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The external genitalia in juvenile *Caiman latirostris* differ in hormone sex determinate-female from temperature sex determinate-female

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ABSTRACT

The broad-snouted caiman (*Caiman latirostris*) is a crocodilian species that inhabits South American wetlands. As in all other crocodilians, the egg incubation temperature during a critical thermo-sensitive window (TSW) determines the sex of the hatchlings, a phenomenon known as temperature-dependent sex determination (TSD). In *C. latirostris*, we have shown that administration of 17-β-estradiol (E_2) during the TSW overrides the effect of the male-producing temperature, producing phenotypic females (E_2SD-females). Moreover, the administration of E_2 during TSW has been proposed as an alternative way to improve the recovery of endangered reptile species, by skewing the population sex ratio to one that favors females. However, the ovaries of E_2SD-female caimans differ from those of TSD-females. In crocodilians, the external genitalia (i.e. clitero-penis structure or phallus) are sexually dimorphic and hormone-sensitive. Despite some morphological descriptions aimed to facilitate sexing, we found no available data on the *C. latirostris* phallus histoarchitecture or hormone dependence. Thus, the aims of this study were: (1) to establish the temporal growth pattern of the phallus in male and female caimans; (2) to evaluate histo-morphological features and the expression of estrogen receptor alpha (ERα) and androgen receptor (AR) in the phallus of male and female pre-pubertal juvenile caimans; and (3) to determine whether the phallus of TSD-females differs from the phallus of E_2SD-females. Our results demonstrated sexually dimorphic differences in the size and growth dynamics of the caiman external genitalia, similarities in the shape and spatial distribution of general histo-morphological compartments, and sexually dimorphic differences in innervation, smooth muscle fiber distribution, collagen organization, and ERα and AR expressions. The external genitalia of E_2SD-females differed from that of TSD-females in many histological features and in the expression of ERα and AR, resembling patterns described in males. Our results alert on the effects of estrogen agonist exposure during TSW and suggest that caution must be taken regarding the use of E_2SD as a procedure for wildlife population management.

1. Introduction

The sex of the offspring of all crocodilian and many turtle species is determined by the environment (Warner, 2011). The incubation temperature of the eggs during the thermo-sensitive window (TSW), a critical window of embryo development, is the main factor that

Abbreviations: ANOVA, analysis of variance; AR, androgen receptor, ATZ, atrazine; BM, body mass; DAB, diaminobenzidine; DDT, dichlorodiphenyltrichloroethane; E_2, 17β-estradiol; E_2SD, estrogen-induced sex determination; EDCs, endocrine-disrupting compounds; END, endosulfan; ERα, estrogen receptor alpha; H&E, hematoxylin and eosin; IHC, immunohistochemistry; PAS, periodic acid Schiff; PBS, phosphate buffered saline; PicH, picrosirius counterstained with Harris hematoxylin for polarized light; RIA, radioimmunoassay; r_S, spearman correlation coefficient; α-SMA, smooth muscle alpha actin; SVL, snout-vent length; T, testosterone; TL, total length; TSD, temperature-dependent sex determination; TSW, thermo-sensitive window; UV, ultraviolet

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determines the sex of the progeny. This process is known as temperature-dependent sex determination (TSD) (Gilbert, 2000; Lang and Andrews, 1994). Estrogens also play a critical role in the sex differentiation of crocodilians and turtles. The administration of 17β-estradiol (E_2) during the TSW overrides the effects of the male incubation temperature, producing phenotypic females (Crain et al., 1997; Crews et al., 1996; Milnes et al., 2002; Stoker et al., 2003). This effect has been defined as sex reversal or estrogen-induced sex determination (E_2SD) (Crews et al., 1991; Holleley et al., 2016; Tousignant and Crews, 1994; Wibbels et al., 1991, 1992). Moreover, the administration of E_2 during the TSW has been proposed as an alternative way to improve the recovery of endangered reptile species, by skewing the population sex ratio to one that favors reproductive females (Crews and Wibbels, 1993a,b).

Caiman latirostris (broad-snouted caiman) is a species widely distributed in wetlands and rivers of South America. This species is a crocodilian with TSD, in which incubation at 30 °C produces 100% female hatchlings, while incubation at 33 °C produces 100% male offspring (Stoker et al., 2003). Estrogens also influence caiman sex determination: eggs incubated at 33 °C (male-producing temperature) but exposed to E_2 produce 100% phenotypic female offspring (E_2SD) (Stoker et al., 2003; Beldoménico et al., 2007). However, previous results have shown that E_2SD-female caimans differ from TSD-females in that the ovaries of 10-day-old E_2SD-females show altered follicle dynamics, lacking type III follicles (Stoker et al., 2003, 2008) and in the fact that, at the juvenile stage, E_2SD-females exhibit higher incidence of multi-oocyte follicles than TSD-females (Stoker et al., 2008). Besides, in E_2SD-female caimans, the gonad mRNA expression of *sox9*, a gene associated with testis development, remains at male levels, differing from the levels observed in TSD-females (Durando et al., 2013). It has been recently reported that both TSD- and E_2SD-*C. latirostris* female embryos have morphologically identified ovaries, but that these ovaries exhibit different expression patterns of molecules associated with ovarian development and function (Canesini et al., 2018).

In crocodilians, the external genitalia (i.e. clitero-penis or phallus) are composed of an unpaired organ that, due to its differences in size and color, is considered a sexually dimorphic structure. In males, the phallus serves functions of intromission and insemination into the female cloaca during copulation. Despite some morphological descriptions aimed to facilitate sexing (Allsteadt and Lang, 1995; Nuñez-Otaño et al., 2010), we found no data on the female crocodilian phallus histoarchitecture or data related to its functions.

In alligators, the size of the phallus is androgen-dependent (Gunderson et al., 2004). This dependence has been used to study environmental pollution and the anti-androgenic properties of different substances (Milnes et al., 2005). Moreover, exposure to androgens may induce alterations in penis development in females, as occurs in other vertebrate species (Orlando and Guillette, 2007). It has also been demonstrated that estrogens and estrogen-like pollutants, such as Dichlorodiphenyltrichloroethane (DDT), can effectively reduce the size of the phallus of juvenile males of *Alligator mississippiensis* (Guillette et al., 1996, 1999). Therefore, the development of the crocodilian phallus is largely dependent on gene regulatory controls, which in turn, are targets of hormone signaling (Gredler et al., 2014; Miyagawa et al., 2009). Alterations in the levels of endogenous hormones or exposure to endocrine-disrupting compounds (EDCs) can lead to subtle or gross irreversible organizational effects on the crocodilian reproductive system (Guillette et al., 1999; Stoker et al., 2003, 2008, 2011; Orlando and Guillette, 2007; Rey et al., 2009; Durando et al., 2013, 2016; Galoppo et al., 2016, 2017).

The aims of this study were: (1) to establish the temporal growth pattern of the phallus in male and female caimans; (2) to evaluate histomorphological features and the expression of estrogen receptor alpha (ERα) and androgen receptor (AR) in the phallus of males and female pre-pubertal juvenile caimans; and (3) to determine whether the phallus of females differs from the phallus of sex reversed pre-pubertal juvenile female caimans (E_2SD-females).

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 (American Society of Ichthyologists and Herpetologists, 2004) and in full compliance with the Institutional Committee of Bioethics in Animal Care and Use of the Universidad Nacional del Litoral, Santa Fe, Argentina.

C. latirostris eggs were collected shortly after oviposition from seven nests (mean clutch size: 34; range: 25–44 eggs per nest; n = 238) randomly selected from a region located in a protected area (Natural Reserve "*El Cachapé*") in the Chaco Province, Argentina. This region is characterized by low anthropogenic intervention. It is situated far upstream of urbanized, industrial and farming areas, which minimizes the exposure to sewage or agriculture and/or feedlot run-off. To establish the developmental stage of the embryos, one egg from each clutch was opened in the field. Since *C. latirostris* TSD takes place after embryo developmental stage 20 (Stoker et al., 2003) and, in order to warrant recent oviposition, only the clutches with embryos at stages lower 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 transfer and incubation. Eggs were transported to the laboratory and distributed into two groups such that half of the eggs from one clutch were incubated at a constant temperature of 30 °C (female-producing temperature) and the other half at a constant temperature of 33 °C (male-producing temperature) (Beldoménico et al., 2007; Stoker et al., 2003). Opaque eggshell banding development was used to confirm embryo viability (Stoker et al., 2003). Temperature was monitored by HOBO temperature loggers (Onset Computer, Pocasset, MA, USA) and by daily recording of incubator electronic thermometer readings. Eggs were maintained at approximately 90% humidity. To minimize clutch effect, eggs from each clutch were distributed in different experimental groups. Each group had a maximum of two siblings. Forty-four caimans were included in this study whereas the others were assigned to the experiments of Canesini et al. (2018). According to the embryo stage of development when collected, and based on our experience on embryo developmental dynamics at each incubation temperature, one egg from each clutch was opened to verify that embryos had reached developmental stage 20, and treatments were applied topically as previously described (Stoker et al., 2003). Eggs incubated at 30 °C (female-producing temperature) received 50 μL absolute ethanol (Control females) whereas eggs incubated at 33 °C (male-producing temperature) received either 50 μL of absolute ethanol (Control males) or 1.4 ppm E_2 (Sigma, St. Louis, USA) dissolved in 50 μL of absolute ethanol (E_2 SD-females).

At hatching, all hatchlings were individually marked using two numbered tags (National Band and Tag Co., Newport, KY, USA) and then held in housing facilities under environmental and sanitary –controlled conditions. Air and water thermal ranges (28.0 ± 2.0 °C and 26.0 ± 2.0 °C, respectively), humidity (60–65%), dark-light cycles (lights on from 06:00 to 20:00 h), UV light pulses (15 min per day), air renewal (every 15 min), water renewal (daily) and caiman stocking density were controlled. Caimans were fed three times a week with premium low fat ground beef (FRIAR S.A., Santa Fe, Argentina) supplemented with vitamins (TetraFauna, Blacksburg, USA), calcium (Laboratorios Cicarelli, Santa Fe, Argentina) and phosphorus (Laboratorios Cicarelli, Santa Fe, Argentina). Housing facilities and animal feeding have previously been described in detail (Zayas et al., 2011; Durando et al., 2016). The biometric parameters body mass (BM), total length (TL) and snout-vent length (SVL) were recorded monthly until animals were euthanized (at late post-hatching or at the

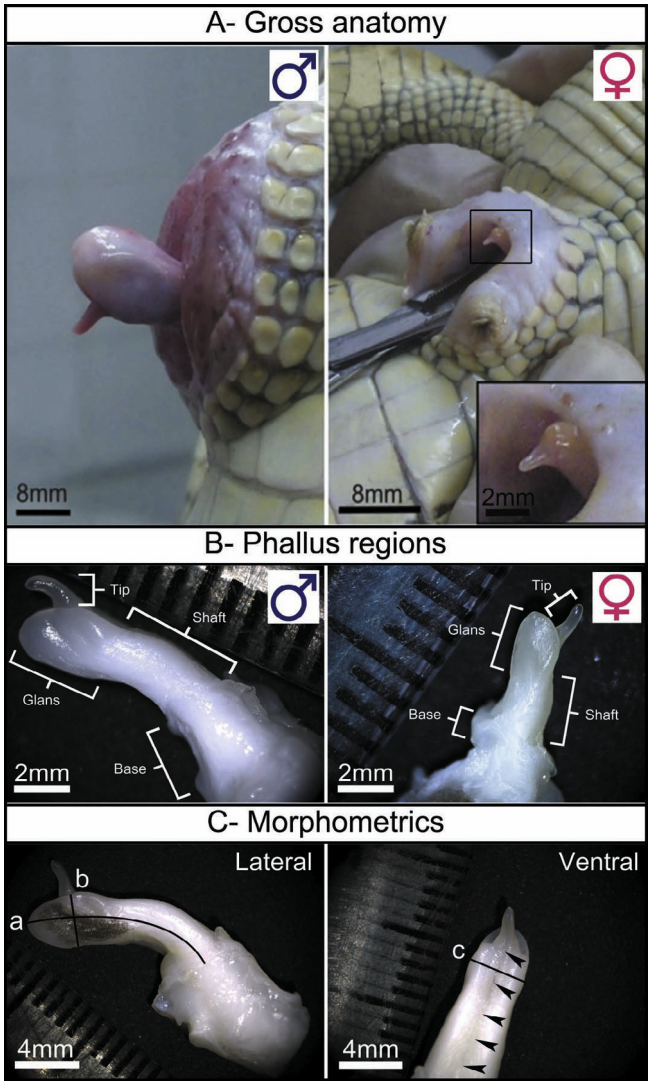


Fig. 1. Gross anatomy and morphometric parameters of the phallus of *Caiman latirostris* (broad-snouted caiman). A. Representative photographs of the male and female phallus (Modified from Zayas, 2013). B. Phallus macroscopic regions. C. Morphometric measures: a. length of the phallus, b. glans height, c. glans width. Arrowheads: sulcus. The samples analyzed came from pre-pubertal juvenile *C. latirostris* that did not differ in their biometric parameters.

pre-pubertal juvenile stage).

2.2. Sample processing

At necropsy, the phallus was manually extruded, dissected and immediately fixed in 10% phosphate-buffered formalin (pH 7.4) for 6 h at room temperature. Fixed tissues were stored in 70% ethanol until morphometric assessment (Section 2.3) and then dehydrated, cleared in xylene (Biopack, Buenos Aires, Argentina), and embedded in paraffin (Biopack, Buenos Aires, Argentina).

2.3. Phallus gross anatomy and assessment of growth dynamics

The gross anatomy of the phallus was described by observing the phalli extruded from juvenile caimans before dissection (Fig. 1) and using formaldehyde-fixed phalli stored in 70% ethanol by simple observation and using a binocular stereomicroscope (Carl Zeiss; Oberkochen, Germany). Based on data published by Moore and Kelly (2015), the phallus structure was anatomically divided into three macroscopic sections: base, shaft and glans (Fig. 1).

2.4. Phallus histoarchitecture

Serial transverse sections (5 µm) of each region (base, shaft and glans) were stained with Hematoxylin and Eosin (H&E) (Biopur, Rosario, Argentina), Periodic Acid Schiff (PAS) (Biopur, Rosario, Argentina), Sirius Red (Direct Red 80, Sigma-Aldrich, Argentina) in picric acid solution (picrosirius), and counterstained with Harris hematoxylin (Picrosirius, PicH) for polarized light microscopy (Lattouf et al., 2014; Galoppo et al., 2016) to establish phallus histoarchitecture and morphometrics. Peripheral nerves were identified on PAS-stained sections (Geneser, 1999), lymphocyte aggregates were identified on H&E-stained sections, and the epithelial height of the sulcus groove was evaluated in H&E-stained histological sections.

2.5. Immunohistochemistry

To identify the muscle cell immunophenotype and to better establish the distribution and organization of muscle fibers, the expression of smooth muscle alpha actin (α-SMA) and desmin was evaluated by immunohistochemistry (IHC). The expression pattern of AR and ERα was assessed by IHC as well. Briefly, to ensure antigen integrity, tissues were immediately fixed upon removal. The fixative, fixation times and paraffin embedding technique are detailed in Section 2.2. To avoid protein deterioration, tissue sections were stored for a maximum of 30 days in a desiccator at 4 °C. Sections were hydrated through a series of alcohols, microwaved in 10 mM citrate buffer (pH 6) for antigen retrieval, and treated to prevent endogenous peroxidase and nonspecific binding 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 diaminobenzidine (DAB) (Sigma–Aldrich, Buenos Aires, Argentina) as a chromogen substrate. Sections were lightly counterstained with Mayer 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 by the antibody-antigen complex (pre-adsorbed antibody). The specificity of ERα and AR antibodies has been previously tested by Western blot. An oviductal protein extract from a juvenile pre-pubertal female *C. latirostris* was used for anti-ERα (LETH-ER-202y) validation, whereas a testicular protein extract from a juvenile pre-pubertal male *C. latirostris* was used for anti-AR (LETH-AR-280y) validation (Varayoud et al., 2012; Galoppo et al., 2017, respectively).

2.6. Testosterone assay

Serum levels of testosterone (T) were determined by radioimmunoassay (RIA) using T, [1,2,6,7-3H (N)] (PerkinElmer Life And Analytical Sciences, Inc., Boston, MA, USA) and a specific antibody provided by Dr. G.D. Niswender. Steroids were extracted from 200 µL of serum with 2 ml of ethyl ether (Merck, Buenos Aires, Argentina). The extraction procedure was repeated three times. The percent recovery of extraction was calculated by the addition of a fixed amount of tracer to dextran coated charcoal stripped caiman serum and was 92%. The detection limit was 13 pg/ml. Quality control standards consisting of a pool of caiman serum and pooled human serum of known hormone concentrations were run in every assay. The interassay coefficient of variation was 6.79%.

2.7. Image and data analysis

2.7.1. Macroscopic morphometrics

Images were recorded using a Spot Insight V3.5 color video camera

(Diagnostic Instruments, USA) attached to the stereomicroscope Stemi 305 (ZEISS, Argentina). Image J software (NIH, U.S.A.; https://imagej.nih.gov/ij) was used to obtain phallus morphometrics. Phallus morphometric measures, including length, glans height (lateral glans width) and glans width (ventral glans width) were recorded (Fig. 1). Phallus morphometric measures and caiman biometric parameters were correlated to evaluate phallus growth dynamics. To this aim, data from both pre-pubertal juvenile and late post-hatching caimans were plotted.

2.7.2. Histoarchitecture

Images were recorded using a SPOT color video camera (Diagnostic Instruments Inc., USA) attached to an Olympus BH2 microscope (Olympus Optical, Tokyo, Japan). Images were analyzed using the Image Pro-Plus 4.1.0.1 system (Media Cybernetics, Silver Spring, MD, USA). The presence of nerves and lymphocyte aggregates was assessed using the image analysis software. The areas occupied by peripheral nerves or lymphocyte aggregates and the total transversal area were manually delimited and automatically calculated. Results are expressed as the percentage of total transversal area occupied by peripheral nerve bundles or by lymphocyte aggregates and as mean ± SEM of nerve bundle transverse area and number of nerve bundles.

2.7.3. Height of the sulcus groove epithelium

Firstly, to define where the sulcus groove epithelium begins, we established the distance from which the external epithelium turns into sulcus groove epithelium. This was done on digitalized images of H&E-stained shaft histological sections by using the Image Pro-Plus 4.1.0.1 system. After measuring samples from all groups (two stained histological sections from each individual), the following mean values were obtained: 350 μm for males and 140 μm for females. Then, the basal and apical edges of the sulcus groove epithelium were manually delimited and the mean epithelial height was automatically calculated. A representative image is shown in Supplementary material (Fig. S1).

2.7.4. Immunohistochemistry

The staining patterns of desmin, α-SMA, and steroid receptors were qualitatively described in all tissue compartments. Based on the histo-functional properties and potential hormone dependence of the epithelia of both the sulcus (protection, secretion, transport) and the cavities (secretion and protection), percentage of AR and ERα immunostained nuclei were quantified in these epithelia. Direct counting of immunostained and negative nuclei was performed using a Dplan 100X objective. Double-blind counting of the percentage of positive cells and intensity score were used. Blood vessels were quantified on α-SMA stained samples of all phallus regions by direct counting using a Dplan 40X objective integrated with a 10x10 reticle grid. Results were reported as percentage of relative area occupied by blood vessels.

The data are reported as the mean ± SEM. To achieve normality, the data were log-transformed (log_{10}). For grouped analysis, ANOVA or Kruskal-Wallis was performed to obtain the overall significance, followed by Bonferroni's or Dunn's as post hoc-test, respectively. $P < 0.05$ was accepted as significant. Spearman correlation coefficient (r_S) was used to establish the correlation between phallus morphometrics and caiman biometric parameters. Statistical differences between slopes were established (F and p values).

3. Results

3.1. Gross anatomy and growth dynamics of the phallus in males and females

The age and biometric parameters of the *C. latirostris* stages studied are summarized in Table 1. As shown in Fig. 1, in spite of size differences, the gross anatomy of the phallus of pre-pubertal juvenile male and female caimans is quite similar. The phallus is located in the ventral wall of the cloaca, and, based on gross anatomy; it can be divided into three regions: base, shaft and glans (Fig. 1B). At the ventral side of the phallus, a medial groove, called sulcus, runs along the shaft and the glans (Fig. 1C right panel). The distal end of the phallus consists of a bulbous glans, which exhibits a semicircular cavity with a tip that protrudes like a finger or pyramidal structure.

Phallus morphometric parameters changed as a function of age in both males and females, and these growth dynamics showed no differences between sexes at the earlier developmental stages. The phallus of males and females was undistinguishable with the naked eyes, before animals attained a TL near 50 cm. During the study period, the male phallus exhibited a sustained increase in size along with animal growth, whereas the female phallus showed little to no changes associated with caiman growth. Differences between curve slopes reflected different phallus growth patterns between male and female pre-pubertal juvenile caimans (Fig. 2). Testosterone levels explain, at least in part, these similarities and differences (Table 3).

3.2. Phallus histoarchitecture

As shown in Fig. 3, despite phallus region and some region-associated characteristics, the male and female caiman phallus is comprised of three major compartments. Based on homology with mammalian external genitalia, these compartments were called external epithelium, corpus spongiosum and corpus cavernosum.

The different types of epithelia observed in the external epithelium are illustrated in Figs. 4 and 5. The main epithelial type is stratified squamous epithelium (Fig. 5A). The flat surface of covering epithelium is interrupted by convolutions and invaginations, while, in the basal layers, finger-like columns of epithelial cells are observed (Fig. 4A and C). The epithelial surface also presents dome-like cells, which are neither squamous nor columnar. These cells allow distention and are characteristic of transitional epithelium (Fig. 4D). Besides, the external stratified squamous epithelium and the transitional epithelium, a ciliated pseudo-stratified epithelium is present (Fig. 5C). The sulcus groove is lined by a stratified epithelium, which progressively changes from stratified to ciliated, pseudo-stratified and cylindrical, in the deep sulcus. The cavity epithelium is stratified as the external epithelium; however, it lacks invaginations or projections. As shown in Fig. 5, PAS staining complemented the epithelial characterization and revealed histo-functional features. In the shaft region of the phallus, the height of the sulcus groove epithelium was significantly higher in females than in males [(36.98 μm ± 2.25 (n: 10) and 27.14 μm ± 1.35 (n: 13), respectively $p < 0.005$]).

Although not comprising a major component of the basic phallus histoarchitecture, lymphocyte aggregates can be observed as rounded structures comprised of small, highly basophilic, mononuclear cells frequently associated with epithelial tissues (Fig. 4B). The area occupied by lymphocyte aggregates showed no differences between sexes at the regions evaluated [0.74 ± 0.30 (n: 9) *vs.* 0.51 ± 0.32 (n: 7), males and females respectively, p: 0.3695 at the shaft region]; [0.17 ± 0.07 (n: 9) *vs.* 0.23 ± 0.25 (n: 7), males and females respectively p: 0.7012 at the glans region].

At the most proximal part of the base, the male phallus also presents a pair of ducts, identified as ductus deferens. In the medial part of the base, these ductus deferens are surrounded by the deep sulcus, and, in the most distal part of the base, they fuse with the sulcus to form a single duct (Fig. 6, upper panel). In post-pubertal caimans, semen flows from the ductus deferens into the sulcus (Fig. 2S). The ductus deferens has a pseudo-stratified ciliated epithelium and a star-shaped lumen surrounded by a layer of smooth muscle fibers. PAS-positive substances can be observed in the ductus epithelia. In the female phallus, the ductus deferens is absent, and thus, only the deep sulcus is observed (Fig. 6, lower panel).

From the shaft to the glans, the sulcus groove is surrounded by a pair of fibrous bodies, called the corpus spongiosum. In the male phallus, expression of α-SMA and desmin revealed that muscle bundles

Table 1
Primary antibodies used for IHC.

Antibody	Animal source	Supplier	Specificity
Anti-Smooth muscle α-actin (α-SMA clone 1)	Monoclonal mouse	Novocastra (Newcastle upon Tyne, UK)	Rey et al. (2009) Galoppo et al. (2016)
Anti-Desmin (Clone DE-R-11)	Monoclonal mouse	Novocastra (Newcastle upon Tyne, UK)	Rey et al. (2009) Durando et al. (2016)
Anti-AR (LETH-AR 280Y)	Polyclonal rabbit	ISAL, Santa Fe, Argentina	Galoppo et al. (2017)
Anti-ER (LETH-ER 202Y)	Polyclonal rabbit	ISAL, Santa Fe, Argentina	Varayoud et al. (2012) Durando et al. (2016)

in the corpus spongiosum run parallel to the sulcus groove, while, in the female phallus, the spatial distribution of α-SMA- and desmin-positive cells looks less organized and the immunostaining intensity is weaker than in males (Fig. 7, upper panel). The corpus spongiosum exhibited not only smooth muscle bundles, but, as it is highly vascularized, α-SMA and desmin-expressing smooth muscle cells were observed on the blood vessel walls. α-SMA expression in the vascular wall was more intense than desmin expression (Fig. 7, lower panel). We found no differences in the vasculature of the phallus between male and female caimans [4.82 ± 0.36 (n: 9) *vs.* 4.95 ± 0.11 (n: 7), males and females respectively, p: 0.8121 at the base]; [4.53 ± 0.61 (n: 9) *vs.* 4.39 ± 0.58 (n: 8), males and females respectively, p: 0.8048 at the shaft]; [5.01 ± 0.64 (n: 9) *vs.* 7.11 ± 0.39 in the glans (n: 9), males and females respectively, p: 0.1892 at the glans]. Few collagen fibers were present among the muscle fibers, as revealed by the PicH stain when observed under polarized light (Fig. 8). Transversally, most of the organ internal volume is comprised of a large area rich in collagen fibers called the corpus cavernosum. In PicH-stained sections observed under polarized light, highly birefringent and densely packed collagen fiber bundles are observed in the male phallus, in contrast with the poorly birefringent and loosely packed bundles present in the female phallus (Fig. 8).

At the glans region, the corpus spongiosum becomes less muscular and spreads toward the corpus cavernosum, which remains as a collagen-rich region surrounding the glans cavity. Empty structures, similar to large blood vessels, but lacking either defined borders or α-SMA- or desmin-positive walls, were found in the proximities of the remaining corpus cavernosum. Such structures were called caverns.

As illustrated in Fig. 9C, the female phallus has significantly higher percentages of areas occupied by nerve bundles than the male phallus. These differences were mainly due to a higher number of structures in the female phallus (Fig. 9A and B).

3.3. Protein expression of hormone receptors

AR and ERα were widely expressed among different tissue compartments of the phallus. Cytoplasmic expression was observed in muscle bundles, sulcus epithelium, cavity epithelium and invaginations of the external epithelium. Nuclear expression was restricted to the sulcus and cavity epithelia in both males and females. In males, AR and ERα were also expressed in the epithelium of the ductus deferens. The cytoplasmic and nuclear expressions of ERα and AR were higher in the male than in the female phallus. Results are shown in Fig. 10.

3.4. E_2SD-female phallus

Gross anatomy and phallus morphometrics of the clitoris showed no differences between TSD- and E_2SD-females (Table 4).

At the pre-pubertal juvenile stage, the phallus size of E_2SD-females was similar to that of TSD-females. Despite, the slope was steeper in E_2SD- than in TSD-females and the correlation between phallus parameters and caiman biometrics was better in E_2SD- than in TSD-females; the phallus growth dynamics did not differ (Fig. 11).

Besides similarities in their gross anatomy and size, at histoarchitecture level, the phallus of E_2SD-females looks quite similar to that of TSD-females. The phallus of both E_2SD- and TSD-females lacks the ductus deferens, a paired structure that characterizes the base of the male phallus. Regarding the epithelial height of the sulcus groove, sexually dimorphic differences remained and no differences were observed between TSD- and E_2SD-females [36.98 ± 2.25 μm (n: 10) *vs.* 44.89 ± 4.79 μm (n: 7)]. However, subtle but significant differences were observed. The presence of blood vessels was higher in E_2SD-

Table 2
Biometrics and phallus morphometrics of *Caiman latirostris* males and females.

		Late post-hatching		Pre-pubertal Juvenile	
	Parameters	Males n = 4	Females n = 4	Males n = 13	Females n = 10
	Age (months)	3.00 ± 0.00	3.00 ± 0.00	14.79 ± 0.64	13.06 ± 0.72
Caiman biometrics	Total length (cm)	61.00 ± 2.04	57.20 ± 2.24	81.72 ± 1.93	79.19 ± 1.99
	Snout-vent length (cm)	31.93 ± 0.99	27.78 ± 1.11	39.33 ± 0.88	38.00 ± 0.99
	Body mass (g)	869.30 ± 99.82	612.50 ± 85.09	2132.10 ± 245.73	1999.00 ± 184.90
Phallus morphometrics	Length (mm)	11.58 ± 0.91	5.72 ± 0.53*	22.82 ± 2.65	6.59 ± 0.43**
	Glans height (mm)	3.85 ± 0.32	2.14 ± 0.22*	6.18 ± 0.67	2.28 ± 0.13**
	Glans width (mm)	3.73 ± 0.23	1.83 ± 0.10*	6.04 ± 0.68	2.42 ± 0.14**

Males and females were TSD. Results are expressed as Mean ± SEM. *Indicates significant differences at $p < 0.05$ and **indicates significant differences at $p < 0.0005$ by Mann Whitney *U* test.

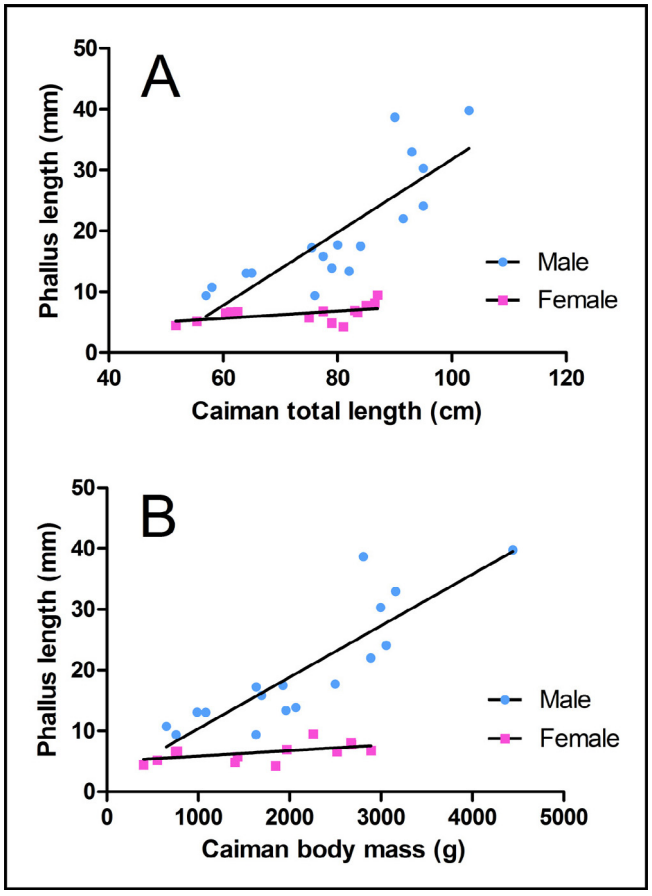


Fig. 2. Phallus growth dynamics in *C. latirostris* males and females. A. Correlation between phallus length and total length (TL) (r^S = 0.8964 and r^S = 0.6440, male and female respectively), B. Correlation between phallus length and body mass (BM) (r^S = 0.9118 and r^S = 0.6403, male and female respectively). The slopes significantly differed between males and females in the phallus length *versus* TL (F = 17.32; p = 0.0003), and in the phallus length *versus* BM (F = 19.97; p = 0.0001). BM < 1000 g or TL < 65 cm, caimans at the late post-hatching stage.

females than in TSD-females [8.22 ± 0.54 (n: 6) *vs.* 4.95 ± 0.11 (n: 7) E_2SD-females and TSD-females respectively, p: 0.0098 at the base]; [8.30 ± 0.77 (n: 7) *vs.* 4.39 ± 0.58 (n: 8) E_2SD-females and TSD-females respectively, p: 0.0012 at the shaft]; [11.35 ± 1.73 (n: 8) *vs.* 7.11 ± 0.39 (n: 9) E_2SD-females and TSD-females respectively, p: 0.0434 at the glans]. Both TSD- and E_2SD-females exhibited a non-defined radial muscle fiber orientation, whereas, like males, not only abundant muscle fibers but also muscle fibers reaching the deep sulcus were observed (Fig. 7, upper panel). The peripheral innervation of the phallus was significantly lower in E_2SD-females than in TSD-females, and these innervations in E_2SD-females were similar to those of males (Fig. 9). Therefore, the sexually dimorphic pattern exhibited by the peripheral innervation is lost in the E_2SD-female phallus.

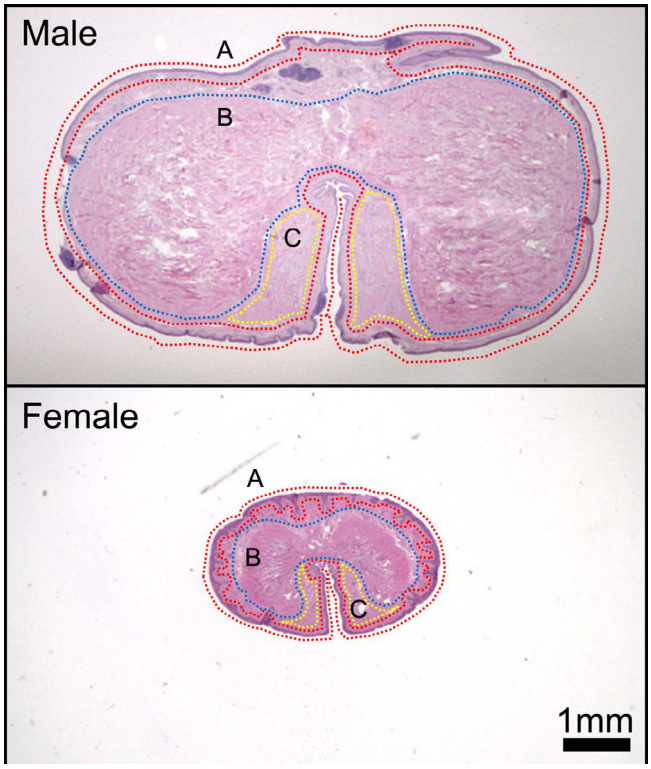


Fig. 3. Histological compartments of the *C. latirostris* phallus. Representative transverse sections of the phallus shaft from *C. latirostris* pre-pubertal juvenile males and females. Dotted lines surround each compartment. Red line: external epithelium (A). Blue line: corpus cavernosum (B). Yellow line: corpus spongiosum (C). H&E stained sections.

The spatial distribution of AR- or ERα-positive cells in the E_2SD-female phallus was similar to that in the TSD-female phallus. However, both the percentage of cells expressing AR and ERα and the immunostaining intensity were higher in the E_2SD-female phallus than in the TSD-female phallus. Percentage of positive immunostained epithelial cells for sexual steroid receptors in the E_2SD-female phallus did not differ from those in the male phallus (Fig. 10B), and once again, the sexually dimorphism was lost.

4. Discussion

In the present study, we demonstrated sexually dimorphic differences in the size and growth dynamics of the external genitalia of *C. latirostris*. Our results showed similarities between males and females in the shape and spatial distribution of general histo-morphological compartments but sexually dimorphic differences in innervation, smooth muscle fiber distribution, collagen organization, and ERα and AR protein expressions. The external genitalia of E_2SD-females differed from those of TSD-females in many histological features and in the expression of ERα and AR, resembling the patterns described in males.

Table 3
Testosterone circulating levels in *C. latirostris* males and females.

	TSD-Males	TSD-Females	E_2SD-Females	References
Early post-hatching	–	66.3 ± 13.1** n = 8	60.9 ± 17.5 n = 11	Stoker et al. (2008)
	165.8 ± 20.5* n = 12	–	–	Rey et al. (2009)
Late post-hatching	–	167.6 ± 4.7 n = 7	152.1 ± 11.0 n = 7	Stoker et al. (2008)
	326.1 ± 86.8 n = 4	202.9 ± 79.7 n = 4	175.6 ± 19.2 n = 4	This manuscript
Pre-pubertal Juvenile	1138.0 ± 192.4 n = 7	251 ± 103.4 n = 7	714.3 ± 234.9 n = 7	This manuscript

* Testosterone levels are reported as pg/ml.
** Results are expressed as mean ± SEM.

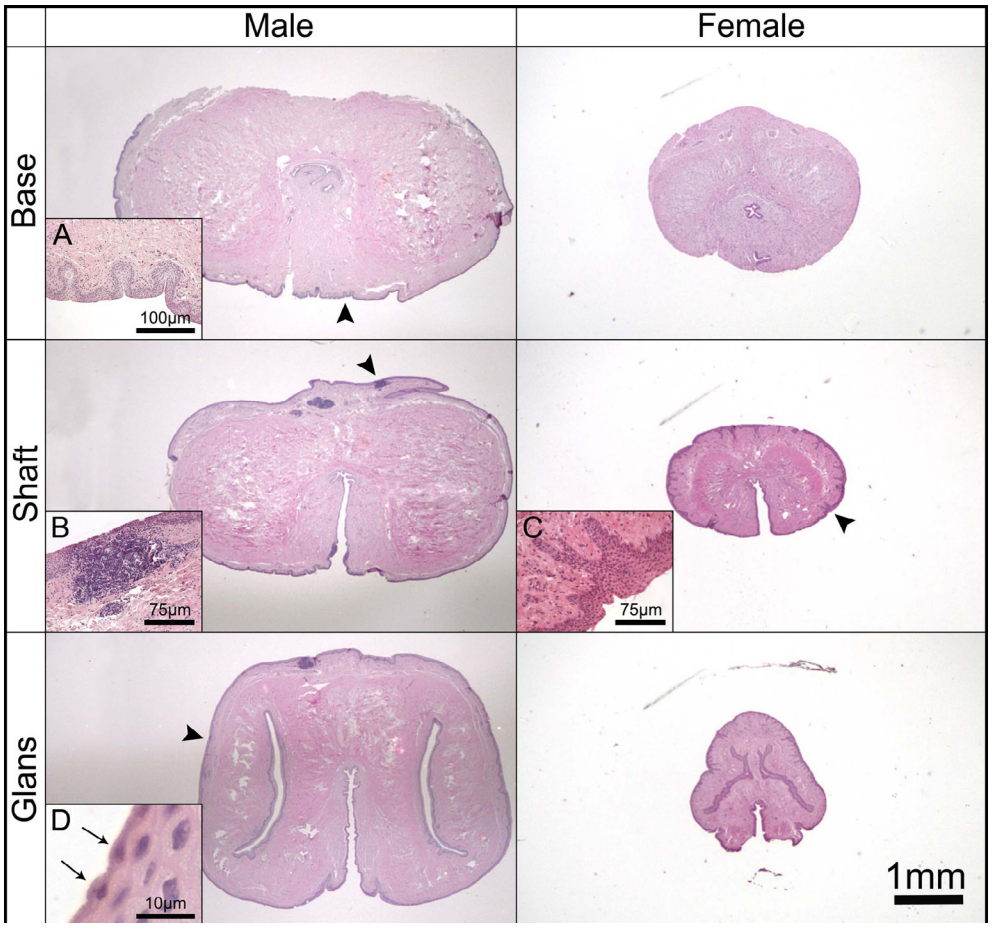


Fig. 4. Histoarchitecture of the male and female phallus. Representative photomicrographs of histological sections of the three regions of the phallus (base, shaft and glans). Features of the covering epithelia and lymphocyte aggregates are shown in higher magnification (Insets). A: invaginations, B: lymphocyte aggregate, C: finger-like columns of epithelial cells, D: transitional epithelium exhibiting characteristic "dome cells". Arrowheads indicate enlarged areas. H&E stained sections.

External genitalia develop through a combination of hormone-independent, hormone-dependent and endocrine/environmental influences (Zhou et al., 2002; Miyagawa et al., 2009; Blaschko et al., 2012). In concordance with testosterone levels, the phallus growth dynamics are sexually dimorphic: in males, the steep slope of the growth curve indicates that the phallus growth during the pre-pubertal juvenile stage still correlates with animal growth, whereas in pre-pubertal juvenile females, the growth curve of the phallus reaches a plateau, suggesting that the organ has already or is about to reach its maximum size. On the other hand, the slope of the phallus growth curve and the testosterone levels in E_2SD-females suggest that the clitoris size could increase; moreover, the correlation coefficients with biometric parameters in

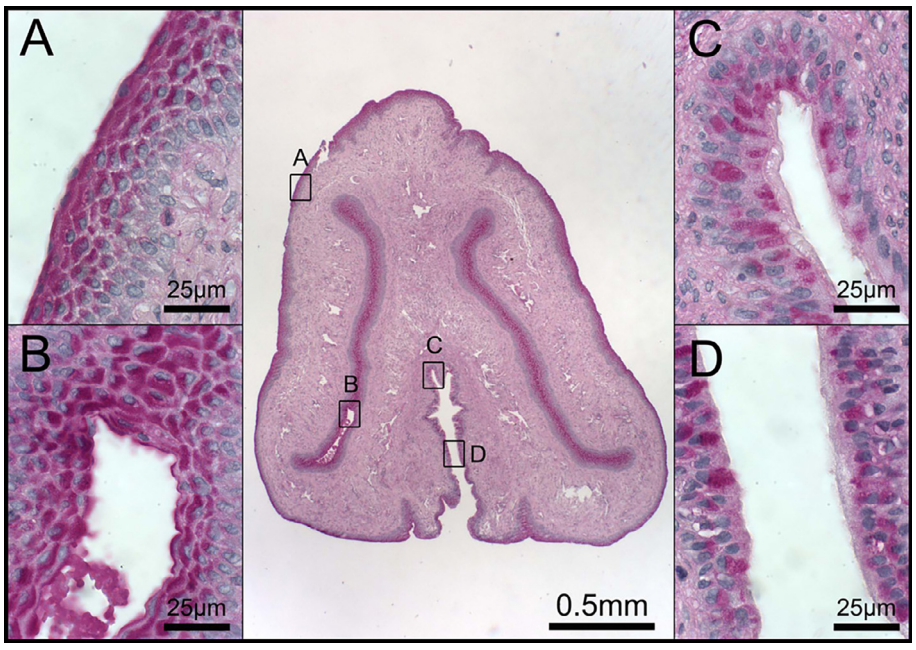


Fig. 5. Epithelial histofunctional features. Stratified squamous external epithelium (A), showing a continuous apical PAS staining pattern. Stratified secretory epithelium of the cavity (B) showing discontinuous apical PAS staining pattern; PAS-positive secretion is observed in the lumen. Simple ciliated columnar epithelium (C, right) and pseudo-stratified ciliated epithelia (C, left and D) at the sulcus. Secretory cells show basal nuclei and PAS-positive storage secretion. Transverse section at the glans region of the phallus from a juvenile pre-pubertal female stained with PAS.

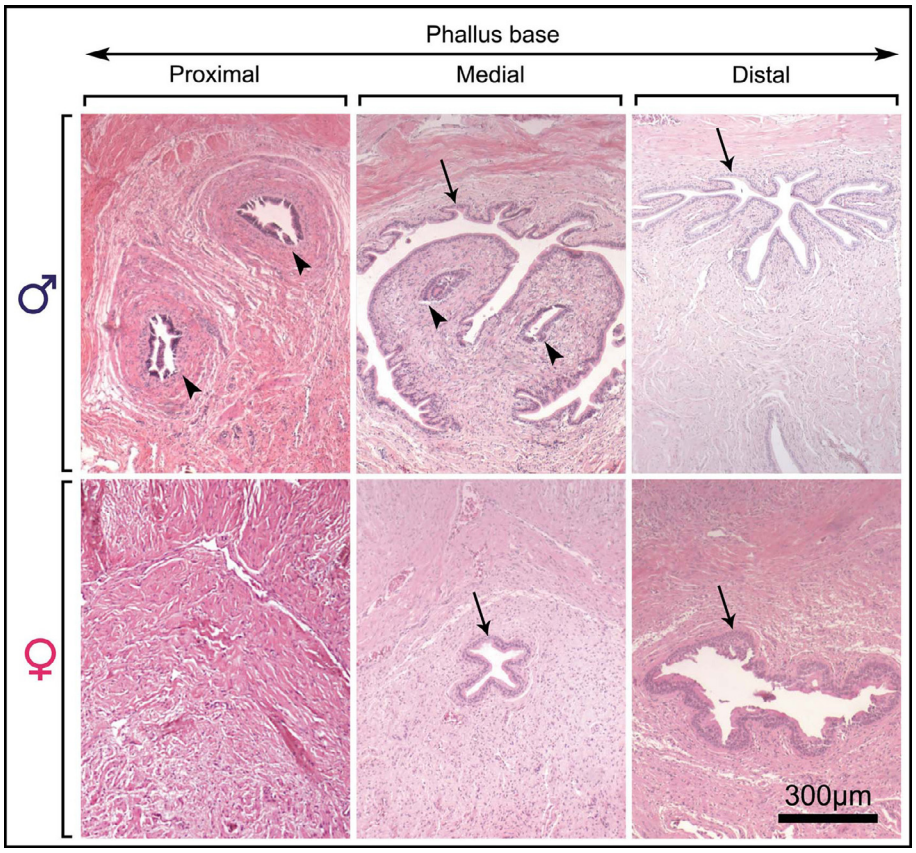


Fig. 6. Differences between males and females at the phallus base. In the male phallus, a pair of ductus deferens (arrowheads) is present at the most proximal level of the base. Towards the medial level of the base, the deep sulcus (arrows) surrounds the ductus deferens. At the most distal level of the base, both ductus deferens fuse with the deep sulcus. In females, the deep sulcus (arrows) is observed at the medial and distal levels of the phallus base. Representative photomicrographs of transverse sections at three different levels of the base region of the male and female phallus. H&E stained sections.

these females were close to those exhibited by males. However, during the study period, differences between slopes of TSD-females and E_2SD-females were not significant. The phallus size is the most reliable anatomic characteristic used to differentiate *C. latirostris* males from females (Nuñez-Otaño et al., 2010). Adult *C. latirostris* also present sexual dimorphism in the upper region of the cranium, which is the part of the head exposed when animals rest on the water surface; however, the discrimination rate of this anatomical feature is not applicable for management purposes (Verdade, 2003). Alterations in the growth dynamics of the E_2SD-female phallus could lead to erroneous sexing during field studies and caiman population monitoring.

In the present work, we characterized, for the first time, the histological features of the male and female phallus of *C. latirostris*. Crocodilian phalli are composed of three regions (base, shaft and glans), each with its particular histomorphological, and possibly histofunctional, features. The main histological differences among the three regions are the presence of ductus deferens in the base of the male phallus and the cavities of the glans in males and females. In males, semen from the ductus deferens enters the most proximal part of the sulcus at the base of the phallus (Cabrera et al., 2007; Moore et al., 2012). While the sulcus is an open groove, the contraction of muscle bundles surrounding this structure would create a closed duct where semen can move through without risk of leaking. Peristalsis of muscle cells could aid the seminal movement during insemination (Moore and Kelly, 2015). The sexually dimorphic differences in spatial distribution and organization of smooth muscle fibers described here support functional differences. Besides the transport of sperm in males, another function of the phallus is the production of bioactive muco-substances. The presence of mucin-producing epithelia has been described in the phallus of male *A. mississippiensis*, suggesting the role of mucins in sperm suspension and/or capacitation (Moore et al., 2012). In the sulcus epithelium of the male phallus of *C. latirostris*, PAS-positive storage secretions were frequently observed. The crocodilian phallus is hidden inside the cloaca. This position constantly exposes the phallus to both the endogenous microflora found in urine and feces and the environmental microbes beyond the cloacal vent. The role of mucins as mucosal barriers against infections has been previously described (Lagow et al., 1999). Thus, the PAS-positive secretions observed in the invaginations of the external epithelium of the phallus of both males and females may play such a role. Other immunological barriers, such as lymphocyte aggregates underlying the phallus epithelium, were present in both males and females In the phallus of male *A. mississippiensis*, lymphocyte aggregates have been described associated with cloacal lesions or pathological conditions (Govett et al., 2005); however, it has been proposed that the presence of these aggregates may be common under normal conditions and needed to prevent infections (Moore et al., 2012). Our observation of lymphocyte aggregates in the phallus agrees with the latter, since the *C. latirostris* individuals studied here were healthy and raised in sanitary controlled conditions. In turtles and mammals, the stiffness of the phallus is achieved through peri-vascular muscle relaxation and engorgement of the corpus cavernosum vascular spaces, whereas, in crocodilians, the corpus cavernosum is comprised of collagen fibers arranged in orthogonal geometries that result in an erect structure that is resistant to bending during copulation (Kelly, 2013; Gredler et al., 2014; Gredler, 2016). In agreement, highly birefringent and densely packed collagen fibers were observed in the phallus of *C. latirostris* males. Besides collagen, blood flow also plays a role during phallus erection in crocodilian males. The caverns at the glans fill up with blood during erection and inflate the glans, changing its general shape. Johnston et al. (2014) proposed that the inflation of the phallic glans of *Crocodylus porosus* forms a seal within the female cloacal opening to lock the phallus into position, reduce retrograde loss of semen, and prevent the mixing of semen with potentially contaminated water. In the present study, we found no differences in the vasculature of the phallus between *C. latirostris* males and females; in contrast, poorly birefringent and loosely packed

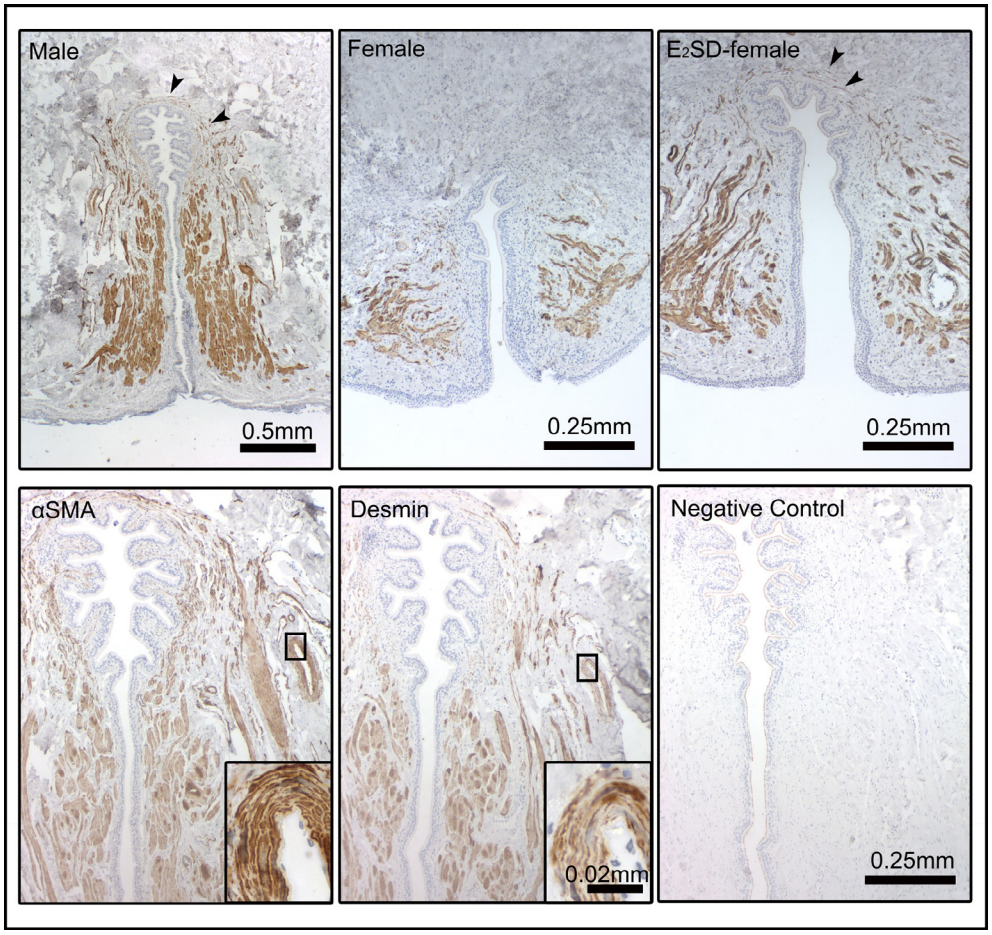


Fig. 7. Expression pattern of desmin and alpha smooth muscle actin (αSMA) in the phallus of *C. latirostris* males and females. Upper panel: Differences and similarities of muscle cell distribution in the phallus of males, TSD-females and E_2SD-females. Muscle fibers, revealed by desmin, exhibited a sexual dimorphic distribution pattern. In males, muscle bundles in the corpus spongiosum run parallel to the sulcus groove, reaching the periphery of the deep sulcus (Arrowheads). In TSD-females, desmin-positive cells never reach the deep sulcus and look less organized; besides, immunostaining intensity was weaker than in males. Like TSD-females, E_2SD-females exhibited no defined radial muscle fiber orientation whereas; like males, abundant muscle fibers that also reach the deep sulcus are observed. Lower panel: Representative microphotographs of consecutive sections of the male phallus shaft, showing expression patterns of αSMA and desmin. The insets show immunostaining differences between α-SMA and desmin in smooth muscle cells on the blood vessel walls. IHC developed by DAB and counterstained with Mayer's hematoxylin. Negative Control: representative histological section run in the same IHC assay in which the primary antibody was omitted.

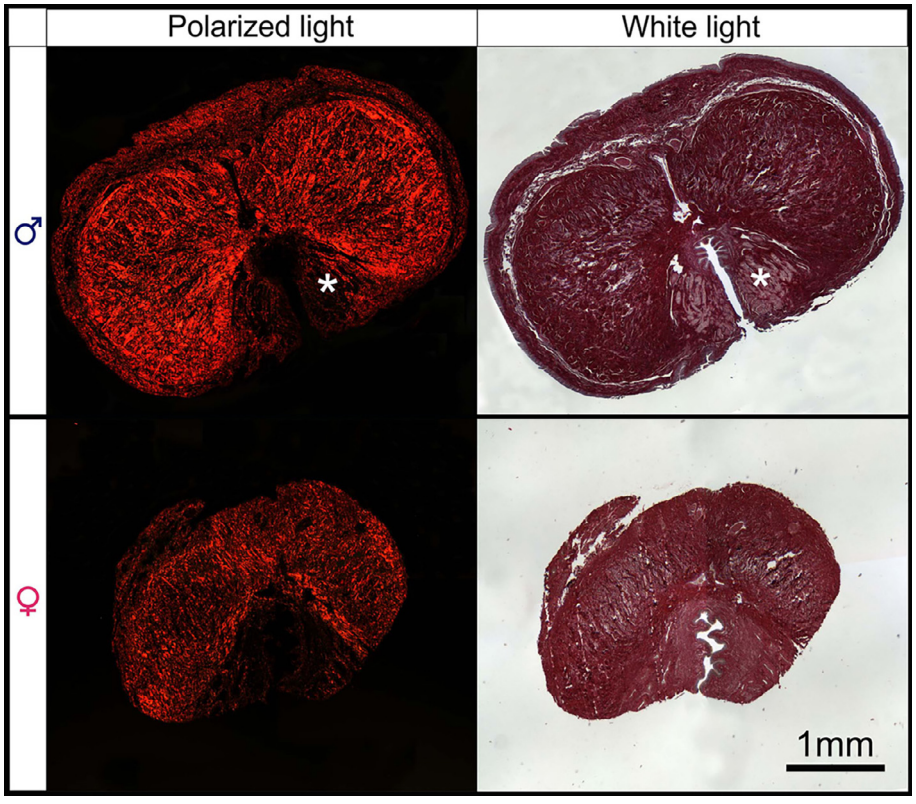
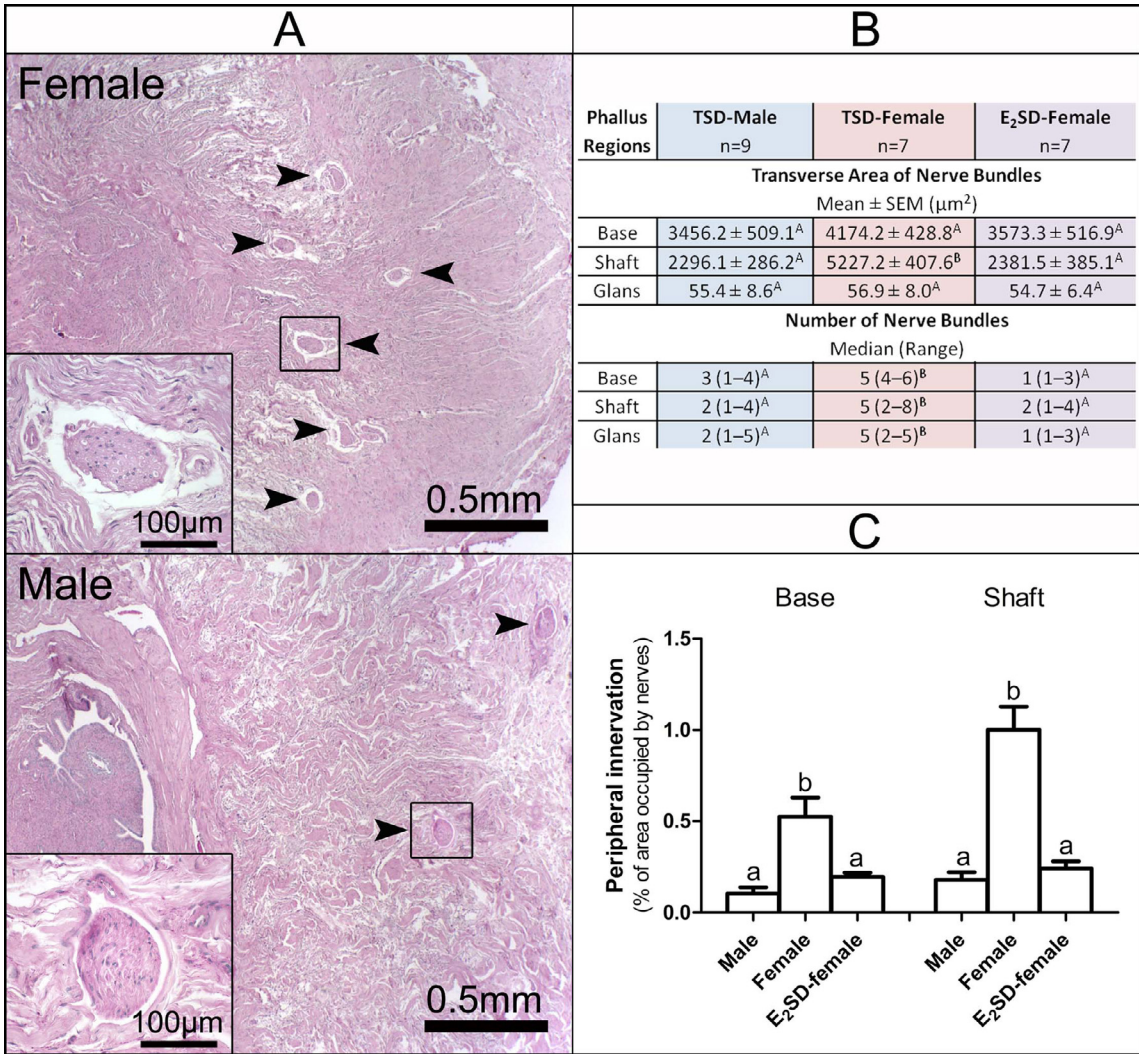


Fig. 8. Collagen fiber organization in the phallus of *C. latirostris* males and females. The male phallus showed intense birefringence, indicating highly organized collagen fibers, whereas the female phallus showed poorly birefringent and loosely packed collagen bundles. The asterisks indicate the zone of the corpus spongiosum, rich in muscle fibers. Few collagen fibers among the muscle bundles are observed under polarized light. Transverse sections of the phallus shaft of males and females stained with Picrosirius-hematoxylin and observed under polarized and white light are shown.



Phallus Regions	TSD-Male n=9	TSD-Female n=7	E_2SD-Female n=7
Transverse Area of Nerve Bundles			
Mean ± SEM (µm²)			
Base	3456.2 ± 509.1^{A}	4174.2 ± 428.8^{A}	3573.3 ± 516.9^{A}
Shaft	2296.1 ± 286.2^{A}	5227.2 ± 407.6^{B}	2381.5 ± 385.1^{A}
Glans	55.4 ± 8.6^{A}	56.9 ± 8.0^{A}	54.7 ± 6.4^{A}
Number of Nerve Bundles			
Median (Range)			
Base	3 (1–4)^{A}	5 (4–6)^{B}	1 (1–3)^{A}
Shaft	2 (1–4)^{A}	5 (2–8)^{B}	2 (1–4)^{A}
Glans	2 (1–5)^{A}	5 (2–5)^{B}	1 (1–3)^{A}

Fig. 9. Sexual dimorphic pattern of the peripheral innervation of the phallus. A: Representative photomicrographs showing nerve bundles (arrowheads and insets) at the base region of the male and female phallus. PAS-stained sections. B: Table summarizing the quantification results as transverse area of nerve bundles or number of nerve bundles. C: Graph representing peripheral phallus innervation expressed as percentage of total area occupied by nerves at the base and shaft regions. Sexual dimorphism is lost betwen males and E_2SD-females. Different superscripts on ranks (B) or columns (C) indicate statistical differences established by Kruskal Wallis test followed by Dunn's post-test at $p < 0.05$. Results are expressed as mean ± SEM (C).

collagen fibers were present in the female phallus when compared to the male phallus. Taking into account the role of collagen in crocodilian male phallus stiffness, our results suggest that the clitoris of *C. latirostris* achieves tumescence, but not rigidity during sexual arousal.

Female sexual arousal results in a combination of vaso-congestive and neuromuscular events, which include increased clitoris, size (Berman et al., 2003). In female mammals, the presence of nerves in the external genitalia is associated with sex arousal (Martin-Alguacil et al., 2007) and biological rewards such as pleasure during copulation (Toesca et al., 1996; Martin-Alguacil et al., 2007). The peripheral nerve bundles observed in the phallus of *C. latirostris* females were more numerous and larger than those observed in the male phallus. Whether the profuse innervation of the female phallus is associated with sex arousal and/or involved in a neuroendocrine response needed for successful copulation remains unknown. The nerve bundles present in the phallus of E_2SD-females were fewer and smaller than those observed in TSD-females and similar to those observed in males, suggesting that E_2 did not override the effect of incubation temperature on phallus innervation. The consequences of this reduced innervation are unknown; however, experimentally induced reduction of nerve supply by tissue damaging in the mammalian female genitalia has been associated with sexual dysfunction (Moszkowicz et al., 2011).

The reproductive tissues of *C. latirostris* males and females are highly sensitive to the effects of EDCs such as endosulfan (END), atrazine (ATZ) and bisphenol A (Stoker et al., 2003, 2008; Rey et al., 2009; Durando et al., 2013, 2016). Prenatal exposure to estrogens or EDCs modifies ovarian follicular dynamics and hormonal steroid levels in postnatal female caimans (Stoker et al., 2008). Recent results have revealed that early postnatal exposure to EDCs alters the temporal and spatial expression pattern of histofunctional differentiation biomarkers in the oviduct later in life (Galoppo et al., 2017). Since *C. latirostris* can be naturally exposed to EDCs (Stoker et al., 2011, 2013), the search for knowledge about the mechanism of action of estrogens on this species is of particular interest both to assess the impact of EDCs on *C. latirostris* populations and to better characterize *C. latirostris* as a bioindicator of ecosystem health.

As already mentioned, the mechanisms leading to external genitalia development are sensitive to endocrine and environmental influences (Zhou et al., 2002; Miyagawa et al., 2009; Blaschko et al., 2012) and it has been described that estrogens and androgens act as mitogens and growth-promoters in steroid-sensitive tissues (Hess-Wilson et al., 2006; Radhi, 2016). In the present study, expression of AR and ERα was

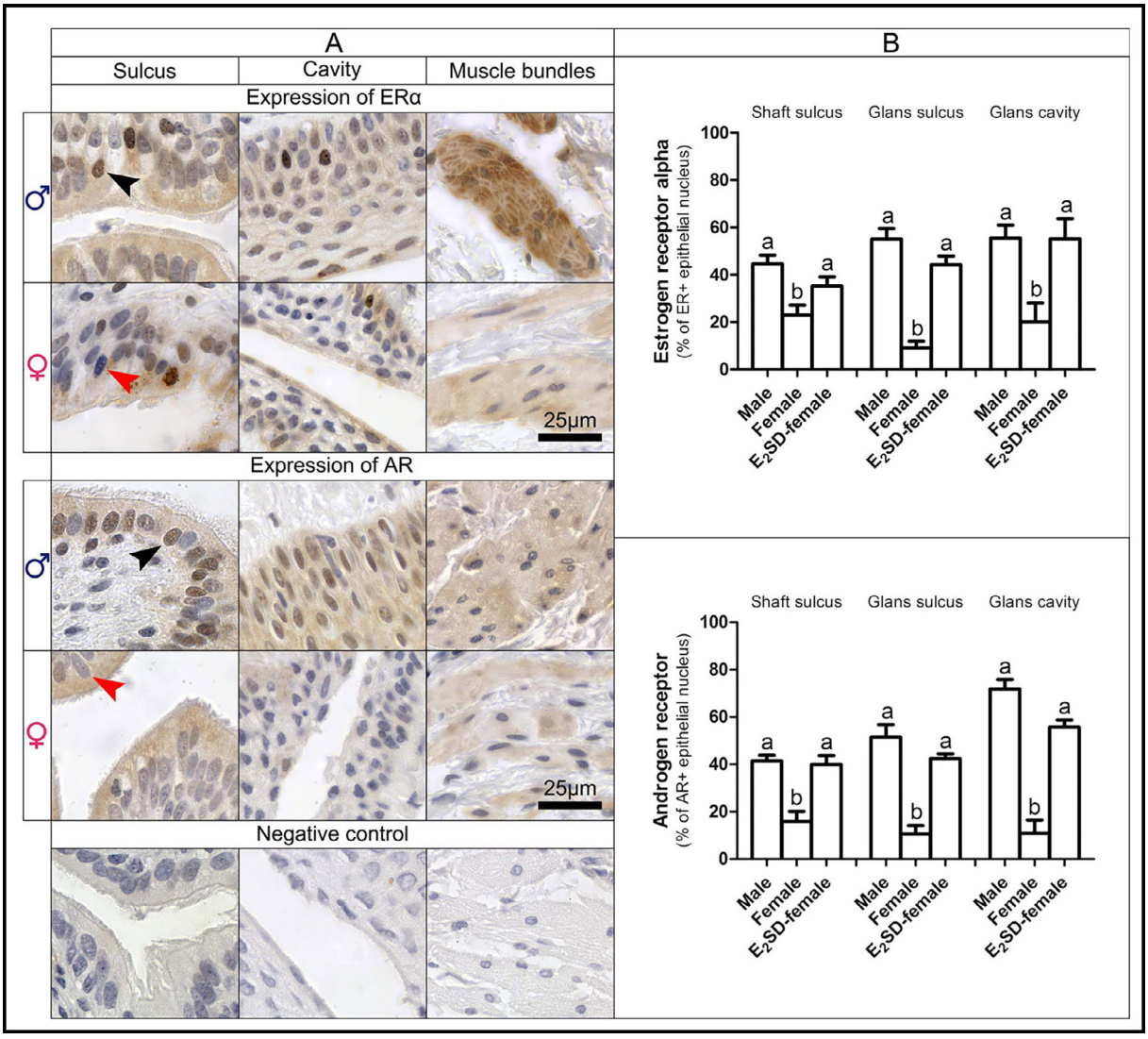


Fig. 10. Expression of sexual steroid hormone receptors in the phallus of *C. latirostris* males and females. A: Representative images from the male and female phallus, showing sexual dimorphic expression patterns of ERα and AR. Positive nuclear immunostaining (Black arrowheads). Negative nuclei (Red arrowheads). Cytoplasmic AR and ERα expression is observed in muscle bundles, sulcus epithelium and cavity epithelium, whereas nuclear AR and ERα expression is restricted to sulcus and cavity epithelia. Negative controls were performed replacing the primary antibody with pre-absorbed serum. IHC revealed with DAB and counterstained with Mayer hematoxylin. B: Percentage of AR and ERα positive immunostained epithelial cells in the sulcus and glans cavities of males, females and E_2SD-females. The sexual dimorphism exhibited by ERα and AR are lost between males and E_2SD-females. Results are expressed as mean ± SEM. Bars with different superscripts denote statistical difference by Kruskal Wallis followed by Dunn's post-test at $P < 0.05$.

observed in the epithelia of the sulcus and cavity and in the smooth muscle fibers and was higher in the external genitalia of males than in that of females at the pre-pubertal juvenile stage. The latter could define an increased sensitivity to hormones in males and the subsequent hormone-mediated growth of the pubertal male phallus. Androgens can also induce sensitization to calcium, a biochemical phenomenon whereby smooth muscle tissues exhibit increased contractile force under restricted calcium concentration (González-Montelongo et al., 2010). On the other hand, the expression of ERα in the reproductive tract has been reported to play an important role in ciliated epithelial cell differentiation (Okada et al., 2004), and the ciliary length and beat frequency (Li et al., 2017). Thus, the expression of AR in the smooth

Table 4
Ratio between phallus morphometrics and caiman biometrics.

Parameters	TSD-Males n = 13	TSD-Females n = 10	E_2SD-Females n = 7
Phallus Length/TL	0.2392 ± 0.0205^{a}	0.0888 ± 0.0059^{b}	0.0795 ± 0.0033^{b}
Glans height/TL	0.0670 ± 0.0048^{a}	0.0297 ± 0.0009^{b}	0.0312 ± 0.0013^{b}
Glans width/TL	0.0680 ± 0.0045^{a}	0.0307 ± 0.0019^{b}	0.0269 ± 0.0013^{b}
Phallus Length/BM	0.0099 ± 0.0007^{a}	0.0061 ± 0.0008^{ab}	0.0046 ± 0.0006^{b}
Glans height/BM	0.0029 ± 0.0002^{a}	0.0020 ± 0.0003^{a}	0.0019 ± 0.0003^{a}
Glans width/BM	0.0029 ± 0.0002^{a}	0.0021 ± 0.0003^{ab}	0.0016 ± 0.0002^{b}

Results are expressed as Mean ± SEM. Different superscripts indicate significant differences at $p < 0.05$ of the log-transformed ratio by ANOVA followed by Bonferroni's post-test. Caiman biometric parameters, TL: total length; BM: body mass.

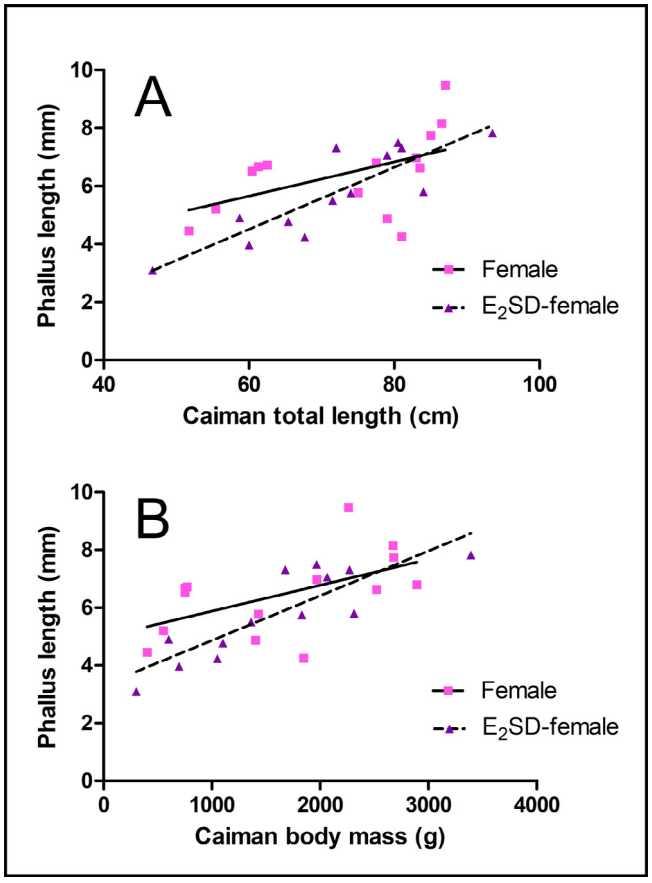


Fig. 11. Phallus growth dynamics in *C. latirostris* TSD-female and E_2SD-female. A. Correlation between phallus length and caiman TL (r^S = 0.6440 and r^S = 0.8693, TSD-female and E_2SD-female respectively), B. Correlation between phallus length and caiman BM (r^S = 0.6403 and r^S = 0.8599, TSD-female and E_2SD-female respectively). No statistical differences were found in the slopes between TSD-females and E_2SD-females in Phallus length *versus* TL (F = 1.50719; p = 0.2325) as in Phallus length *versus* BM (F = 2.00302; p = 0.1716). BM < 1000 g or TL < 65 cm, caimans in late post-hatching stage.

muscle fibers of the corpus spongiosum of the male phallus may warrant muscle contraction even under low calcium levels, whereas the expression of ERα may be associated with an easier transport of gametes through the sulcus lumen, both phenomena involved in a successful copulation. Regarding the hormone dependence of the female phallus, in the mammalian female genitalia, the expression of AR has been associated with sexual arousal and blood flow (Traish et al., 2002).

Percentages of AR and ERα positive immunostained epithelial cells in the phallus of E_2SD-females were higher than those in the phallus of TSD-females and similar to those of the male phallus, suggesting that prenatal exposure to E_2 could exacerbate the response of external genitalia to endogenous hormones and/or to estrogenic agonists later in life (Luque et al., 2018).

In laboratory rodents, alterations in both the androgenic and estrogenic pathways may lead to abnormal external genitalia development (revised by Blaschko et al., 2012). Since the sexually dimorphic characteristics of the phallus, established during sexual differentiation, were modified in E_2SD-females, alterations due to exposure to EDCs could not be ruled out. Preliminary results from our laboratory showed that *in ovo* exposure to EDCs such as ATZ or END modified the phallus size in male and female *C. latirostris* specimens (Zayas et al., 2010). Therefore, disrupting the sexual dimorphic growing pattern of the phallus may lead to inaccurate sexing at field population monitoring and could compromise the reproductive biology of these individuals and their population size. Sexual dimorphism occurs even though the sexes have virtually identical DNA sequences. Thus, sexual dimorphism must, in most cases, arise due to epigenetic mechanisms (Connallon and Knowles, 2005; Rinn and Snyder, 2005). Since epigenetic mechanisms are involved in EDC effects (Altamirano et al., 2017; Milesi et al., 2017; Vigezzi et al., 2016), future studies will address this issue to better understand the alterations observed in the sexually dimorphic patterns in the external genitalia of *C. latirostris* E_2SD-females.

The present results indicate the risk of adverse effects associated with estrogenic agonist exposure during the TSW and suggest that caution must be taken on using E_2SD as a tool for wild reptile population recovery.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.10.003.

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