tract; GAM, gonad-adrenal-mesonephros; IHC, immunohistochemistry; PAS, periodic acid Schiff; PBS, phosphate buffered saline; α-SMA, smooth muscle alpha actin; Wnt, Wingless-related integration site; Wnt-7a, Wingless related integration site family member 7a; Wnt-5a, Wingless related integration site family member 5a.

1. Introduction

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Humans and wildlife are daily exposed to manmade contaminants classified as endocrine-disrupting compounds (EDCs) (Luque et al., 2018). Since aquatic environments are among their main recipients, aquatic and semi-aquatic organisms could be exposed to EDCs throughout their lives. Exposure to EDCs during early life stages raises particular concern since organogenesis and tissue maturation processes are active and can be easily affected by small changes in the levels of key regulatory molecules (Bergman, 2012; Durando et al., 2016).

The female reproductive tract (FRT), named oviduct in oviparous species, is a sensitive organ to exposure with EDCs. Environmentally occurring EDCs have been shown to cause detrimental effects on the oviduct of different aquatic organisms such as *Xenopus tropicalis* (Gyllenhammar et al., 2009; Kvarnryd et al., 2011; Pettersson et al., 2006; Porter et al., 2011), *Marisa cornuarietis* (Oehlmann et al., 2006; Oehlmann et al., 2000), and *Pomacea canaliculata* (Giraud-Billoud et al., 2013). In *Alligator mississippiensis*, natural exposure of eggs to EDC-polluted water alters the ovarian-oviductal axis response to follicle stimulating hormone in prepubertal alligators (Moore et al., 2012). Interestingly, experimental *in ovo* exposure to the estrogen receptor selective agonist 4,4´,4´´-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol, in embryos of *Alligator mississippiensis*, has been found to induce enlargement of the Müllerian Duct with precocious development of glands and connective tissue differentiation (Doheny et al., 2016).

Plasticizers, pharmaceutical products and natural hormones classified as EDCs have been found present in aquatic environments. The pseudo-persistent plasticizer Bisphenol A (BPA) (Bergman et al., 2012) has been recognized as an EDC with estrogenic and antiandrogenic activity (Shelby, 2005). Microplastics have been shown to be both sources and vectors for the exposure of aquatic organisms to BPA (Chen et al., 2019; Liu et al., 2019; Wu et al., 2020). BPA has been found in

surface waters at levels up to 56 µg/L and in effluents from wastewater treatment plants at levels up to 370 µg/L (Corrales et al., 2015).

Regarding natural hormones, the endogenous sex steroid 17β-estradiol (E_2) is among the environmentally occurring estrogens daily released to the environment through effluents from the cattle industry and municipal sewage treatment plants (Gonzalez et al., 2020; Kolpin et al., 2002; Valdes et al., 2015). The environmental half-life of E_2 depends on the presence of metabolically active microorganisms (Xuan et al., 2008) and the presence of oxygen. Its levels in surface waters range from non-detectable up to 23.9 ng/L (Gorga et al., 2015; Ma et al., 2016; Valdes et al., 2015; Wang et al., 2018).

The broad-snouted caiman (*Caiman latirostris*) is a semi-aquatic oviparous archosaur species widely distributed in wetlands and rivers of South America. Since this species spends most of its life in the aquatic environment, it can be exposed to waterborne pollutants throughout its lifetime. We have previously demonstrated that *C. latirostris* is highly sensitive to the effects of estrogenic and anti-androgenic EDCs (Durando et al., 2016; Stoker et al., 2008; Tavalieri et al., 2020). We have also shown that early postnatal exposure to the EDCs E_2 and BPA alters the temporal pattern of oviduct differentiation (Galoppo et al., 2017).

There is evidence that the development of the oviduct is regulated, among other factors, by proteins of the Wnt signaling pathway in mice (Cooke et al., 2013; Li and Winuthayanon, 2017), sheep (Alarcón et al., 2020; Hayashi and Spencer 2006) and chicken (Lim et al., 2013) and by the Forkhead box family in humans (Kelleher et al., 2019), mice (Jeong et al., 2010; Kelleher et al., 2017) and sheep (Alarcón et al., 2020). Wnt proteins participate in oviductal adenogenesis in mice and sheep (Alarcón et al., 2020; Mericskay et al., 2004; Miller et al., 1998), in the

organization and proliferation of oviductal muscle fibers in mice and rats (Cooke et al., 2013; DiRenzo et al., 2016; Mericskay et al., 2004), and in the growth and differentiation of the myometrium in mice (Ma and Sassoon, 2006). Among the different members of the Wnt signaling pathway, Wnt-7a and Wnt-5a have been shown to play an important role in postnatal uterine morphogenesis in mice and sheep (Cooke et al., 2013; Hayashi and Spencer, 2006; Jeong et al., 2009; Mericskay et al., 2004; Miller et al., 1998). Indeed, Wnt-5a could be related to gland development and growth of the muscle layer. The Wnt signaling pathway can be regulated by endogenous estrogens in mice (Miller et al., 1998; Tepekoy et al., 2015), and exposure to xenoestrogens can alter the expression of Wnt proteins in mice and sheep (Alarcon et al., 2020; Ingaramo et al., 2016; Miyagawa et al., 2011; Sassoon, 1999). Another molecule implied in FRT development is β-catenin, which can exert its effect by functioning as a transcriptional co-activator of target genes in humans and mice (Komiya and Habas, 2008; Miller and McCrea, 2010). Alternatively, β-catenin can bind to E-cadherin to form the cadherin complex, a regulator of cell-cell interactions that plays an important role in maintaining the integrity of the epithelium in human and mice (Nelson and Nusse, 2004; Tian et al., 2011).

In the neonatal mouse uterus, the differentiation and development of glands are regulated by Forkhead box protein A2 (FoxA2), a member of the forkhead transcriptional factor family (Cooke et al., 2013; Jeong et al., 2010; Kelleher et al., 2019; Kelleher et al., 2017). Studies in transgenic mice and human atypical endometrial hyperplasia have shown that FoxA2 expression is regulated by Wnt signaling pathway proteins and *vice versa* (Connelly et al., 2018; Kimura-Yoshida et al., 2007; Villacorte et al., 2013; Yu et al., 2009).

The aim of this study was to determine the molecular mechanism behind the altered differentiation of the oviduct in caimans exposed to BPA and E_2. To accomplish this purpose, we

analyzed the ontogenic changes in Wnt-7a, Wnt-5a, β-catenin and FoxA2 from the neonatal to the prepubertal juvenile stage in non-exposed caimans, and assessed the effects of early postnatal exposure to BPA and E_2.

2. Material and Methods

2.1. Animals and treatments

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 (ASIH, 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 (Approved Protocol Certificate 03/17).

Caiman latirostris eggs were collected shortly after oviposition from six nests randomly selected in a protected natural area (Natural Reserve "*El Cachapé*") in Chaco province, Argentina. To establish the developmental stage of the embryos, one egg from each clutch was opened in the field. To warrant recent oviposition, only the clutches with embryos at stages earlier than 15 were transported to the laboratory (Canesini et al., 2018). Prior to removal from the nest, the upper surfaces of the eggs were marked with a graphite pencil to keep the original orientation during both moving and incubation. Eggs were transported to the laboratory and incubated at 30 °C (female-producing temperature), as previously described (Beldomenico et al., 2007; Stoker et al., 2003). Embryo viability was confirmed based on opaque eggshell banding development (Ferguson, 1985). Temperature was monitored by HOBO temperature loggers (Onset Computer, Pocasset, MA, USA) and by daily recording the readings of the electronic thermometer of the incubator. Eggs were maintained at approximately 90% humidity. Upon hatching, neonates were individually identified, weighed, measured and housed in controlled conditions. The housing

facilities have been previously described in detail (Durando et al., 2016; Tavalieri et al., 2019; Zayas et al., 2011). To minimize clutch effect, eggs from each clutch were distributed across different experimental groups according to the experiment they were assigned to. Each group had a maximum of two siblings.

2.1.1. Experiment I: Ontogenic changes in molecules involved in vertebrate oviduct differentiation

To analyze the ontogeny of changes of Wnt-5a, Wnt-7a, β-catenin, FoxA2, smooth muscle alpha actin (α-SMA) and desmin, the oviducts from 28 caimans were studied at four developmental stages: neonatal, early postnatal, late postnatal and prepubertal juvenile (Galoppo et al., 2016) (Table 1). Caimans were euthanized with sodium pentobarbital. 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 oviduct-GAM complexes were dissected and immediately fixed for histological studies. At the late postnatal and juvenile stages, 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 *Alligator mississippiensis* (Girling, 2002) and *C. latirostris* (Galoppo et al., 2017; 2016). The region of choice for this study was the uterine tube, the oviductal region where the albumen is secreted. Since the albumen contains growth factors, antimicrobial peptides, nutrients and other substances essential for embryo development (Palmer and Guillette, 1991; Cox and Guillette, 1993), we considered it a structurally and functionally relevant region of the archosaurian oviduct.

Table 1. Biometric characteristics and number of animals used in Experiment I.

Developmental stage	n	Age	BM	SVL
Neonatal	7	10 days post-hatch.	45.71 ± 4.31 g	9.29 ± 1.98 cm
Early postnatal	7	40 days post-hatch.	61.43 ± 5.86 g	12.29 ± 2.98 cm
Late postnatal	7	90 days post-hatch.	165.57 ± 11.19 g	18.50 ± 0.41 cm
Prepubertal juvenile	7	12–31 months post-hatch.	1960.43 ± 567.07 g	37.93 ± 3.00 cm

n describes the number of individuals used in this analysis (biological replicates). BM: Body mass; SVL: Snout-to-vent length. The results are expressed as the mean value ± standard deviation (SD).

2.1.2. Experiment II: Effects of postnatal exposure to xenoestrogens on the expression of molecules involved in the development of the caiman oviduct

Fifty-one caimans were injected with BPA (Aldrich, Milwaukee, WI, USA) 1.4 ppm (n=11) or 140 ppm (n=11), 17β-estradiol (E_2) (Sigma-Aldrich, St Louis, MO, USA) 0.014 ppm (n=11) or 1.4 ppm (n=11), or vehicle (VEH) (corn oil) (n=7) on postnatal days 26 and 33. Since doses were calculated based on body weight, animals were weighed 24 h before every injection. Injections were administered subcutaneously in the dorsal side of the right hind leg. The experimental groups were the control (injected with VEH), E_2 0.014, E_2 1.4, BPA 1.4 and BPA 140. The

higher doses of both E_2 (1.4 ppm) and BPA (140 ppm) were applied as reference doses known to cause effects when administered *in ovo*, since both override the temperature effect on *C. latirostris* sex determination (Stoker et al., 2008; Stoker et al., 2003). The lower doses of E_2 (0.014 ppm) and BPA (1.4 ppm) were 100 times lower than the reference doses. Indeed, the BPA dose of 1.4 ppm is lower than the no-observable-adverse-effect level established by the regulatory agency of the USA (FDA, 2014) and previously considered an environmentally relevant dose (Stoker et al., 2003). The treatments were applied at the early postnatal stage. Previous results suggest that at this stage, the developing oviduct could respond to xenoestrogens (Galoppo et al., 2016). *C. latirostris* hatchlings spend most of the time in an aquatic environment and, during their first month of life, energy intake is mainly through the remaining yolk sac. In this context, and to ensure the effective dose, we chose the subcutaneous route of administration. The caimans were euthanized on postnatal day 40 (7 days after the second subcutaneous dose).

2.2. Sample processing

At necropsy, GAM complexes and oviducts were manually extruded, dissected and immediately fixed in 10% 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, Buenos Aires, Argentina). Serial transverse sections were obtained as described below. When used in immunostaining techniques, tissue sections were stored for a maximum of 30 days in a desiccator at 4 °C to avoid protein deterioration. The oviduct and liver from a prepubertal juvenile caiman were extracted and maintained at -80 °C for Western Blot analysis (supplementary material).

2.3. Microtomy

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Each paraffin-embedded uterine tube region was sliced into 5µm-thick sections. For each endpoint to be studied, a series of three tissue sections separated 150 µm from each other was obtained for each individual animal.

2.4. Analysis of histofunctional score

Adenogenesis is a critical process that characterizes the postnatal differentiation of the oviduct and relies on a plethora of histological changes used to define a histofunctional score. Briefly, a series of histomorphological features considered signs of adenogenesis, previously defined in Galoppo et al., (2016) (Figure 4), were identified and counted in each stained oviduct section from control caimans at different developmental stages. The number of times that each of these features was observed (n) in each oviduct sample was recorded, the overall mean value (x) and standard deviation (SD) were calculated for each feature and four different ranks were defined: $n=0$; $0 < n \leq SD/2$; $SD/2 < n \leq x$; and ~~($n > x$)~~. The partial scores (i.e. the scores obtained for each feature) were stated as: 0 when the feature was absent ($n=0$), 1 when the number of observations was $0 < n \leq SD/2$, 2 when the number of observations was $SD/2 < n \leq x$, and 3 when the number of observations was higher than the mean value ($n > x$). The final score for each oviduct sample was determined by the sum of the partial scores. Here, we analyzed each histomorphological feature individually to find which ones characterize each developmental stage and which ones, if any, are specifically impacted by BPA and E_2 exposure. For this purpose, the oviduct sections were stained with Periodic Acid Schiff (PAS) (Biopur, Rosario, Argentina) and counterstained with Mayer's hematoxylin. Picrosirius staining with Harris hematoxylin was also used (Biopur, Rosario, Argentina). The stained sections were observed along the whole epithelial extension using a light microscope. The different histological features used to establish the histofunctional score were identified, the number of times that each of these features was observed was recorded

and a partial score was assigned for each feature in each oviductal section. The partial score for each animal was calculated as the average score between the three consecutive sections analyzed.

2.5. Immunohistochemistry

To characterize the myometrium and better establish the distribution and organization of muscle fibers, the expression of desmin and α-SMA was revealed by immunohistochemistry (IHC). IHC was also performed to evaluate the localization of β-catenin, Wnt-7a, Wnt-5a and FoxA2. Briefly, sections were hydrated through a series of alcohols and microwaved in 10 mM citrate buffer (pH 6) for antigen retrieval. To prevent endogenous peroxidase activity and nonspecific binding, the sections were treated with methanol/H_2O_2 and 2% (v/v) normal horse serum in 0.01 M phosphate-buffered saline (PBS), respectively. Primary antibodies were incubated overnight in a humid chamber at 4 °C. The antibody characteristics are summarized in Table 2. On the second day, after incubation with biotin-conjugated secondary antibodies, reactions were developed using a streptavidin-biotin peroxidase method and 3,3´diaminobenzidine tetrahydrochloride hydrate (DAB) (Sigma–Aldrich, Buenos Aires, Argentina) as a chromogen substrate. Sections were lightly counterstained with Mayer's hematoxylin and mounted with a glass coverslip for light microscopy. All the IHC assays included samples from different experimental groups, an inter-assay control, and a negative control. Negative controls were performed by replacing the primary antibody with non-immune serum (Sigma-Aldrich) or with the antibody-antigen complex (pre-adsorbed antibody).

The specificity of newly used antibodies was tested by Western Blot analysis (Supplementary Data).

Table 2. Primary antibodies used for IHC.

Antibody	Animal source	Dilutions used	Supplier	Specificity
Anti- α-SMA (clone 1)	Monoclonal Mouse	1:50	Novocastra (Newcastle upon Tyne, UK)	Rey et al., 2009 Galoppo et al., 2016
Anti-Desmin (Clone DE-R-11)	Monoclonal Mouse	1:80	Novocastra (Newcastle upon Tyne, UK)	Rey et al., 2009 Durando et al., 2016
Anti-β-catenin (β-catenin E5 SC 7963)	Monoclonal Mouse	1:1600	Santa Cruz Biotechnology	This manuscript
Anti-Wnt-7a	Polyclonal Rabbit	1:400	ISAL, Santa Fe, Argentina (Vigezzi et al., 2016)	This manuscript
Anti-Wnt-5a	Polyclonal Rabbit	1:400	ISAL, Santa Fe, Argentina (Vigezzi et al.,	This manuscript

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			2016)	
Anti-FoxA2	Polyclonal Rabbit	1:2000	ISAL, Santa Fe, Argentina (Alarcon et al., 2020)	This manuscript

α-SMA: Smooth muscle α actin; ISAL: Instituto de Salud y Ambiente del Litoral; Wnt-7a: Wingless-related integration site family member 7A; Wnt-5a: Wingless-related integration site family member 5a; FoxA2: Forkhead box protein A2.

2.6. Image analysis

All the evaluations were performed in three sections separated 150 µm from each other. Images of immunostained GAM complexes and oviducts were recorded using a SPOT color video camera (Diagnostic Instruments Inc., USA) attached to an Olympus BH2 microscope (Olympus Optical, Tokyo, Japan). Images covering the whole subepithelial area (desmin, α-SMA and Wnt-5a assessment) and the whole epithelial compartment (β-catenin and FoxA2 assessment) were captured and analyzed. Images were analyzed using different tools (spatial and light intensity calibration, area measurement tools, thresholding, image conversion, among others) provided by the Image J software (NIH, USA; https://imagej.nih.gov/ij). The results are reported as the average effects of the three histological sections analyzed for each animal.

2.6.1 Assessment of oviductal desmin and α-SMA localization

The presence of muscle cells expressing α-SMA and desmin was assessed in the subepithelial compartment (the histological region that spreads from the basal membrane of the luminal epithelium towards the serosa layer and includes the subepithelial stroma and the muscle layer). The total transversal area of the subepithelial compartments was manually delimited and the areas occupied by α-SMA and desmin were automatically calculated. Results are expressed as the ratio between the percentage of total transversal area occupied by desmin and the percentage of total transversal area occupied by α-SMA. The thicknesses of the α-SMA and desmin expression region were automatically calculated for each oviduct to be used as reference to determine subepithelial stromal and muscular areas. The maximum thickness of α-SMA or desmin expression was considered as the muscle thickness.

2.6.2 Assessment of Wnt-7a localization

Wnt-7a localization was evaluated in the whole epithelial compartment. Double-blind differential counting of immunostained and negative nuclei was performed in each of the three oviductal sections using a Dplan 100X objective. The percentage of Wnt-7a positive epithelial nuclei was calculated for each oviductal section. For statistical analyses, the average percentage of Wnt-7a positive epithelial nuclei was used.

2.6.3 Assessment of Wnt-5a localization

Wnt-5a localization was evaluated separately in two different sub-compartments of the subepithelium (subepithelial stroma and muscle layer). Given that the muscle layer is not present at the neonatal stage, only the subepithelial stromal expression of Wnt-5a was determined for this developmental stage.

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The stromal and muscle subepithelial regions were manually delimited and Wnt-5a expression was determined following the same procedure previously explained for α-SMA and desmin assessment. The limits of the different sub-compartments were set as follows: since IHC of the different molecules was performed on consecutive sections of a given sample, the muscle thickness obtained from α-SMA- and desmin-immunostained sections was used to delimit the muscle region in Wnt-5a-immunostained sections. The subepithelial stroma was delimited as the area that spreads from the basal membrane to the muscle layer. Results are expressed as the percentage of the total stromal or muscle area occupied by Wnt-5a.

2.6.4 Assessment of β-catenin localization

β-catenin expression was evaluated in the epithelial compartment by integrated optical density (IOD) as a linear combination between the average gray intensity of the gray-converted digitalized images and the relative area occupied by β-catenin. The relative area was delimited between the basal border of the epithelium and the mean epithelial height, previously described in Galoppo et al. (2016). Since the intensity of β-catenin expression is different between the luminal epithelium and the epithelium of buds (epithelial bud-like structures that characterize the beginning of gland morphogenesis), β-catenin expression was differentially assessed in the luminal epithelium, showing no evidences of gland morphogenesis, and in the epithelium of buds. In both cases, the area of reference was manually delimited between the basal border of the epithelium and the mean epithelial height. The epithelial areas occupied by β-catenin were automatically calculated. Since IOD is a dimensionless parameter, the results are expressed as arbitrary units.

2.6.5 Assessment of FoxA2 localization in the luminal and glandular epithelia

Since FoxA2 localization differs between the luminal and glandular epithelia, it was evaluated by IOD in both epithelial sub-compartments (luminal and glandular) separately using digitalized images converted to gray scale. The total epithelial area was manually delimited following the apical and basal borders. The area occupied by FoxA2 and the intensity of the immunostaining were automatically calculated. Results are expressed as arbitrary units.

2.7. Statistical analysis

The data are reported as the median ± range. When the variables exhibited normal distribution, analysis of variance (ANOVA) was performed to obtain the overall significance, followed by Tukey´s post-test. For non-normal distribution, Kruskal–Wallis analyses were performed to obtain the overall significance, followed by the Dunn's post-test to establish differences between groups. In the case of the comparison between the expression of β-catenin in the luminal epithelium and the buds, results were analyzed by the Mann-Whitney test. In all cases, $P < 0.05$ was accepted as significant.

3. Results

3.1. Experiment I

3.1.1. Ontogeny of changes in key molecules involved in the postnatal oviduct differentiation

3.1.1.1. Wnt7a spatial and temporal oviduct expression pattern

Wnt-7a exhibited both non-nuclear and nuclear localization in the oviduct luminal epithelium (Figure 1). Its levels of expression gradually increased from the earlier developmental stage

towards the more advanced stages, and differed significantly between the neonatal and prepubertal juvenile stages, being intermediate at the early and late postnatal stages (Table 3).

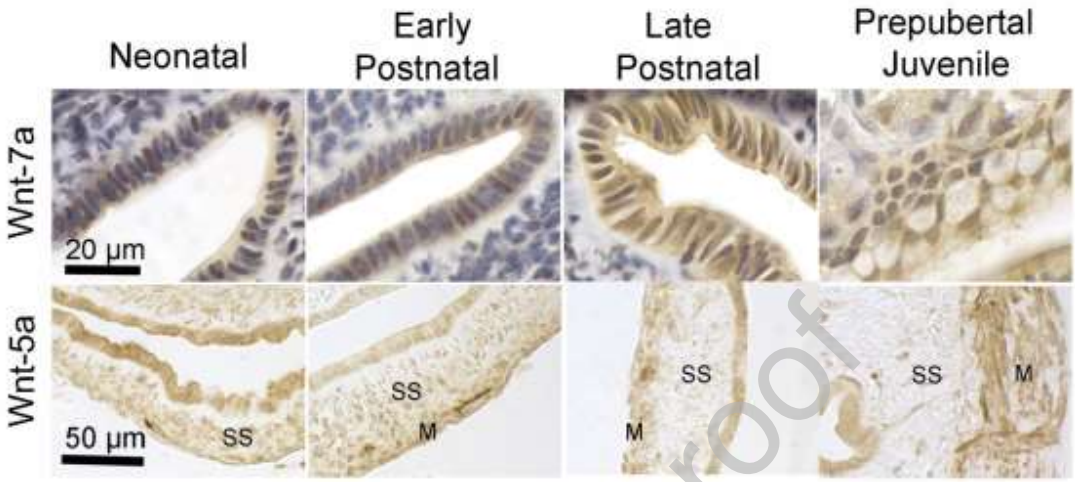

Figure 1. Ontogenic changes in the localization of Wnt-7a, and Wnt-5a proteins in the oviduct of *C. latirostris*. First row: Wnt-7a localization in the oviduct epithelium. Increasing expression can be observed from the neonatal to the prepubertal juvenile stages (left to right). Second row: Wnt-5a expression in the subepithelial stroma (SS) and muscle layer (M). IHC developed with DAB. Oviduct sections in the first and third rows were counterstained with Mayer's hematoxylin. The oviduct sections shown in the second row were not counter-stained to better visualize changes at the different developmental stages.

3.1.1.2. Wnt-5a spatial and temporal oviduct expression pattern

Wnt-5a exhibited cytoplasmic expression in the epithelial and the subepithelial compartments. At the earlier developmental stages, Wnt-5a was expressed in the subepithelial compartment without a defined pattern. As oviduct differentiation took place, the immunostaining pattern became gradually regionalized to the outer part of the subepithelium, i.e., the muscle layer (Figure 1). The percentage of muscle area occupied by Wnt-5a gradually increased from the early postnatal to the prepubertal juvenile stage (Table 3). In the subepithelial stroma, Wnt-5a expression remained without significant changes throughout the different developmental stages.

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However, the homogeneity of Wnt-5a stromal expression increased as the developmental stage advanced and a decreasing tendency was observed.

Table 3. Ontogeny of changes in molecules involved in the postnatal differentiation of the oviduct.

Developmental stage	Epithelial Wnt-7a (% of positive epithelial nuclei)	Subepithelial -Stromal Wnt-5a (% of area occupied by Wnt-5a positive cells)	Muscular Wnt-5a (% of area occupied by Wnt-5a positive cells)	Epithelial β-catenin (IOD)	Epithelial FoxA2 (IOD)
Neonatal (n=7)	42.95[a] (38.04-48.19)	7.42 (1.41-16.19)	NA	23.86[a] (10.18-28.56)	0.93[a] (0.06-1.68)
Early postnatal (n=7)	55.50[ab] (33.50-78.20)	4.07 (0.89-11.39)	19.34[a] (2.68-37.63)	12.35[b] (6.74-16.77)	2.14[ab] (0.05-5.80)
Late postnatal (n=7)	48.05[ab] (31.20-	4.38 (2.61-6.62)	26.66[ab] (16.50-	11.88[b] (4.54-17.68)	5.39[b] (1.51-7.69)

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	90.12)		39.58)		
Prepubertal Juvenile (n=7)	68.95[b] (52.70-72.80)	2.09 (1.27-4.17)	41.46[b] (19.47-45.02)	13.45[b] (5.87-17.63)	1.32[a] (0.64-3.64)

Results are expressed as the median (minimum value- maximum value). n describes the number of individuals used in this analysis (biological replicates). IOD: integrated optical density. Different superscripts indicate significant differences between developmental stages by ANOVA or Kruskal-Wallis followed by Tukey's or Dunn's post-test, respectively, at $P < 0.05$. NA: not applicable. Since the oviducts of neonatal caimans do not express desmin, the ratio could not be calculated at this stage.

3.1.1.3. β-catenin spatial and temporal oviduct expression pattern

β-catenin was expressed in the cytoplasm and the basolateral membrane of the luminal epithelial cells, being more intense in the latter (Figure 2). No nuclear expression was observed. At the neonatal stage, the intensity of the basal β-catenin immunostaining was homogeneous throughout the whole basal border, whereas at later developmental stages, short segments of the basal border with low expression of β-catenin were observed. These slightly stained segments were commonly associated with buds (Figure 2). Taking into account that the differences in β catenin expression are related to the intensity of the expression rather than to the area of β-catenin expression, β catenin expression was evaluated by IOD, as described previously, in different segments of the luminal epithelium in immune-stained sections. The expression levels of β-catenin decreased from the neonatal to the early postnatal stage. From that stage onwards, β-catenin expression remained relatively similar during the subsequent developmental stages

(Table 3). At the neonatal or early postnatal stage, buds were rarely present, whereas at the late postnatal and prepubertal juvenile stages, this feature became relevant (Figure 5). For this reason, the differential expression of β-catenin on the epithelium of buds was assessed at the late postnatal and prepubertal juvenile stages. No changes in β-catenin expression were observed in the epithelium of buds between the late postnatal and prepubertal juvenile stages. However, when comparing β-catenin expression in the different epithelial regions for the same developmental stage, we found that β-catenin expression in the epithelium of buds was significantly reduced (Figure 2).

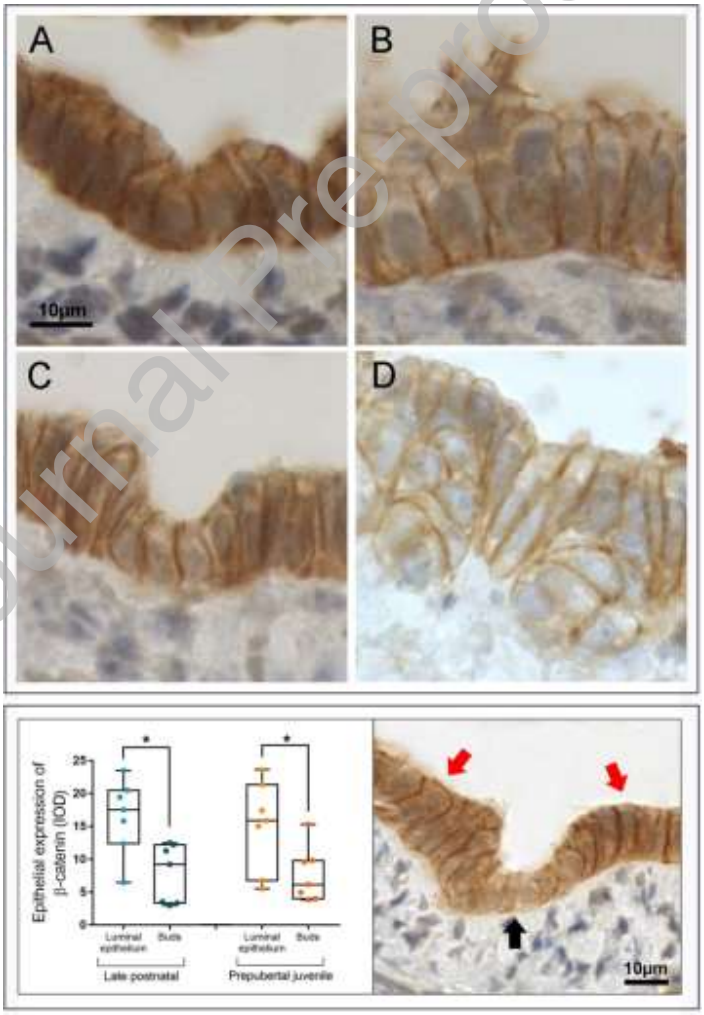

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Figure 2. Ontogenic changes in the oviductal expression pattern of β-catenin. Upper panel: Representative photomicrographs showing β-catenin epithelial expression in oviducts of neonatal (A), early postnatal (B), late postnatal (C) and prepubertal juvenile (D) *C. latirostris* caimans. Lower panel: Graph showing changes in the intensity of β-catenin expression in the oviduct epithelium. The boxes represent the expression of β-catenin measured by IOD. Results are expressed as median ± interquartile range. The asterisks show statistical differences between the groups linked by brackets by the Mann-Whitney test at $P < 0.05$. Right figure: Representative photomicrograph from the *C. latirostris* oviduct showing different intensity of β-catenin expression in non-budding luminal epithelium (red arrows) and epithelium of buds (black arrow). IHC developed by DAB and counterstained with Mayer's hematoxylin.

3.1.1.4. FoxA2 spatial and temporal oviduct expression pattern

FoxA2 oviduct expression was restricted to the epithelium and exhibited cytoplasmic expression. At the neonatal, early postnatal, and late postnatal stages, FoxA2 expression presented a granular dot-like pattern at the luminal border of the epithelium (Figure 3). At the juvenile stage, the immunostaining intensity of FoxA2 expression in the luminal epithelium decreased and a granular pattern was observed in the glands (Figure 3).

Since changes in FoxA2 expression were based on the intensity of immunostaining in the luminal epithelium, this factor was evaluated by IOD. FoxA2 expression in the luminal epithelium increased from the neonatal to the late postnatal stage. At the early postnatal stage, FoxA2 expression was intermediate, not significantly different from that at the neonatal stage or at the late postnatal stage. At the prepubertal juvenile stage, the pattern of distribution of FoxA2 expression was different between the luminal and glandular epithelium, being more intense in the latter. The luminal epithelial expression of FoxA2 at the prepubertal juvenile stage significantly decreased to values similar to those observed at the neonatal stage (Table 3).

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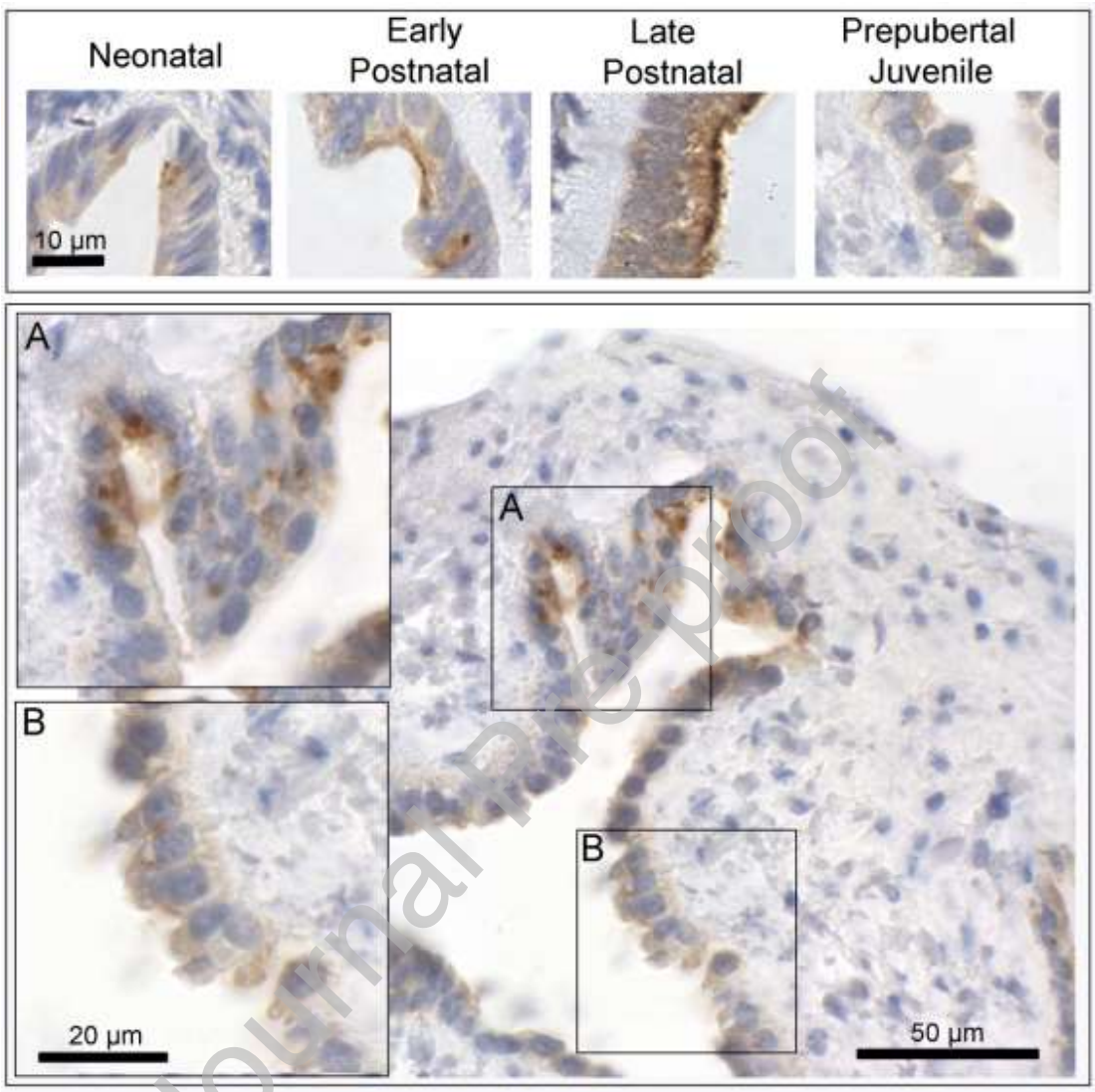

Figure 3. Distribution pattern of FoxA2 protein expression in the oviduct of *C. latirostris*. Upper panel: Representative photomicrographs showing FoxA2 expression in the oviduct epithelium of *C. latirostris* at different stages of development. FoxA2 expression in the luminal epithelium increases from the neonatal to the late postnatal stage. Lower panel: Distribution pattern of FoxA2 protein expression in the oviduct of juvenile prepubertal *C. latirostris*. At this stage, a gland-related regionalization of FoxA2 expression is observed. A: Glandular epithelial expression of FoxA2. B: Luminal epithelial expression of FoxA2. IHC developed with DAB and counterstained with Mayer's hematoxylin.

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3.1.2. Ontogeny of histofunctional features used in the scoring system

As previously shown, the differentiation of the oviduct is characterized by changes in histological features that determine a developmental stage-related increase in the histofunctional score (Galoppo et al., 2016). Among the characteristics taken into account to build the histofunctional score, some are more closely related to gland morphogenesis, while others are particularly related to luminal epithelial hyperplasia (Figure 4). When analyzing each individual feature separately, we found that the early postnatal and late postnatal stages differed not only in the number of subepithelial projections and buds but also in the partial scores due to those features. The most drastic changes, in both the total and partial scores were observed between the late postnatal and the prepubertal juvenile stages (Figure 5).

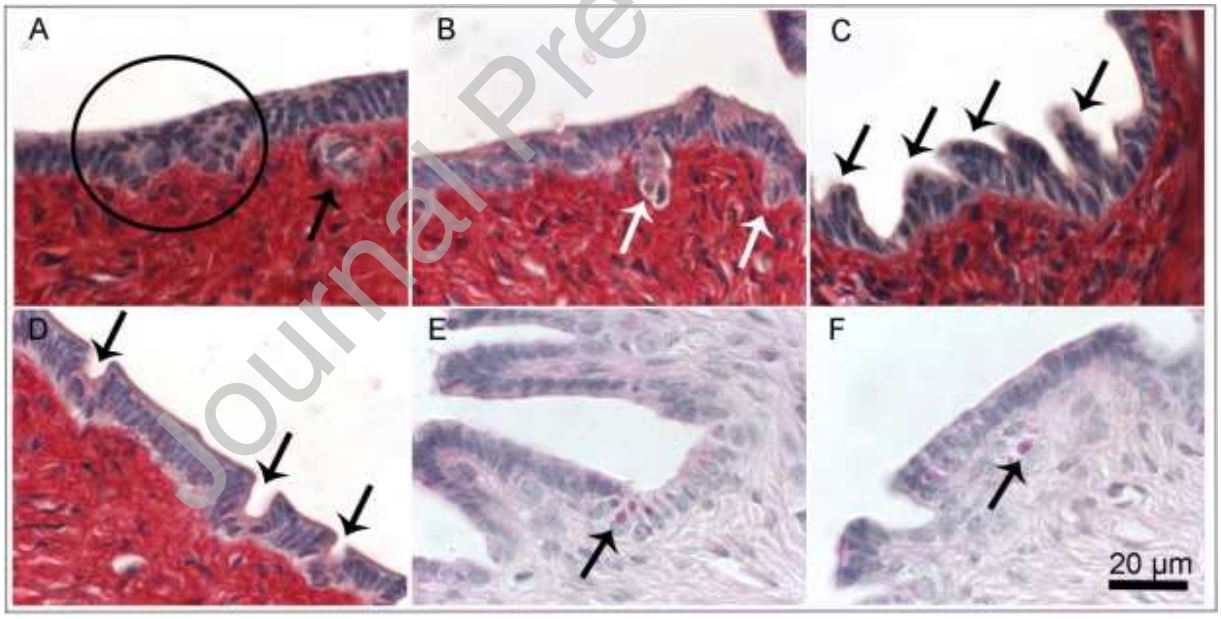

Figure 4. Histomorphological features used to define the histofunctional score for *C. latirostris* oviducts. Representative photomicrographs showing: A: epithelial disorder (encircled) and epithelial cell cluster in the subepithelium (arrow); B: subepithelial projection; C: Protrusions; D: Buds; E: Intra-epithelial periodic acid Schiff (PAS) (+) cells; F: Subepithelial PAS (+) cells. Oviductal sections used in photomicrographs A-D were stained with

Picrosirius and counter-stained with Harris hematoxylin. Samples used in photomicrographs E and F were stained with PAS and counter-stained with Mayer´s hematoxylin.

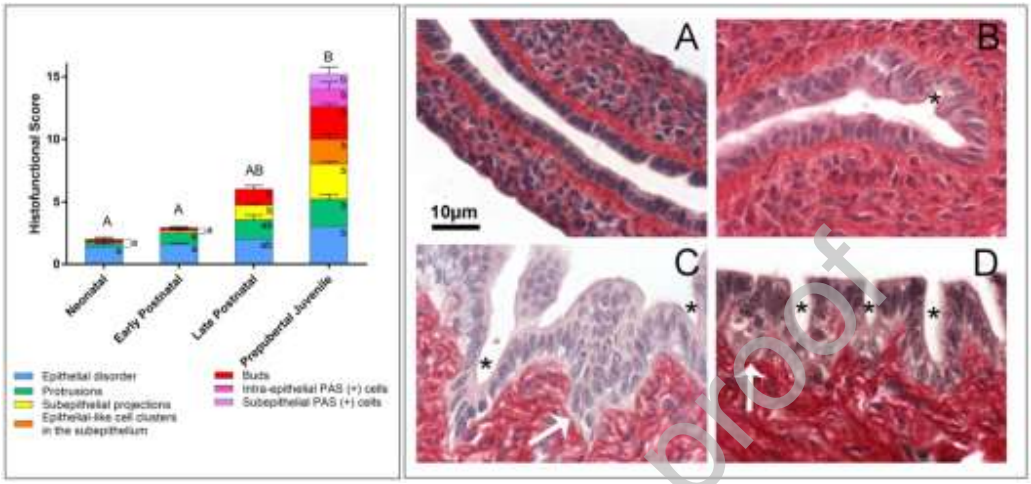

Figure 5. Histomorphological features used as markers of postnatal oviduct differentiation. Left panel: Graph showing changes in the histofunctional score as a consequence of ontogenic variations in the partial scores. The bars represent the total histofunctional score. The stacked colored bars represent the partial score assigned for each histological feature. Results are expressed as mean ± SEM. Bars with different capital superscripts denote statistical differences in the histofunctional score between the different developmental stages. Different lower-case letters in each color section in the bars denote statistical differences in the partial score between the different developmental stages. Kruskal-Wallis Test followed by Dunn's post-test at $P < 0.05$. Right panel: Representative photomicrographs showing histomorphological features that characterize the neonatal (A), early postnatal (B), late postnatal (C) and prepubertal juvenile (D) developmental stages. The asterisks show the presence of buds. The arrows show subepithelial projections. Samples were stained with picrosirius stain and counterstained with Harris hematoxylin.

3.1.3. Ontogeny of changes in the desmin/α-SMA ratio

The desmin/α-SMA ratio significantly increased from the early postnatal to the late postnatal stage, but then showed no changes from the late postnatal stage to the prepubertal juvenile stage

(Figure 6). Since the oviduct of neonatal caimans does not express desmin, the ratio could not be calculated at this stage and was thus omitted from the graph in Figure 6.

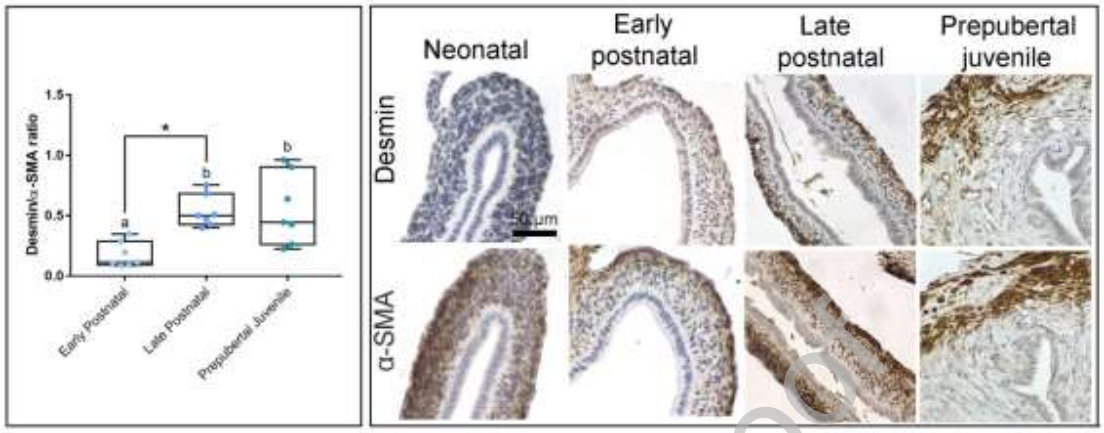

Figure 6. Changes in the desmin/α-SMA ratio in the oviduct of *C. latirostris* at the different developmental stages. Left panel: Graph showing ontogenic changes in the desmin/α-SMA ratio. The boxes represent the ratio values. Results are expressed as median ± interquartile range. The superscripts denote significant differences between the experimental groups. Results analyzed by Kruskal-Wallis followed by Dunn's post-test at $P < 0.05$. Right panel: Representative photomicrographs showing differential expression and distribution of desmin and α-SMA in the oviduct of *C. latirostris* at different developmental stages. IHC developed with DAB and counterstained with Mayer's hematoxylin.

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3.2. Experiment II

3.2.1. Effects of postnatal xenoestrogen exposure on molecules involved in oviduct differentiation

Early postnatal exposure to E_2 (both doses) as well as that to the higher dose of BPA significantly down-regulated the nuclear expression of Wnt-7a in the luminal epithelium of the oviduct (Table 4). In addition, early postnatal exposure to BPA (both doses) as well as that to the

higher dose of E_2 decreased Wnt-5a expression in both the subepithelial stroma and the muscle layer of the oviduct (Table 4).

Early postnatal exposure to the higher doses of E_2 or BPA significantly down-regulated the epithelial expression of β-catenin, while the higher dose of BPA up-regulated the epithelial expression of β-catenin in the caiman oviduct (Table 4). Since buds at this developmental stage are rarely found and, as shown later, the treatments did not significantly change the number of buds and the partial score due to this feature (Figure 8), differential β-catenin expression in the epithelium of buds was not assessed at this developmental stage.

Early postnatal exposure to the higher doses of E_2 or BPA significantly down-regulated the epithelial expression of FoxA2 in the caiman oviduct (Table 4, Figure 7).

Table 4. Effects of postnatal xenoestrogen exposure on molecules involved in oviduct differentiation.

Experimental group	Epithelial Wnt-7a (% of positive nuclei)	Subepithelial Stromal Wnt-5a (% of area occupied by Wnt-5a positive cells)	Muscular Wnt-5a (% of area occupied by Wnt-5a positive cells)	Epithelial β-catenin (IOD)	Epithelial FoxA2 (IOD)

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Control (n=7)	55.50 (33.50-78.20)	4.07 (0.88-11.39)	19.34 (2.68-37.63)	12.35 (6.74-16.77)	2.14 (0.05-5.80)
E_2 0.014 (n=11)	21.60* (9.20-44.01)	1.62 (0.00-9.29)	5.88 (2.04-16.33)	12.25 (4.05-23.78)	2.31 (0.76-4.57)
E_2 1.4 (n=11)	28.50* (8.93-42.40)	1.34* (0.41-2.04)	6.19* (1.84-11.00)	6.16* (0.88-10.89)	0.54* (0.04-1.65)
BPA 1.4 (n=11)	42.34 (24.71-66.86)	1.08* (0.34-2.34)	3.95* (0.37-7.65)	17.69* (5.18-23.69)	2.13 (0.27-4.24)
BPA 140 (n=11)	36.17* (10.31-57.23)	1.38* (0.02-4.74)	4.00* (1.82-9.02)	5.17* (2.02-9.71)	0.56* (0.01-2.52)

Results are expressed as the median (minimum value- maximum value). n describes the number of individuals used in this analysis (biological replicates). IOD: integrated optical density. Asterisks indicate significant differences between each exposure group and the control by ANOVA or Kruskal-Wallis followed by Tukey's or Dunn's post-test respectively at $P < 0.05$.

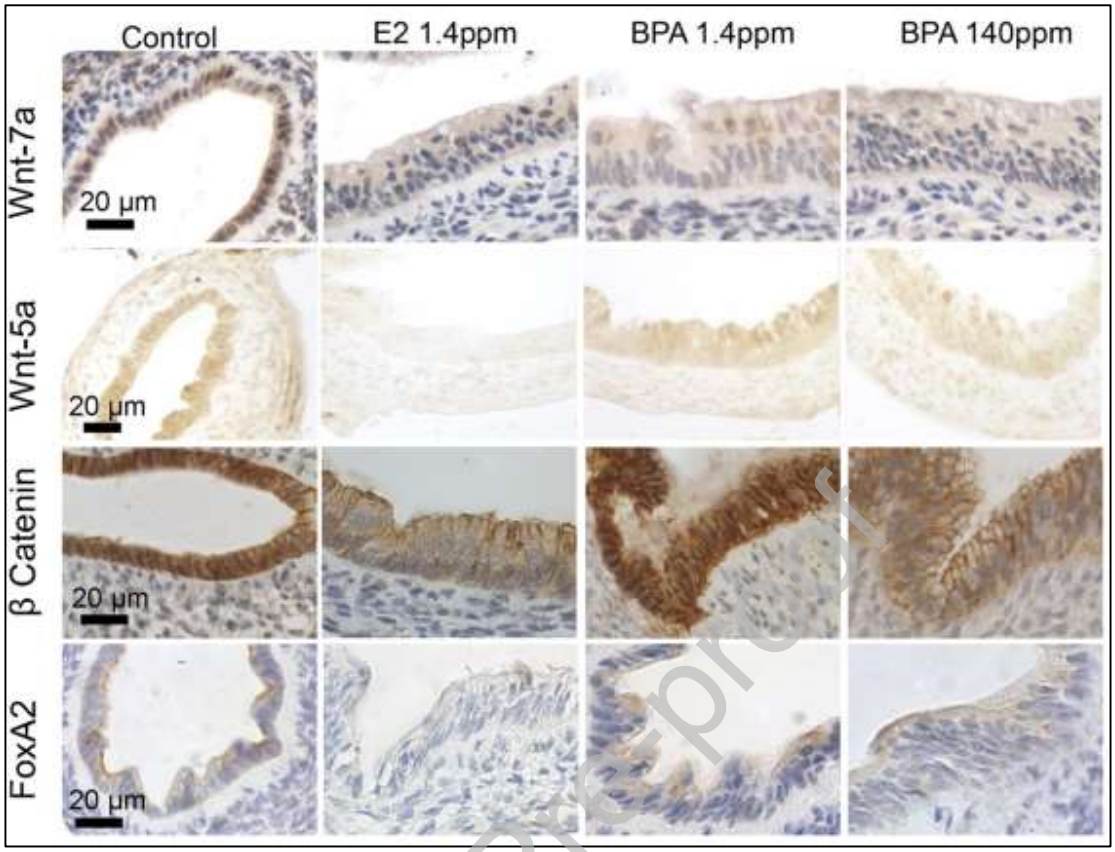

Figure 7: Effects of postnatal exposure to the xenoestrogens BPA and E_2 on the oviductal expression of Wnt-7a, Wnt-5a, β-catenin and FoxA2 in *C. latirostris*. Since the treatment with E_2 0.014 ppm induced changes only in Wnt-7a epithelial expression in a way similar to E_2 1.4 ppm, representative photomicrographs of this experimental group were omitted. IHC developed with DAB and counterstained with Mayer's hematoxylin (first, third and fourth rows). Photomicrographs in the second row were not counter-stained to better visualize the changes in subepithelial Wnt-5a expression as a consequence of BPA and E_2 exposure.

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3.2.2. Effects of postnatal xenoestrogen exposure on the histofunctional features used in the scoring system

Early postnatal exposure to the higher doses of BPA and E_2 significantly increased the histofunctional score in caimans. We also found that the main feature modified by the exposure

to the higher doses of E_2 and BPA was epithelial disorder. Briefly, the oviductal luminal epithelial cells, especially at early stages of postnatal development, are organized in a palisade-like arrangement, in simple columnar or pseudostratified epithelium. Epithelial disorder is defined as the presence of rounded-nuclei and rounded or polyhedral cells immersed in simple or pseudostratified luminal epithelium without apparent organization (Galoppo et al., 2016). Caimans treated with the higher doses of BPA also showed an increased number of intra-epithelial PAS (+) cells. Interestingly, caimans treated with the lower dose of BPA showed an increased number of subepithelial projections, but no differences in the total score (Figure 8).

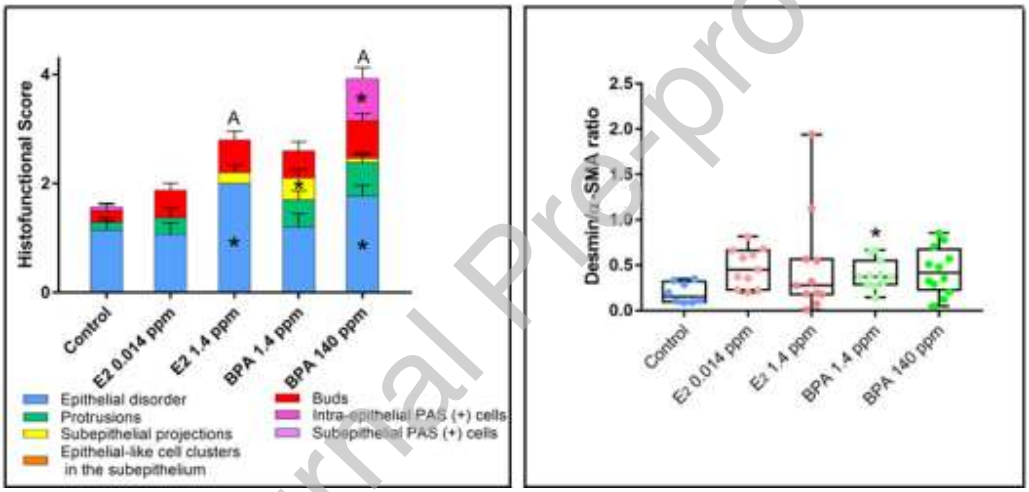

Figure 8: Effects of postnatal exposure to the xenoestrogens BPA and E_2 on the histofunctional features used as markers of oviduct differentiation. Left graph: Effect of BPA and E_2 exposure on the histofunctional score as a consequence of xenoestrogen-induced changes in partial scores. The bars represent the histofunctional score. The stacked colored bars represent the partial score assigned to each histological feature. Results are expressed as mean ± SEM. Capital superscripts denote statistical differences between the histofunctional score of the different experimental groups and the control group. Asterisks in the stacked colored bars denote statistical differences in the partial score assigned to each feature between the treated groups and the control group. Right graph: Effect of BPA and E_2 exposure on the desmin/α-SMA ratio. The boxes represent the ratio values. Results are expressed as median

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± interquartile range. The asterisk above the box denotes statistical difference with the control group. Results analyzed by Kruskal-Wallis followed by Dunn´s post-test at $P < 0.05$.

3.2.3. Effects of postnatal xenoestrogen exposure on the desmin/α-SMA ratio

The ratio between desmin expression and α-SMA expression in the subepithelial muscle layer was evaluated as a marker of oviduct maturation. Postnatal exposure to the lower dose of BPA increased the desmin/α-SMA ratio. Although no statistically relevant differences were observed between control caimans and caimans treated with E_2 (both doses) and the higher dose of BPA, the desmin/α-actin ratio showed an increasing tendency in these treated groups (Figure 8). When comparing the desmin/α-SMA ratio obtained for caimans treated with E_2 and BPA with the ratio that characterized the different developmental stages, we observed that the ratio of treated animals did not differ from that of late postnatal caimans.

4. Discussion

4.1. Ontogenic changes in the Wnt signaling pathway in the caiman oviduct

Previously, we have demonstrated that the exposure to the xenoestrogens E_2 and BPA can alter the postnatal differentiation of *C. latirostris* oviduct (Galoppo et al., 2017). The Wnt signaling pathway and its role in FRT differentiation have been extensively studied in many mammalian species, including mice, rats, sheep and humans (Ghosh et al., 2017; Hayashi and Spencer, 2006; Hayashi et al., 2011; Kelleher et al., 2019; Mericskay et al., 2004; St-Jean et al., 2019). In addition, in archosaur species such as chickens, the Wnt signaling pathway is involved in the development of gonads (Bae et al., 2013; Smith et al., 2008), oviduct (Bae et al., 2014; Lim et al., 2013), feathers (Lin and Yue, 2018; Lin et al., 2018) and neural development (Kumar et al., 2019; Matsushita et al., 2019). However, as far as we know, no evidence of the Wnt pathways

regulating FRT differentiation had been previously reported in crocodilian species. In the present study, we observed gradual increasing levels of epithelial expression of Wnt-7a from the neonatal to the prepubertal juvenile stage but no changes in Wnt-5a at the subepithelial stromal sub-compartment. In the mouse FRT, Wnt-7a and Wnt-5a are mutually regulated in a feedback loop (Mericskay et al., 2004). Firstly, Wnt-7a would maintain high levels of molecules involved in cell differentiation, including Wnt-5a, and would thus be required as a prerequisite to generate the glandular compartment. Secondly, Wnt-5a would inhibit Wnt-7a expression at specific points of the luminal epithelium, promoting the formation of invaginations and, eventually, glands in such points (Mericskay et al., 2004). In our model, the role of Wnt-7a in adenogenesis seems to be in consonance with that described for murine FRT given that the increasing levels of Wnt-7a expression are accompanied by histofunctional changes that evidence an active adenogenesis process (Galoppo et al., 2016). However, the increasing levels of Wnt-7a are not related to the subsequent increases in stromal Wnt-5a as described for the mouse FRT suggesting that, throughout the developmental stages here studied in *C. latirostris*, Wnt-5a would not be regulated by Wnt-7a and would not be directly related to FRT gland morphogenesis.

In the present work, we also determined the levels and pattern of expression of β-catenin. This protein showed a membrane-associated pattern of expression that decreased in intensity in the segments of the luminal epithelium participating in budding. In mammals, β-catenin, as a part of the cadherin complex, controls cell-cell adhesion (Lodish et al., 2003; Tian et al., 2011) and endothelial migration (Nelson and Nusse, 2004; Tian et al., 2011). Indeed, in mammals, a fine balance between the tightening and loosening of cell-cell interactions at specific sites allows the invagination of epithelial cells into the subjacent stroma (Cooke et al., 2013; Tian et al., 2011), which eventually leads to the bud outgrowth that precedes gland formation. Similarly, in *C.*

latirostris, the reduced levels of membrane-associated expression of β-catenin in the buds of late postnatal and prepubertal juvenile oviducts may lead to the loosening of cell-cell adhesion, and increased cell migration and may be a prerequisite for budding and gland outgrowth. In mammals, FoxA2 is a factor involved in gland differentiation (Jeong et al., 2010; Kelleher et al., 2017). In the oviduct of *C. latirostris*, FoxA2 is expressed in the luminal epithelium from the neonatal to the late postnatal stage. At the prepubertal juvenile stage FoxA2 is also expressed in the glands. However, FoxA2 expression in the luminal epithelium is lower than that in the glandular epithelium, suggesting that the expression pattern of this factor may be reorganized as the oviduct matures, within the observed ages. In mammals, FoxA2 regulates the differentiation of the glandular epithelium from the luminal epithelium in the acini of the gland (Cooke et al., 2013). Although the role of FoxA2 in the luminal epithelium remains unclear, the reorganization of its pattern of expression in the glandular epithelium in *C. latirostris* was associated with the proposed model of oviduct gland differentiation in mammals.

The differentiation of the oviduct is characterized by changes in histofunctional features that can be measured by a histofunctional score, as reported in Galoppo et al. (2016). In the present work, we found that the increased histofunctional score from the early postnatal to late postnatal stage was due to the presence of subepithelial projections and buds. Bud formation is described as the first step in gland morphogenesis (Hinck and Silberstein, 2005). Since gland formation requires epithelial cells to move into the subepithelial compartment, subepithelial projections can be considered as signs of gland development. The temporal association between the increasing expression of Wnt-7a and the presence of buds and subepithelial projections from the early postnatal to the late postnatal stage adds supporting evidence of the role in Wnt-7a on gland formation.

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Our results showed that the desmin/α-SMA ratio increased significantly from the early to the late postnatal stage. Since the proportion of desmin and α-SMA changes as developmental stages advance, here we propose the desmin/α-SMA ratio as a marker of oviduct maturation. The increase in the desmin/α-SMA ratio was observed in a scenario of increasing levels of Wnt-7a in the epithelium and of Wnt-5a in the muscle layer, suggesting a relationship between these signaling proteins and muscle development. In the murine oviduct, members of the Wnt signaling pathway are required for the organization of the smooth muscle layers (Cooke et al., 2013). Specifically, it has been proposed that Wnt-5a could have a role in muscle cell proliferation in mammals (DiRenzo et al., 2016). Thus, Wnt-5a may also play a role in muscle growth and organization in *C. latirostris*.

Our results support evidence of the involvement of a cross-talk of signaling pathways that intersect to coordinate growth, cell differentiation, migration, and cell-cell interactions necessary for the normal development of the *C. latirostris* oviduct.

4.2. Effects of postnatal xenoestrogen exposure on the Wnt signaling pathway in the oviduct

Previously, we have shown that exposure to E_2 and BPA alters the temporal pattern of oviduct development, suggesting a precocious differentiation process (Galoppo et al., 2017). Similarly, Doheny et al (2016) informed that *in ovo* exposure to an estrogen receptor agonist induced precocious development of glands in the Müllerian Duct of *Alligator mississippiensis.* In the subepithelial compartment, early postnatal exposure to BPA 1.4 ppm significantly increased the values of the desmin/α-SMA ratio. Although exposure to E_2 (both doses) and to the higher dose of BPA did not significantly change the desmin/α-SMA ratio, it is noteworthy that the ratio

reached a value similar to that observed at the late postnatal stage. Consequently, our results suggest that postnatal exposure to E_2 and BPA promotes oviduct differentiation at subepithelial level. In this context, it is remarkable that, contrary to what was expected, the Wnt-5a expression levels in the muscle layer in animals treated with BPA (both doses) as well as in those treated with E_2 1.4 ppm were decreased. Although Wnt-5a could have a role in muscle cell proliferation, studies *in vitro* and *in vivo* in humans (Teveroni et al., 2017), rabbit (Stamatiou et al., 2011), and rats (Zhang et al., 2011) have shown that muscle growth and differentiation are regulated by estrogens. Besides, in humans and rats, BPA can induce vascular smooth muscle cell proliferation (Gao et al., 2019). Consequently, the increased desmin/α-SMA ratio in E_2- and BPA-treated caimans, despite the decreased levels of Wnt-5a, could be explained by an estrogenic effect.

Exposure to the higher dose of BPA and both doses of E_2 caused a significant decrease in Wnt-7a levels. These results coincide with previous studies that showed decreased expression levels of Wnt-7a in the FRT of neonatal mice exposed to other xenoestrogenic substances such as diethylstilbestrol and mixtures of polychlorinated biphenyls (Carta and Sassoon, 2004; Ma and Sassoon, 2006; Miller et al., 1998). As mentioned before, Wnt-7a acts in the first steps of gland morphogenesis. Consequently, the increasing levels of Wnt-7a shown in the ontogenic study could be taken as a pro-adenogenic stimulus. In our model, postnatal exposure to E_2 and BPA decreased the expression of Wnt-7a, suggesting that this exposure could be acting as an anti-adenogenic stimulus. Indeed, it has been reported that inhibition or ablation of Wnt-7a leads to disruption of adenogenesis in the FRT of mammals (Hayashi et al., 2011; Mericskay et al., 2004). These results seem to be conflicting with those previously reported by our group, which, based on the histofunctional score, suggested that exposure to E_2 and BPA advanced oviduct

development and differentiation (Galoppo et al., 2017). To address this issue, we analyzed the histofunctional score in terms of the effect of the different treatments on each individual feature considered to build it. We found that the increased histofunctional score induced by E_2 and BPA exposure was related to increased epithelial disorder and intraepithelial PAS positivity. The development of the oviduct is a process that could be associated with histofunctional features such as luminal epithelial hyperplasia (epithelial disorders, protrusions and intraepithelial PAS positivity) and others more closely related to gland morphogenesis itself (presence of buds, subepithelial projections, clusters of epithelial-like cells in the subepithelial compartment and subepithelial PAS positivity). The increasing values of the histofunctional score observed between the early and late postnatal stages were mainly due to increased features related to gland morphogenesis (presence of buds and subepithelial projections), whereas the increase in the histofunctional score observed as a consequence of E_2 or BPA exposure was due to features related to luminal epithelium hypertrophy (epithelial disorder and intra-epithelial PAS positivity). In vertebrate species, xenoestrogen-induced epithelial hypertrophy of the FRT has been associated with former adenogenesis impairment in advanced developmental (Berg et al., 2001; Carpenter et al., 2003; Gray et al., 2001). These hypertrophy-related qualitative changes in the histofunctional features occur in the context of decreased levels of Wnt-7a. Consequently, altered levels of Wnt-7a could lead, at least in part, to a loss of coordination of the histofunctional changes that are necessary for normal adenogenesis and oviduct development.

Postnatal exposure to the higher doses of E_2 and BPA downregulated β-catenin expression, while that to the lower dose of BPA had the opposite effect. The decreasing levels of β-catenin observed at the intercellular junctions may indicate loosening of cell-cell interactions, which is one of the first processes that lead to the formation of glands. Therefore, decreased epithelial

levels of β-catenin would facilitate gland epithelium outgrowth from the luminal epithelium. However, the histofunctional features related to these treatments are those related to epithelial hypertrophy rather than to gland morphogenesis. The discordance between histomorphological evidence and β-catenin expression could reflect the loss of coordination in the events that characterize the adenogenic process aforementioned (Andrews et al., 2012; Furuse et al., 2006; Zhou et al., 2013). Our results are in concordance with studies that have informed an association between exposure to BPA and altered development of glands in different organs of other vertebrate species (Folia et al., 2013; Mandrup et al., 2016; Perrot-Applanat et al., 2018). Finally, in our model, exposure to the higher doses of E_2 and BPA decreased the expression of FoxA2 in the luminal epithelium. As previously discussed, FoxA2 is essential for FRT glandular development (Jeong et al., 2010). Taking this into account, our result supports the anti-adenogenic effect of early postnatal exposure to E_2 and BPA. Interestingly, the same exposures that significantly decreased the levels of expression of FoxA2 also decreased the levels of expression of β-catenin. Since FoxA2 expression is also known to be upregulated by β-catenin (Villacorte et al., 2013; Yu et al., 2009), alterations in FoxA2 expression may be explained, at least in part, by the decreasing levels of β-catenin due to xenoestrogen exposure. Decreased levels of gene and protein expression of FoxA2 as a consequence of EDC exposure during critical periods of development have been previously reported in mice and lambs (Alarcon et al., 2020; Burns et al., 2013). Indeed, Burns et al. (2013) showed an association between decreased uterine gland count and low gene expression of FoxA2 in mice. This finding suggests that disruption of FoxA2 expression likely contributes to decreased gland count, which, in turn, could have long-lasting reproductive effects during critical periods of development.

5. Conclusions

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The differentiation of the oviduct of *C. latirostris* is completed postnatally and is characterized by histofunctional changes regulated by proteins of the Wnt signaling pathway, FoxA2 and β-catenin. Early postnatal exposure to the xenoestrogens E_2 and BPA altered the levels of these molecules involved in oviduct differentiation and modified the temporal pattern of histofunctional changes that characterize the postnatal differentiation of the oviduct of *C. latirostris*. These modifications could impair the coordination of the morphogenetic events necessary for proper gland formation. Since both E_2 and BPA can be found as environmental pollutants in aquatic ecosystems, additional studies with exposures that more closely simulate environmental scenarios could shed some light on the effects of environmental water pollution on the reproductive health of exposed organisms.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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