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Research paper

Bisphenol A disrupts the temporal pattern of histofunctional changes in the female reproductive tract of *Caiman latirostris*

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ABSTRACT

Recently, we have described the ontogeny of histofunctional differentiation changes in the oviduct of *Caiman latirostris*. The expression of estrogen receptor alpha and progesterone receptor shows that the caiman oviduct could be a target of the action of xenoestrogens such as the widely environmentally present Bisphenol A (BPA), early in life. The aims of this study were: to complement oviduct characterization by establishing the ontogenetic changes in androgen receptor (AR) expression and assessing the effects of early postnatal exposure to 17-β-estradiol (E2) or BPA on the histofunctional features of the oviduct. AR was expressed in all the stages studied. The spatial pattern of AR immunostaining changed from neonatal to juvenile caimans. In the luminal epithelium, changes were at the subcellular level, from cytoplasmic to nuclear. In the subepithelium, although both cytoplasmic and nuclear AR expression was observed, changes were mainly at tissue level, from the subepithelial compartment to the outer muscular layer. The oviduct was highly sensitive to E2 and BPA at the early postnatal developmental stage. E2- and BPA-exposed caimans showed increased luminal epithelial height and higher proliferative activity. Changes in histomorphological features (measured by a scoring system), steroid hormone receptors, collagen remodeling and muscle-associated proteins suggest a precocious oviduct histofunctional differentiation in E2- and BPA-exposed caimans. The modification of the temporal pattern of oviductal biomarkers suggests that organizational changes could impair *C. latirostris* reproductive health later in life. The alterations in the caiman female reproductive tract exposed to BPA highlight the importance of preserving aquatic environments from plastic pollution.

1. Introduction

Caiman latirostris is a South American crocodilian species widely distributed in Argentina. Like in all crocodilians, the sex of *C. latirostris* offspring is determined by the incubation temperature (temperature-dependent sex determination), and influenced by sex steroid hormones (Stoker et al., 2003). We have previously found that, female and male caiman reproductive tissues are highly sensitive to the effects of endocrine disrupting compounds (EDCs) (Stoker et al., 2003; Stoker et al., 2008; Rey et al., 2009, Durando et al., 2013, Durando et al., 2016). Prenatal exposure to EDCs modifies ovarian follicular dynamics and hormonal steroid levels in postnatal female caimans (Stoker et al., 2008). We have also found that male caimans exposed to low doses of Bisphenol A (BPA), one of the most important plasticizers, exhibit altered gonadal histomorphology accompanied by decreased testosterone levels and/or disrupted expressions of sex-determining genes (Stoker et al., 2003; Rey et al., 2009, Durando et al., 2013, Durando et al., 2016).

In oviparous species, such as *C. latirostris*, a prominent function of the oviducts is to provide the egg white proteins and eggshell to ovulated eggs. Thus, we have recently assessed the relationship between the burden of organochlorine compounds, which behave as EDCs, in caiman eggs and eggshell features (Stoker et al., 2013). Our results suggest a direct effect of the exposure to organochlorine compounds on the mother's oviductal functions, evidenced by decreased eggshell porosity (Stoker et al., 2013).

Abbreviations: AR, androgen receptor; BPA, Bisphenol A; BrdU, bromodeoxyuridine; DAB, diaminobenzidine; E2, 17-β-estradiol; EDCs, endocrine disrupting chemicals; ERα, estrogen receptor alpha; FRT, female reproductive tract; GAM, Gonadal-Adrenal-Mesonephros; IHC, immunohistochemistry; IOD, integrated optical density; PAS, Periodic Acid Schiff; PR, progesterone receptor; TGF-β, transforming growth factor beta; α-SMA, alpha smooth muscle actin.

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To better understand the effects of EDC exposure on oviduct physiology, we have also described the histomorphological features of the oviduct of *C. latirostris* and established the ontogeny of changes in biomarkers of histofunctional differentiation from the neonatal to the pre-pubertal juvenile stage (Galoppo et al., 2016). We confirmed that, like in other vertebrates, the female reproductive tract (FRT) of caimans -the oviduct in oviparous species- shows postnatal development and differentiation (Galoppo et al., 2016, Gray et al., 2001, Massé et al., 2009). Among the multiple factors that regulate the postnatal development of the FRT, ovarian steroid hormones play a key role. Thus, we also established the spatial and temporal patterns of estrogen receptor alpha (ERα) and progesterone receptor (PR) expression in the oviduct of *C. latirostris* (Galoppo et al., 2016).

Androgens and their receptors also are involved in the postnatal development of the FRT. Androgens regulate the expression of genes involved in the patterning along the anteroposterior axis during critical periods of the development of the FRT (reviewed by Massé et al., 2009). Moreover, it has been proposed that androgens can exert their action synergistically with estrogen and/or progesterone (Mika et al., 1987), promoting normal growth and development of all oviduct segments (Joensuu et al., 1992). Based on this and since little is known about the occurrence and role of androgen receptors (AR) during oviduct development in reptile species (Rhen et al., 2003; Selcer et al., 2005; Liu et al. 2016), the first aim of this study was to complement caiman oviduct characterization by establishing the ontogenetic changes in AR protein expression.

Humans and wildlife are daily exposed to contaminants which have the potential to interfere with their endocrine system by acting as EDCs (Colborn et al., 1993; Bergman et al., 2012). Plasticizers, which are the most common plastic additives, are often not covalently bound to the plastic matrix, and can thus slowly diffuse out of plastics, leading to wide environmental contamination (Mathieu-Denoncourt et al., 2015). BPA, which, as mentioned above, is one of the most important plasticizers, is recognized as an EDC with estrogenic and antiandrogenic activity (National Toxicology Program, 2008). Microplastics are considered new emerging pollutants in aquatic ecosystems and have also raised concern regarding their endocrine disruptive effects (Eerkes-Medrano et al., 2015; Muñoz-de-Toro, 2015).

At embryonic stages, caimans may be exposed to contaminants by maternal transfer or exposure through the eggshell. After hatching, exposure continues through the remaining yolk sac, diet and aquatic environment. Caimans spend a large part of their lives in the water, are long-lived animals, and are at the top of the food web. All these make them particularly susceptible to EDC exposure. We have previously found that *in ovo* exposure to 17-β-estradiol (E2) and BPA is able to disrupt estrogen-sensitive events both in caiman embryos and juvenile caimans (Stoker et al., 2003, Stoker et al., 2008; Durando et al., 2013, Durando et al., 2016). However, the sensitivity of caimans to early postnatal xenoestrogen exposure has not been investigated.

Early phases of development are critical windows of sensitivity to exposure to hormones or EDCs (Bergman et al., 2012), and this exposure may result in effects that will remain throughout life. These effects, called organizational, could be subtle and do not become apparent until sexual maturity (Guillette et al., 1995). An example of this is the effect of embryonic exposure to E2 on quail uterine morphology and adenogenesis, evident only at sexual maturity (Berg et al., 2001a). It has also been demonstrated that, in turtles, *in ovo* exposure to E2 blocks the development of the Müllerian ducts, causes hypertrophy of the differentiated portion and prevents further differentiation of the Müllerian duct in the caudal region (Dodd and Wibbels, 2008) and that in quails, dietary exposure to E2 causes oviductal gland atrophy (Yamashita et al., 2010). On the other hand, exposure to BPA at different stages of development causes embryonic oviduct malformation in quails (Berg et al., 2001b) and reduces glandular density and thickness of the tunica mucosa in adult hens (Yigit and Daglioglu, 2010). More extensive studies conducted in rats have shown that BPA exposure is related to lower expression of genes related to the differentiation of the anteroposterior axis, the downregulation of progesterone receptor (PR) and estrogen receptor alpha (ERα) (Varayoud et al., 2011; Vigezzi et al., 2016), increased density of glands with squamous metaplasias, deregulated expression of E2-regulated genes, altered glandular proliferation and α-actin expression, and increased abnormalities in the luminal and glandular epithelium (Vigezzi et al., 2015).

In caimans, the oviduct expression pattern of ERα and PR shows that, even at early postnatal developmental stages, the oviduct could be a target of xenoestrogen action (Galoppo et al., 2016). Therefore, the second aim of this study was to assess the effects of early postnatal exposure to E2 or BPA on histofunctional features of the oviduct of *C. latirostris*.

2. Material and methods

2.1. Animals, treatments and sample processing

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 Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina.

2.1.1. Ontogeny of AR expression in the caiman oviduct

To establish temporal and spatial patterns of AR expression in the caiman oviduct, archived paraffin-embedded samples were used. Samples came from intact caiman females euthanized when they reached the neonatal (n = 10), early postnatal (n = 15), late postnatal (n = 14) or juvenile (n = 7) developmental stages (Galoppo et al., 2016). At the neonatal and early postnatal stages, the caiman oviduct is a thin structure that runs attached to the G onadal–Adrenal–Mesonephros (GAM) complexes. In advanced developmental stages (late postnatal and juvenile), the oviduct was dissected from the GAM complexes, sectioned into three segments (caudal, middle and rostral), immediately fixed in 4% phosphate-buffered formalin (pH 7.4) for 6 h at room temperature and processed separately. Fixed tissues were dehydrated in graded ethanol series, cleared in xylene (Biopack, Buenos Aires, Argentina), and paraffin-embedded.

2.1.2. Effects of early postnatal xenoestrogen exposure on the histoarchitecture and histofunctional features of the caiman oviduct

To assess the effects of early postnatal xenoestrogen exposure on the oviduct, *C. latirostris* eggs were collected shortly after oviposition from seven nests randomly selected from Chaco Province, Argentina. The collection sites were placed upstream of urbanized, industrial and farming areas, to minimize 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, the female-producing temperature (Stoker et al., 2003, 2008). 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). Two to three one-month-old female hatchlings from each nest were injected subcutaneously twice, 7 days apart, with BPA

(Aldrich, Milwaukee, WI, USA) 1.4 ppm (n = 16) or 140 ppm (n = 14); 17β-estradiol (E2) (Sigma-Aldrich, St Louis, MO, USA) 0.014 ppm (n = 16) or 1.4 ppm (n = 15) or vehicle (corn oil, n = 15). Animals were euthanized 7 days after the last treatment injection. Two hours before sacrifice, caimans were injected with bromodeoxyuridine (BrdU; Sigma Chemical, St. Louis, MO, USA; 6 mg/100 g body weight/1.5 ml PBS, ip). After euthanasia, the GAM complexes and oviducts were dissected, fixed and processed as described in Section 2.1.1.

The age for the treatment was chosen considering our previous results regarding the ontogeny of changes in the oviduct of *C. latirostris*. At the early postnatal stage, ERα and PR expression suggests response to xenoestrogens (Galoppo et al., 2016). The higher doses of both E2 (1.4 ppm) and BPA (140 ppm) were applied as reference doses known to cause effects when *in ovo* administered, since both overrode the temperature effect on *C. latirostris* sex determination (Stoker et al., 2003, 2008). E2 (0.014 ppm) and BPA (1.4 ppm) were 100 times lower than 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). *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. Insects and small arthropods are occasionally part of their diet (Borteiro et al., 2008). In this context, and to ensure the effective dose, the subcutaneous route of administration was chosen.

2.2. Oviduct histoarchitecture

For regular histological examination and morphometric evaluation, oviduct serial transverse sections (5 μm) were stained with Picrosirius solution (Stoker et al., 2008) and Harris hematoxylin (P&H) (Biopur, Rosario, Argentina). As shown in Fig. 2S, to identify mucous glands beside the morphological features, we used histochemical stains. 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 rule out non-specific PAS positivity, negative controls were performed by preventing aldehyde formation by skipping the periodic acid pretreatment. Finally, we also performed Alcian blue-PAS staining. In this staining technique, all the acidic mucins are first stained with Alcian blue, and then neutral mucins which are solely PAS positive are stained in magenta. Where mixtures occur, the resultant color will depend on the dominant moiety (staining color varying from purple to blue) (Pearse, 1985). To analyze levels of organization of collagen fibers in the subepithelial compartment, P&H for polarized light microscopy (P&H-pol) was used (Luque et al., 1998). All the evaluations were performed in at least three sections separated 150 μm from each other.

2.2.1. Morphometric analysis

Morphometric analysis was performed on digitalized images of tissue sections captured 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).

To measure luminal epithelial height, the basal and apical edges of the epithelium were manually delimited and the mean epithelial height was calculated (Fig. 2A). Calibration with reference rulers was performed at the beginning of each measurement.

To evaluate collagen remodeling, P&H-pol-stained samples were observed with an optical microscope under polarized light (filter of polarization model BH-POL, Olympus Optical Co., LTD). Images were captured and digitized as described by Rodríguez et al. (2003). By using this method, only orientated collagen molecules appear as brightly birefringent structures. When collagen fibers are not dense and/or not regularly arranged, they are weakly birefringent. Results are expressed as the percentage of total subepithelial area occupied by highly birefringent collagen fibers. A decrease in the intensity of birefringence shows a transformation from a hard and unyielding structure to a soft, swollen and flexible structure denoting collagen remodeling (Fig. 3).

2.3. Biomarkers of histofunctional differentiation: muscle phenotype, cellular proliferation and hormone dependence

Biomarkers were defined according to the Biomarkers Definitions Working Group (2001). The expression of desmin and/or alpha smooth muscle actin (α-SMA) was used to establish not only the muscle phenotype but also the distribution pattern of different muscle cells, which is a sign of histofunctional differentiation. Incorporation of BrdU was used to evaluate proliferative activity and expression of ERα, PR and AR as biomarkers of hormone dependence. The expression of biomarkers was studied by immunohistochemistry (IHC). The antibodies used and the references regarding antibody specificity are listed in Table 1. Tissue sections (5 μm in thickness) were removed from the paraffin and dehydrated in a graded ethanol series. As already mentioned, BrdU was administered 2 h before sacrifice. BrdU incorporation was evaluated by immunohistochemistry; protocol includes microwave pre-treatment for antigen retrieval and acid hydrolysis for DNA denaturation (Kass et al., 2000). To evaluate the expression of desmin, α-SMA and steroid hormone receptors routine protocols were followed (Galoppo et al., 2016). Briefly, tissue sections (5 μm in thickness) were removed from the paraffin and dehydrated in a graded ethanol series. Microwave pretreatment for antigen retrieval was performed. Endogenous peroxidase activity and non-specific binding sites were blocked. Primary antibodies were incubated overnight at 4 °C. After incubation with biotin-conjugated secondary antibodies for 30 min, the reactions were developed using a streptavidin–biotin peroxidase method and

Table 1
Antibodies used for immunohistochemistry.

Primary Antibodies	Supplier	References
Anti-Smooth muscle α-actin (α-SMA clone 1)	Novocastra, Newcastle upon Tyne, UK	Rey et al., 2009
Anti-Desmin (Clone DE-R-11)	Novocastra, (Newcastle upon Tyne, UK)	Rey et al., 2009 Durando et al., 2016
Anti-BrdU (clone 85-2C8)	Novocastra, (Newcastle upon Tyne, UK)	Stoker et al., 2008
Anti-ER (LETH-ER 202Y)	LETH (Santa Fe, Argentina)	Varayoud et al., 2012 Durando et al., 2016
Anti-PR (Code A0098)	DAKO Corp. (Carpinteria, CA, USA)	Galoppo et al., 2016
Anti-AR (LETH-AR 280Y)	LETH (Santa Fe, Argentina)	Specificity of anti-AR was tested (see Supplementary Material and Fig. S1)

diaminobenzidine (DAB; Sigma-Aldrich, Buenos Aires, Argentina) as a chromogen substrate. Each IHC included positive and negative controls. For negative controls, the primary antibody was replaced by non-immune serum (Sigma-Aldrich) or by the antibody-antigen complex (pre-adsorbed antibody). Samples were counterstained with Harris hematoxylin (cellular proliferation) or Mayer's hematoxylin (muscle phenotype and hormone dependence) and mounted with a permanent mounting medium (Eukitt, Sigma-Aldrich, St Louis, MO, USA).

2.3.1. Quantification of protein expression

Desmin and α-SMA expressions were assessed to evaluate the effects of exposures to E2 and BPA on the thickness and organization of the oviductal muscle layers. Results are expressed as the percentage of subepithelial area occupied by desmin- or α-SMA-positive cells.

The proliferative activity of the luminal epithelium was assessed by BrdU incorporation (Kass et al., 2000). Results are expressed as percentage of positive cells.

Oviduct expression of ERα was determined in both the epithelial and subepithelial compartments, whereas PR expression was determined only in the epithelial compartment because of its low subepithelial expression. The expression of both ERα and PR was quantified by image analysis as previously described (Varayoud et al., 2012). Briefly, images of tissue sections were recorded and analyzed using image analysis software. When possible, the expression was evaluated in the epithelial and subepithelial stromal compartments in each image (at least five fields from each compartment were recorded from each slide). The microscope was set up properly for Köehler illumination. Correction of unequal illumination (shading correction) and calibration of the measurement system were performed with a reference slide. The images from immunostained slides were converted to gray scale. By using the Image Pro-Plus 4.1.0.1 system, an automated standard sequence operation was created to measure the integrated optical density (IOD) as a linear combination between the average gray intensity and the relative area occupied by positive cells. Because IOD is a dimensionless parameter, the results are expressed as arbitrary units. AR expression was qualitatively analyzed to establish the spatial distribution pattern of immunostaining (cytoplasmic or nuclear); quantitative evaluation was expressed as percentage of immunostained luminal or glandular epithelial nuclei. Subepithelial AR expression was quantified as percentage of subepithelial area occupied by AR.

2.4. Histofunctional score

C. latirostris oviductal histofunctional differentiation is characterized not only by changes in muscle phenotype, cellular proliferation and steroid hormone receptor expressions but by changes in both histomorphological features and spatial PAS staining pattern. The histofunctional scoring system previously reported (Galoppo et al., 2016) was applied to obtain additional information regarding the effects of xenoestrogen exposure on the differentiation pattern of the oviduct. Briefly, histological features and different spatial distribution patterns of PAS staining were identified (Fig. 3S upper panel). The number of times that each particular feature appeared in an oviduct section was recorded and the score was assigned according to a previously established rank (see Supplemental material for additional information).

2.5. Statistical analysis

The data are reported as the mean ± SEM. When the variables exhibited normal distribution, parametric tests were used. ANOVA was performed to obtain the overall significance, followed by Tukey post-test to establish differences between control and experimental groups. 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 control and experimental groups. $p < 0.05$ was accepted as significant.

3. Results

3.1. Ontogenetic changes in AR protein expression in the caiman oviduct

The spatial pattern of AR immunostaining in the caiman oviduct depended greatly on the developmental stage. In the luminal epithelium, changes in the spatial pattern of AR expression were at the subcellular level. The earliest stages of development were characterized by cytoplasmic AR expression. A gradual increase in nuclear AR immunostaining was observed as developmental stages progressed, being significantly higher at the juvenile stage (Fig. 1). Independently of the developmental stage, most of the ciliated cells showed nuclear AR protein expression (Fig. 1A-D). In the subepithelium, cytoplasmic and nuclear AR expression was observed, whereas changes in the AR spatial pattern were mainly at tissue level. Neonatal and early postnatal stages were characterized by an absent to very low AR expression mainly in outermost tissue layers. At the late postnatal stage, AR expression was observed all over the subepithelial compartment. When the juvenile stage approached, subepithelial expression of AR was restricted to the muscular layer, showing cytoplasmic and nuclear patterns. In the presence of glands (sign of differentiation), the muscular AR immunostaining was more intense than in its absence (Fig. 1 E-H). The epithelium of mucous gland showed nuclear AR expression from levels similar to those exhibited by the luminal epithelium to almost 100%.

3.2. Effects of xenoestrogen exposure on the oviduct histoarchitecture

3.2.1. Luminal epithelial height and proliferative activity

Oviduct luminal epithelial height is a sensitive target of sex steroid hormones. All the doses of BPA and E2 tested increased the oviduct luminal epithelial height in early post-natal *C. latirostris* (Fig. 2). Treatments with the lower doses of E2 and BPA (0.014 and 1.4 ppm) increased the epithelial cell height mainly by increasing the cell size as a result of secretion stored in the apical border, while the higher doses of E2 and BPA (1.4 ppm and 140 ppm, respectively) increased the epithelial height not only by increasing the cell size but also by increasing the number of epithelial cell layers (stratification process) (Fig. 2). These results parallel those of proliferative activity. No changes in luminal epithelial proliferative activity were observed as a consequence of the exposure to the lower doses of E2 and BPA. As mentioned above, the higher doses (E2 1.4 ppm and BPA 140 ppm) not only increased the percentage of proliferating cells but also the number of cell layers, showing earlier changes in proliferative activity.

3.2.2. Subepithelial histoarchitecture

3.2.2.1. Collagen remodeling. Many developmental processes depend on proper epithelium-mesenchyme interactions, with a key role of extracellular matrix components. All the treatments induced an increase in collagen remodeling, revealed by a decrease in the subepithelial area occupied by high birefringent collagen structures (Fig. 3).

3.2.2.2. Muscle-associated proteins. Exposure to E2 and BPA increased the subepithelial area occupied by α-SMA. In addition,

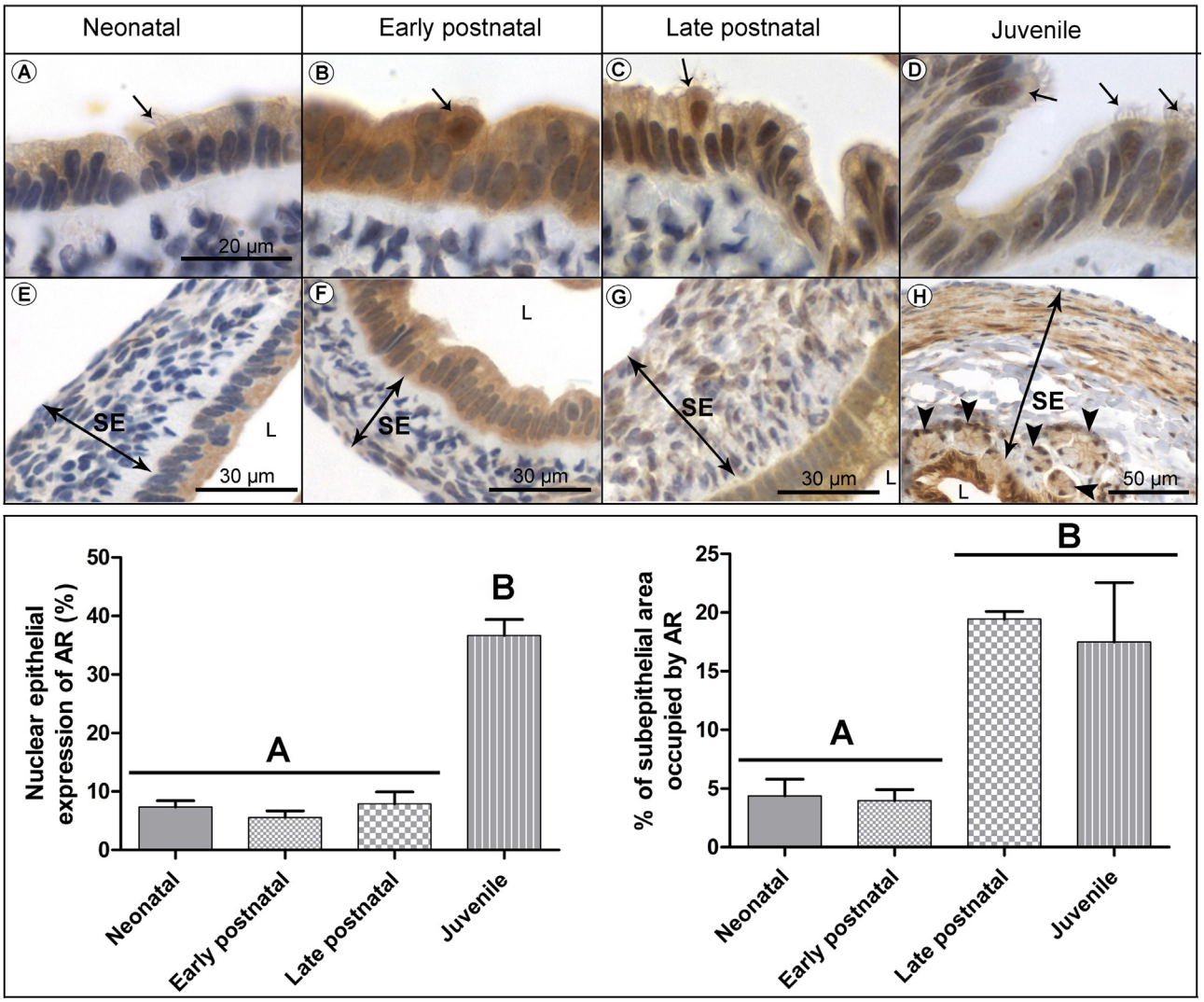

Fig. 1. Ontogeny of AR expression. Upper panel: Representative photomicrographs of oviduct sections from different developmental stages, showing the AR immunostaining pattern in luminal epithelium (A-D) and subepithelium (E-H). Luminal epithelial cells exhibit cytoplasmic and nuclear AR staining. At the juvenile stage, cytoplasmic staining is almost absent, but significant increase in nuclear staining is observed. The neonatal and early postnatal stages are characterized by low subepithelial nuclear and cytoplasmic AR expression (E,F), whereas the late postnatal and juvenile stages are characterized by increased AR expression (G-H). At the late postnatal stage, AR expression is present all over the subepithelium (G), whereas at the juvenile stage, it is restricted to the muscular layer (H). The mucous gland (arrowheads) epithelium expresses nuclear AR (H). All the cells with cilia, independently of the developmental stage, show AR-immunostained nuclei (arrows). IHC developed with DAB and counterstained with Mayers's hematoxylin. SE: subepithelium; L: lumen. Lower panel: Graphs showing changes in nuclear luminal epithelial (left) and total (nuclear and cytoplasmic) subepithelial (right) AR expressions. Different capital letters over individual bars or horizontal lines denotes differences between groups. Kruskal Wallis test followed by Dunn post-test, $p < 0.05$.

a strong expression of α-SMA in the muscle layers that form a ring around the organ was observed in the oviducts of caimans exposed to E2 1.4 ppm or BPA 140 ppm.

In controls, only few isolated cells in the periphery of the organ expressed desmin, whereas in exposed caimans, a clear desmin expression was observed. Moreover, the oviducts of caimans exposed to the higher doses of E2 and BPA exhibited a redistribution of α-SMA-positive cells towards the outer border.

3.2.3. Histofunctional score

Treatments with the higher doses of E2 and BPA (1.4 ppm and 140 ppm respectively) differentially increased the frequencies of budding, epithelial disorders, and intraepithelial PAS staining, leading to a significant increase in the score. Moreover, two animals in the BPA 140 ppm group reached the cut-off value defined to differentiate between preadenogenic and adenogenic oviducts (Fig. 4). Higher scores were achieved mainly due to a significant increase in the presence of budding (see Fig. 3S J).

3.3. Effects of early postnatal xenoestrogen exposure on expression of sex steroid hormone receptors

3.3.1. ERα protein expression

ERα expression in oviductal epithelial and stromal cells of early postnatal control caimans was high. ERα expression in the epithelial compartment increased in the BPA 1.4 ppm group, mainly due to increased labeling intensity ($p < 0.05$), whereas that in the E2 0.014 ppm group showed a trend to an increase. On the other hand, exposure to the higher doses of both E2 and BPA decreased ERα expression levels (Fig. 5). No changes in stromal cell ERα expression were observed as a consequence of xenoestrogen exposure.

3.3.2. PR protein expression

As previously reported (Galoppo et al., 2016), PR expression in oviductal luminal epithelial cells of early postnatal control caimans was high, whereas that in the subepithelium was very low. No changes were observed in xenoestrogen-exposed caimans (Fig. 5).

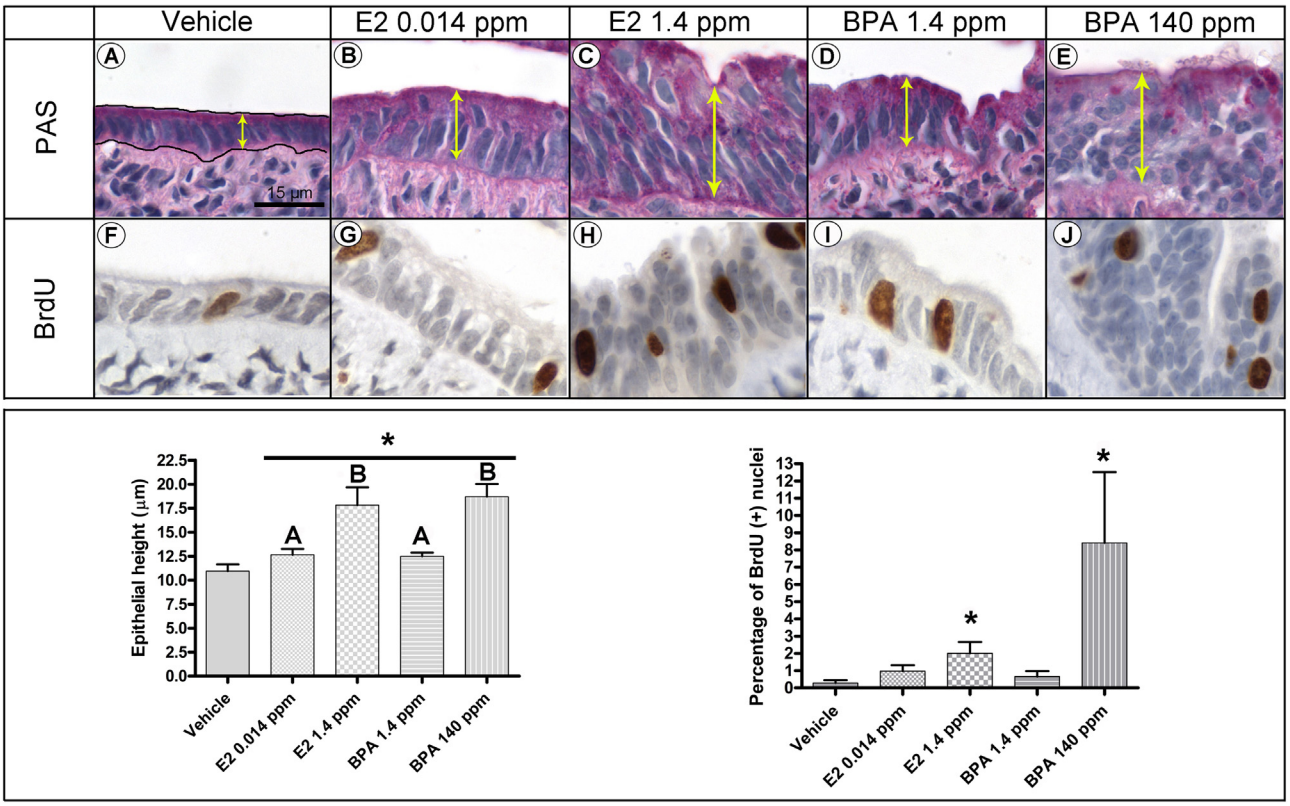

Fig. 2. Effect of xenoestrogen exposure on luminal epithelial height and proliferative activity. Upper panel: Representative photomicrographs from each experimental group showing changes in oviduct epithelial height (A-E) and in proliferative activity (F-J). A-E: PAS stain; F-J: BrdU incorporation, IHC developed with DAB and counterstained with Harris hematoxylin. Lower panel: Graphs representing quantitative changes in epithelial cell height and proliferative activity. Bars report mean ± SEM. Different capital letters over individual bars denote differences between groups treated with different doses of E2 and BPA. Asterisks over individual bars and on horizontal line denote significant differences at $p < 0.05$ between the treatment groups and the control group (vehicle). One-way ANOVA test followed by Tukey post-test.

3.3.3. AR protein expression

Early postnatal exposure to E2 or BPA up-regulated the nuclear expression of AR in the luminal epithelium. Similarly, at subepithelial level, exposure to E2 and to the lower dose of BPA up-regulated AR expression. Thus, the oviduct of early postnatal E2- or BPA-exposed caimans exhibited AR expression levels close to those found in late postnatal and juvenile control caimans. In addition, the spatial distribution pattern of subepithelial AR resembled that described for the late postnatal developmental stage (Fig. 5).

4. Discussion

Experimental exposure of early postnatal *C. latirostris* hatchlings to BPA or E2 induced changes in the oviduct histoarchitecture, biomarkers of histofunctional differentiation, and biomarkers of hormone dependence, leading to a precocious oviduct development and differentiation.

The increase in the luminal epithelial height of the FRT has been used as an endpoint of the action of endogenous estrogens and xenoestrogens in birds (Berg et al., 2001a), mammals (Carpenter et al., 2003) and reptiles (Crain et al., 1999; Doheny et al., 2016). Consistent with these results, in the present study, we observed that postnatal exposure to E2 and BPA increased the oviduct luminal epithelial height in *C. latirostris*. The luminal epithelial hypertrophy induced by postnatal exposure to estrogen-like compounds has been associated with adult impairment of the development of glands in the FRT in ewes (Carpenter et al., 2003) and prenatally exposed quails (Berg et al., 2001a). It has been proposed that alterations in cell shape and cell-cell interaction due to luminal epithelial hypertrophy could prevent invagination (Gray et al., 2001) and thus impair FRT gland development. Interestingly, in the present study, exposure to the higher doses of E2 and BPA (1.4 ppm and 140 ppm, respectively) induced an increase in the epithelial cell height not only by hypertrophy but also by increasing the number of epithelial cell layers as a consequence of increased proliferative activity. These changes could account for future impairment in oviduct gland morphogenesis.

Proper reproductive tract development depends on interactions between the epithelium and the mesenchyme. Our results show that postnatal exposure to E2 and BPA increases the subepithelial area occupied by low birefringent collagen fibers, a feature exhibited when collagen remodeling is present (Rodríguez et al., 2003). Collagen fibers can regulate developmental processes through a variety of mechanisms (reviewed by Rozario and DeSimone, 2010), which include acting as a reservoir of growth factors (Silberstein, 2001; Kanematsu et al., 2004). It is noteworthy that neonatal and early postnatal caiman oviducts, characterized by few histological signs of gland morphogenesis, show highly organized collagen bundles beneath the epithelium (Galoppo et al., 2016). Highly organized collagen fibers have been associated with budding inhibition (Wessels, 1970), probably by binding gland inhibitors as transforming-growth factor beta (TGF-β) (Daniel et al., 1989; Silberstein et al., 1992). Collagen remodeling could lead to a decrease in the levels of naturally occurring TGF-β or other inhibiting factors, thus allowing the budding process. We have previously found that during oviduct postnatal development, collagen remodeling begins at the late postnatal stage along with signs of advanced gland morphogenesis (Galoppo et al., 2016). Results presented here suggest that exposure to EDCs advances

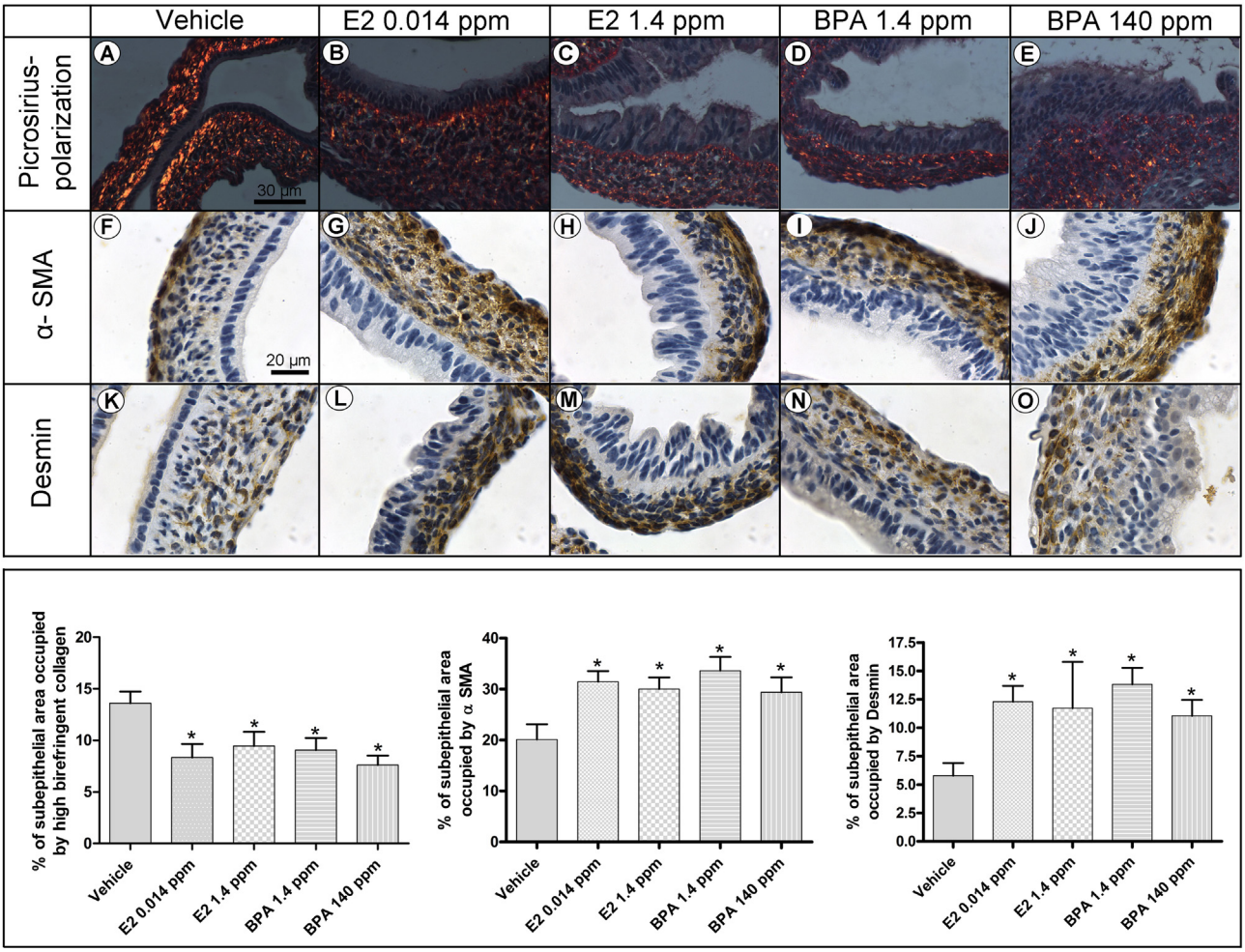

Fig. 3. Effects of xenoestrogen exposure on oviduct subepithelial histoarchitecture. Upper panel: Representative photomicrographs showing changes in collagen birefringence (A-E), α-SMA (F-J) and desmin (K-O). A-E: Picrosirius-polarization stain; F-O: IHC developed with DAB and counterstained with Mayer's hematoxylin. Lower panel: Graphs showing quantitative changes. Bars represent mean + SEM. Asterisks indicate differences between the control group (vehicle) and the treatments by Kruskal Wallis test followed by Dunn's post-test; $p < 0.05$ (collagen birefringence) or one-way ANOVA followed by Tukey post-test, $p < 0.05$ (α-SMA and desmin).

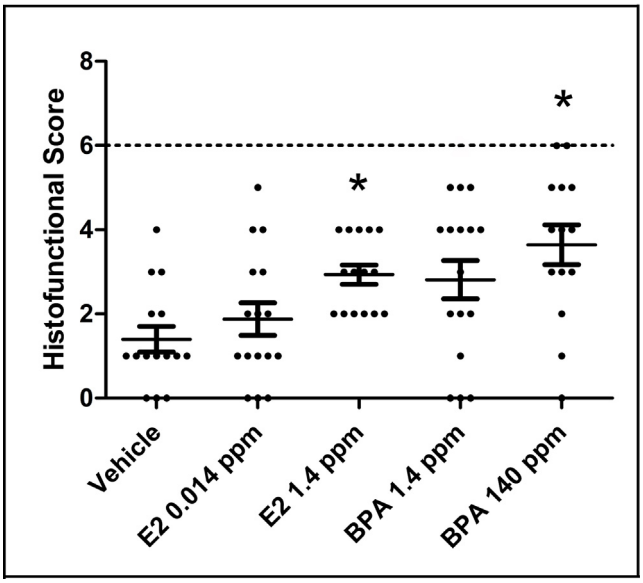

Fig. 4. Effect of xenoestrogen exposure on histofunctional score. The dashed line shows the cut-off value between preadenogenic (less than 6) and adenogenic oviducts. The asterisk denotes statistical differences ($p < 0.05$) between treated groups and the control (vehicle group). Kruskal-Wallis test followed by Dunn's post test.

subepithelial collagen remodeling, modifying the temporal pattern of changes that characterize the postnatal development of the oviduct.

Besides collagen remodeling, exposure to both E2 and BPA increased the percentage of subepithelial area occupied by α-SMA- and desmin-expressing cells, suggesting an enlargement of the muscle cell layer and confirming a pro-myogenic effect of xenoestrogen exposure (Ma and Sassoon, 2006). Since not only muscle cells but also myofibroblasts express α-SMA (Varayoud et al., 2001), both cell types could be increased in the oviductal subepithelium of exposed caimans. Besides the increased expression of α-SMA, changes in the spatial distribution of α-SMA-positive cells towards the outer border were observed, a feature displayed by prepubertal juvenile controls (Galoppo et al., 2016). BPA exposure also enhanced desmin expression at the earlier postnatal stages. As we have previously established that desmin is a marker of organization and maturity of *C. latirostris* oviductal muscle layer (Galoppo et al., 2016), this result suggests an advanced process of growth and differentiation of the muscular layer described by Gray et al. (2001) as a part of the postnatal development of the FRT.

Gland morphogenesis (or adenogenesis) is a multistep process that involves a series of coordinated histofunctional changes which lead to gland outgrowth from the luminal epithelium to the underlying stroma. We have previously established a scoring system to

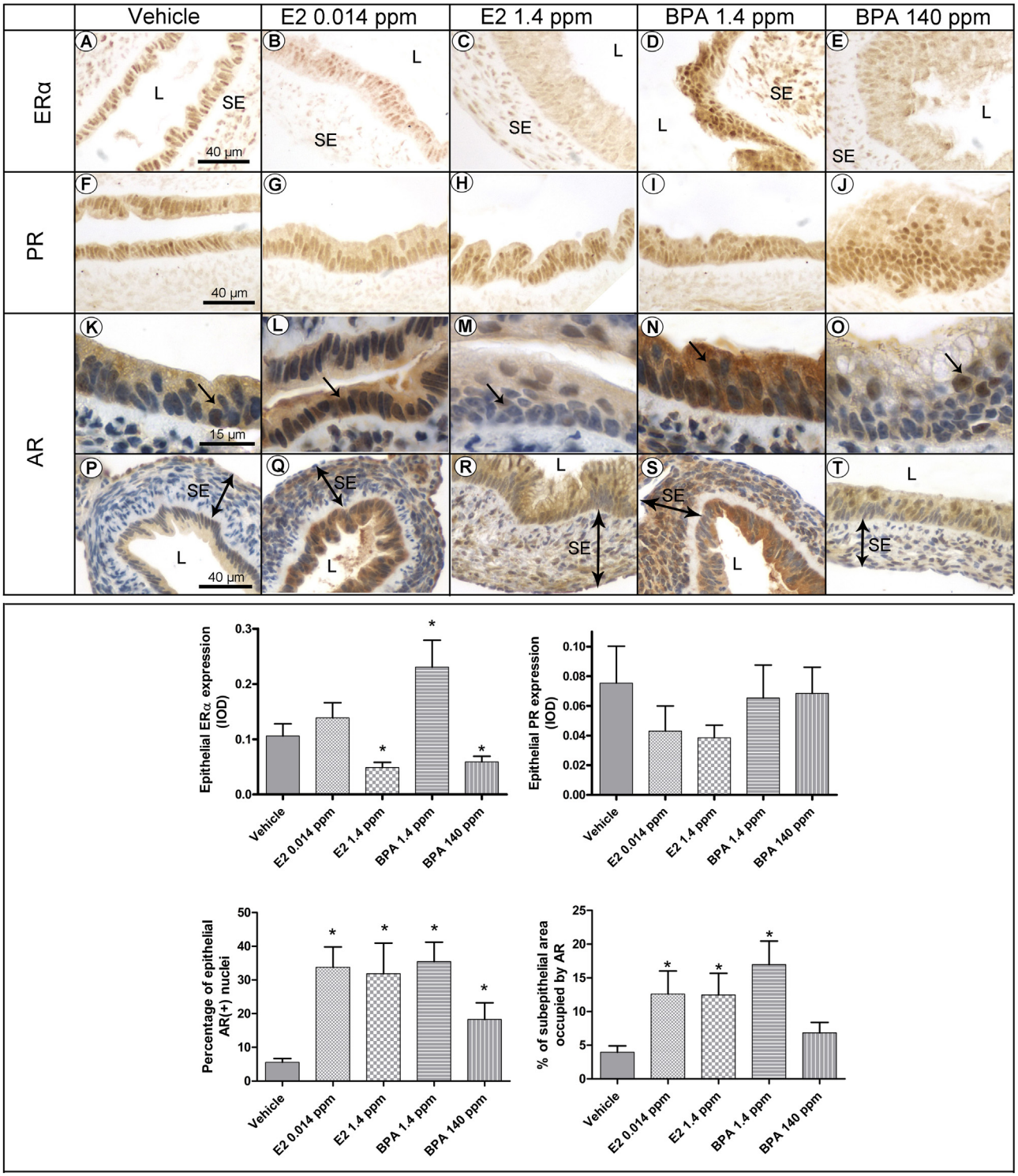

Fig. 5. Effects of xenoestrogen exposure on oviduct steroid hormone receptors. Upper panel: Representative photomicrographs of oviduct ERα, PR and AR expression. ERα and PR, IHC developed with DAB without counterstaining (A-J). AR, IHC developed with DAB and counterstained with Mayer's hematoxylin (K-T). Arrows point positive AR nuclei. SE, subepithelium; L, lumen. Lower panel: Graphs representing changes in ERα, PR and AR. Bars represent mean ± SEM. Asterisks indicate significant differences at $p < 0.05$ between the control group (vehicle) and the treatments. Significant differences were established by one-way ANOVA test followed by Tukey post-test.

distinguish between adenogenic and preadenogenic oviducts (Galoppo et al., 2016). Treatments with the higher doses of both E2 and BPA induced an increase in the histofunctional score. Indeed, in the BPA 140 ppm-exposed group, two early postnatal oviducts reached a score of 6 (cut-off value between adenogenic and pre-adenogenic oviduct), whereas in the control group, the highest score reached was 4.

The reproductive tract in female vertebrates is particularly sensitive to endocrine disruption during perinatal development (Miller et al., 2004). Since one of the pathways by which EDCs exert their action involves ERα, we assessed ERα expression in the oviductal luminal epithelium. In our model, exposures to E2 1.4 ppm and BPA 140 ppm downregulated ERα epithelial expression, resembling the response of ewes to postnatal exposure to

E2 valerate (Carpenter et al., 2003). It has been described that high concentrations of hormones cause downregulation of receptor number (Bergman et al., 2012). This could explain why exposure to the higher doses of E2 and BPA decreased ERα oviductal levels. On the other hand, exposure to BPA 1.4 ppm induced ERα expression. BPA is a xenoestrogen that has shown non-monotonic dose responses in a variety of tissues where the slope of the curve changes sign over the course of the dose-response (Bergman et al., 2012). Therefore, while low-dose BPA exposure induced ERα oviductal expression, high doses did just the opposite. The level of epithelial ERα observed in BPA 1.4 ppm-treated caimans was similar to that observed in late postnatal untreated caimans (Galoppo et al., 2016).

PR is considered an estrogen-induced protein (Schultz et al., 2003), making PR protein expression a marker of exposure to estrogen-like compounds. In avian species, this relationship between E2 and PR oviductal expression is clear (Syvälä et al., 1997). Nonetheless, in reptiles, oviductal PR regulation seems to be complex. In turtles, although high levels of circulating endogenous estrogen during ovulation increase oviductal PR expression (Gist, 2011), E2 treatment has no effect on oviductal PR levels (Giannoukos and Callard, 1996). This suggests an apparent lack of estrogenic regulation of PR in the oviduct of turtles or that other factors –possibly seasonal factors– may be important for PR regulation (Gist, 2011). The apparent lack of response of oviductal PR protein levels to E2 and BPA in *C. latirostris* seems to support this hypothesis.

Little attention has been paid to AR expression in the FRT; however, it has been suggested that androgens play a role in FRT physiology and development (Staub and De Beer, 1997). In the *C. latirostris* oviduct, the earliest developmental stages are characterized by cytoplasmic expression of AR, whereas the juvenile stage is characterized by an increase in the nuclear expression of AR. In the absence of ligand, AR is localized in the cytoplasm, whereas in the presence of ligand, it moves to the nucleus (Shao et al., 2007). Since both estrogen and androgen are able to induce AR expression in mice (Shao et al., 2007), our results suggest that *C. latirostris* endogenous androgen or estrogen circulating levels are not sufficient to induce nuclear AR expression until the juvenile stage of development. It is worth pointing out that juvenile female caimans studied both in our previous study (Galoppo et al., 2016) and here are prepubertal (SVL $\leq$ 40 cm) and thus far from sexual maturity (SVL $\geq$ 65 cm) (Portelinha et al., 2015). AR and its ligand have been related to oviduct protein synthesis and secretion in reproductive turtles (Selcer et al., 2005) and egg-shell components in hens (Kawashima et al., 1999). The increasing levels of luminal epithelial nuclear AR along with high nuclear AR expression in the gland epithelium in the oviduct of *C. latirostris* suggest that, together with ERα, AR may regulate the secretion of egg components. A novel role for AR in maintaining the viability of sperm during sperm storage has been suggested for Chinese soft-shelled turtles (Liu et al., 2016) and bats (Roy and Krishna, 2010). Androgens might play a role in sperm storage through the ciliated epithelium (Liu et al., 2016). Crocodilians also show sperm storage (Gist et al., 2008). Besides, we found that caiman ciliated cells exhibited AR-positive immunostaining. Therefore, androgen through AR could play a similar role in post-pubertal caimans. Exposure to low doses of both E2 and BPA increased nuclear expression of AR, mimicking the estrogen action described for mice (Shao et al., 2007). The levels of nuclear AR expression induced by E2 and BPA are similar to those found in juvenile caimans, suggesting an advanced developmental process induced by these compounds.

Previous results have demonstrated that *in ovo* exposure to BPA causes estrogen-like developmental effects by reversing caiman gonadal sex and altering gonadal histoarchitecture (Stoker et al., 2003). Differences in responses to BPA and E2 in our *in vivo* system were in the order of 100-fold. In contrast, published *in vitro* studies have reported differences in the order of 10,000 or more (Ruan et al., 2015; Song et al., 2006). The present results confirm that the relative estrogenic potency of BPA in neonatally exposed caimans is also significantly higher than expected, according to *in vitro* bioassays. This is especially significant in terms of the impact that environmental BPA could have on the development and reproduction of *C. latirostris* and perhaps on other wildlife species (Muñoz-de-Toro, 2015). Although in the present study BPA treatment mimicked the effects of E2 in terms of oviduct development and differentiation disruption, we could not rule out the possibility of an anti-androgenic effect of BPA (Sohoni and Sumpter, 1998). As previously demonstrated in rodent uterine biomarkers (Varayoud et al., 2008; Bosquiazzo et al., 2010, 2013; Vigezzi et al., 2015), exposure to EDCs at critical stages of development affects the temporal-spatial pattern of caiman oviduct biomarkers, suggesting organizational changes that could alter *C. latirostris* reproductive health later in life. Many questions remain to be answered about possible species differences in terms of sensitivity to environmental estrogens and their long-term effects. However, the alterations reported here in the caiman oviduct exposed to BPA and those previously reported (Stoker et al., 2003, 2008, Durando et al., 2013, 2016, Rey et al., 2009) highlight the importance of preserving aquatic environments from plastic pollution.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ygcen.2017.09.021.

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