Disruption of the developmental programming of the gonad of the broad snouted caiman (Caiman latirostris) after in ovo exposure to atrazine.

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Research Article

Abstract

Environmental exposure to agrochemicals during early stages of development can induce subtle alterations that could permanently affect normal physiology. Previously, we reported that *in ovo* exposure to atrazine (ATZ) disrupts testicular histoarchitecture in postnatal caimans (*Caiman latirostris*). To assess whether such alterations are the result of disruption of gonadal developmental programming, this study aimed to evaluate the expression of histofunctional biomarkers (VASA, ER, PR, PCNA, and aromatase) and genes involved in gonadal development and differentiation (*amh*, *sox-9*, *sf-1* and *cyp19-a1*) in the gonads of male and female caiman embryos and to assess the effect of ATZ exposure on these biomarkers and genes in the gonads of male embryos. Our results suggest that *amh*, aromatase and *sox-9* play a role in sex determination and gonadal differentiation. In male caiman embryos, ATZ exposure increased aromatase expression and altered the temporal expression pattern of *amh* and *sox-9* evidencing an ATZ-induced disruption of gonadal developmental programming. Since the effects of ATZ are consistent across all vertebrate classes, the ATZ-mediated disruptive effects here observed could be present in other vertebrate species.

1. Introduction

Many environmental compounds have been classified as endocrine-disrupting chemicals (EDCs), as they have the potential to interfere with the endocrine system (WHO and UNEP, 2012). Over the last decades, endocrine disruption has been described as a matter of environmental health concern because EDC exposure alters development, reproduction, and endocrine physiology in almost all species studied (Luque et al., 2018; Luque and Muñoz-de-Toro, 2020). The herbicide atrazine (ATZ) is among the environmental substances classified as EDCs due to its estrogenic, anti-androgenic and thyroid-disrupting activity (Elkayar et al., 2022; Galoppo et al., 2020; Hayes et al., 2011). Although the half-life time of ATZ is relatively short when compared to pollutants classified as persistent, and its physicochemical properties do not favor bioaccumulation, the presence of ATZ in groundwater, freshwater, sea water, soil, and animal tissues (Tavalieri et al., 2020) evidences its wide and continuous environmental input as well as its large dispersal rate. Thus, wildlife species that are not target of herbicides can be exposed to ATZ. Since the sexual differentiation of the reproductive system depends upon precise exposure to sex steroid hormones at specific times during development, exposure to low doses of ATZ during these hormone-sensitive critical periods of development can alter the developmental programming and lead to long-term deleterious effects on the adult organism.

The broad snouted caiman (*Caiman latirostris*) is a South American crocodilian species highly sensitive to EDC exposure, widely distributed in wetlands of Argentina, Brazil, Paraguay, and Uruguay. This species shows temperature-dependent sex determination (TSD): embryos developed at 33°C result in phenotypic males, whereas embryos developed at 30°C result in phenotypic females (Stoker et al., 2003). In TSD species, temperature is an environmental factor that initiates a cascade of molecular events that lead the undifferentiated gonad to acquire the female or male phenotype. This process is defined as sex determination (Warner, 2011). Once sex determination is initiated, sex differentiation (defined as the

histomorphological changes that lead to the development of a specialized set of organs) takes place. Sex determination and differentiation involve hormones, hormone receptors, steroidogenic enzymes, and genes such as *amh* (the anti-Müllerian hormone-encoding gene), *sox-9* (which encodes a transcription factor that controls the anti-Müllerian hormone gene), *sf-1* (which encodes the steroidogenic factor 1, which regulates the transcription of key genes involved in sexual development and reproduction), and *cyp19-a1* (which encodes the enzyme aromatase) (Durando et al., 2013; Warner, 2011). Moreover, the expression of proteins such as ATP-dependent RNA helicase (VASA) (to identify germ cells), estrogen receptor (ER) and progesterone receptor (PR) (biomarkers of hormone-dependence), Proliferating Cellular Nuclear Antigen (PCNA) (to assess proliferative activity), and the enzyme aromatase (steroidogenic enzyme) is useful to study gonadal histofunctional differentiation (Canesini et al., 2018). Noteworthy, in TSD species, there is a critical window of time, named thermosensitive period, in which the embryonic sex remains labile and can be modified by environmental cues (Warner, 2011). Such cues may include EDC exposure. In our previous studies in *C. latirostris*, we demonstrated that, when exposed to EDCs such as 17β-Estradiol or bisphenol A during the thermosensitive period, embryos developed at the male-producing temperature (MPT) produced phenotypic females (Stoker et al., 2003). We also found that exposure to ATZ did not affect phenotypic sex (Beldomenico et al., 2007), but induced gonadal alterations in 10-day-old postnatal male caimans (Rey et al., 2009). Considering these findings, in the present study, we hypothesized that ATZ exposure in embryos developed at the MPT induces early modifications in key developmental genes and/or molecules that lead to histomorphological alterations observed in the gonads, long after exposure ended. To test this hypothesis, we firstly studied the expression of key developmental molecules and genes in the gonads of unexposed embryos developed at the MPT or female-producing temperature (FPT) and secondly studied the effect of ATZ exposure on these molecules and genes in the gonads of embryos developed at the MPT.

2. Materials And Methods

2.1. Egg collection and experimental design

Eggs were collected and incubated as described in Canesini et al., (2018). Briefly, *C. latirostris* eggs (n = 120) were collected shortly after oviposition from seven nests randomly selected in a protected area (Natural Reserve "El Cachapé") of Chaco Province, Argentina, and transported to the laboratory. Since the objectives of the present work were to study the molecules and genes involved in the processes of temperature-dependent sex determination and differentiation and to study the effects of ATZ exposure on sex-determination and differentiation-related molecules and genes, eggs were divided into three experimental groups, and embryos were obtained at different developmental stages (Fig. 1). The groups were as follows: Temperature-sex determined females (TSD-Females, embryos (n = 40) obtained from eggs incubated at 30°C, which is the FPT), temperature-sex-determined males (TSD-Males, embryos (n = 40) obtained from eggs incubated at 33°C, which is the MPT), and ATZ-Males (embryos (n = 40) obtained from eggs incubated at the MPT and exposed to ATZ immediately before sex differentiation). To avoid

biased responses to treatment due to clutch-related differences, eggs from each clutch were equally distributed in all experimental groups.

2.2. Assessment of the embryo developmental stage (DS) and treatment

Recently laid eggs allowed us to obtain sexually undifferentiated embryos plausible to be TSD, as well as to minimize environmental egg exposure that could affect experimental results. Before egg collection, recent oviposition was checked by determining the embryo DS by following the criteria previously described (Iungman et al., 2008). Only eggs from nests showing embryos at DS lower than 15 were collected. Once in the lab, the eggs were incubated at 30 or 33°C. Based on our experience in developmental timing at 30°C and 33°C, the DS was checked frequently until stages 20, 22, 24 or 27. Eggs were treated at DS 20 (prior to sex determination). Treatments were applied topically as previously described (Stoker et al., 2003). For ATZ exposure, a single dose of 0.2 ppm of ATZ (96% purity, Icona S.A., Argentina) diluted in absolute ethanol (purity ≥ 99.9%, Merck, Germany) was applied to the eggshell (ATZ-Males). To expose the egg to 0.2 µg of ATZ per kg of egg (0.2 ppm), the volume of the ethanol solution of ATZ was adjusted to the mass of the egg at the moment of treatment. For control groups (TSD-Males and TSD-Females), 50 µL of absolute ethanol (purity ≥ 99.9%, Merck) was applied topically. The dose of ATZ used is considered environmentally relevant since it corresponds with tissue levels of ATZ reported in environmentally exposed fishes (Basopo and Muzvidziwa, 2020; Brodeur et al., 2021; Zhang et al., 2022). Additionally, we have previously demonstrated that the use of ethanol as vehicle causes no effect on caimans (Stoker et al., 2003). After treatment, incubation continued until DS 22, 24 or 27 (Fig. 1).

2.3. Sampling and processing

Twelve animals from each experimental group were euthanized at DS 22, 24 or 27. To obtain the Gonad-Adrenal-Mesonephric (GAM) complexes, different protocols were followed according to the experimental purpose. To study gene expression, immediately after euthanasia, the GAM complexes were dissected using a stereomicroscope (Stemi 305 Zeiss, Carl Zeiss Microscopy, Germany) in RNAse-free conditions. Immediately after dissection, the GAM complexes were placed ventral side up on a cold sterilized aluminium foil in close contact with dry ice to be flash frozen. After flash-freezing, the samples were wrapped in the sterile aluminium foil and stored at -80 ºC until gonad isolation and RNA extraction.

For histomorphological and histofunctional studies, the GAM complexes were fixed in formalin-buffered solution (10% phosphate-buffered formalin, pH 7.4) for 6 h at room temperature, rinsed in phosphate buffer saline solution (PBS pH 7.5), dehydrated in an ascending ethanol series, cleared in xylene, and embedded in paraffin.

2.4. Histomorphological features of the gonad and biomarkers of histofunctional differentiation

For histomorphological studies, 5-µm-thick histological sections of the embryo GAM complex were stained using the trichromic picrosirius/hematoxylin staining technique, as previously described

(Canesini et al., 2018). Serial sections were studied to evaluate the presence of sex-defining structures (Ferguson and Chenier, 1983; Forbes, 1940). All histological evaluations were performed in at least three sections separated 300 μm from each other.

The expression of proteins such as VASA, ER, PR, and PCNA and the enzyme aromatase, useful to study gonadal histofunctional differentiation, was assessed by immunohistochemistry (IHC) using immunoperoxidase staining. IHC was performed as previously described (Canesini et al., 2018; Galoppo et al., 2016; Varayoud et al., 2012). All the antibodies used (Table 1) were previously characterized and used in caiman tissues (Canesini et al., 2018; Galoppo et al., 2016; Varayoud et al., 2012).

Table 1
Antibodies used for immunohistochemistry.

Target protein	Supplier	Primary Antibodies	Animal Source	Dilutions used
PCNA	Novocastra, (Newcastle, UK)	Anti-PCNA (Clone PC-10)	Mouse	1:2000
VASA	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	Anti-VASA H-80 (Code sc67185)	Rabbit	1:200
ERα	LETH-ISAL[a] (Santa Fe, Argentina)	Anti-ER (LETH-ER 202Y)	Rabbit	1:200
PR	DAKO Corp. (Carpinteria, CA, USA)	Anti-PR (Code A0098)	Rabbit	1:300
Aromatase	LETH-ISAL[b] (Santa Fe, Argentina)	Anti-Aromatase (LETH-AROMy)	Rabbit	1:2000
PCNA: Proliferating Cellular Nuclear Antigen; VASA: ATP-dependent RNA helicase; ERα: Estrogen Receptor alpha; PR: Progesterone Receptor. LETH-ISAL: Laboratorio de Endocrinología y Tumores Hormonodependientes- Instituto de Salud y Ambiente del Litoral. [a] Varayoud et al., 2012. [b] Canesini et al., 2018.				

2.4.1. Image analysis and quantification of molecules revealed by IHC

Image analysis was performed on digital images recorded using the ImageJ/Fiji 1.46 software as previously described. Briefly, the whole gonadal area and the immunostained area were determined (Varayoud et al., 2012). The expression levels of ERα, PR, aromatase and PCNA were quantified as the

percentage of the gonadal area occupied by each marker (Canesini et al., 2018). A threshold based on the intensity of the immunostaining was set for the PCNA-positive area (Durando et al., 2016). VASA was assessed qualitatively to describe the spatial distribution of germ cells.

2.5. Expression of key developmental genes in the embryonic gonad

2.5.1. Isolation of gonadal tissue for RNA extraction and gene quantification

Gonadal tissue was isolated from the GAM complex by using the microdissection technique previously described (Lazzarino et al., 2019), modified and adapted for caiman embryo samples. Briefly, GAM samples stored at -80°C were embedded in an OCT cryo-embedding matrix (CRYOPLAST® Biopack, Argentina) and 300-µm-thick GAM serial transverse sections were obtained using a cryostat (Leica CM 1860, Leica Biosystems, IL, USA) at an operating temperature of -20°C. Once obtained, the GAM sections were placed on previously cooled glass sterile slides and observed using a dissecting microscope (Stemi 305, Zeiss, Oberkochen, Germany). The gonads were identified by histological and morphological criteria and then isolated from the rest of the structures of the GAM complex. Sterile stainless steel microdissection needles of 0.5 mm internal diameter were used for the isolation of the gonads of embryos at DS 22, while microdissection needles of 1.0 mm internal diameter were used for the isolation of the gonads of embryos at DS 24 and 27. Gonads were removed bilaterally, and microdissected sections were immersed in total RNA isolation reagent (TRIzol™ Invitrogen, Carlsbad, CA, USA) and stored at -80°C until RNA extraction.

To make sure that all the sections obtained by the microdissection technique belonged to the gonad, the remaining tissue was histologically inspected. The remaining GAM sections were thawed, and the microdissection holes were located. Since the gonadal tissue is surrounded by a layer of connective tissue and shows characteristics that make it perfectly distinguishable from the rest of the structures that constitute the GAM complex, we checked that no tissue outside of the gonad limits had been removed.

Each microdissected gonad from each individual animal (between 10 and 20 punches, depending on the DS of the embryo) was processed and analysed as a single data point, as previously described (Lazzarino et al., 2017, 2019).

2.5.2. RNA extraction, reverse transcription, and real-time quantitative polymerase chain reaction (PCR) analysis

Total RNA was isolated from microdissected gonadal tissue stored at -80°C using TRizol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNA were determined by measuring the absorbance at λ = 260nm and λ = 280nm in a NanoDrop Lite Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Gene quantification was performed using a two-step quantitative reverse transcriptase PCR by the standard curve method (Čikoš et al., 2007). Firstly, equal quantities (0.5 µg) of total RNA from each sample were reverse-transcribed into copy DNA (cDNA) with Moloney Murine Leukemia Virus reverse transcriptase (200 units; Promega, Madison, WI, USA), by using 200 pmol of random primers (Promega, Madison, WI, USA), 20 units of the ribonuclease inhibitor RNAout (Invitrogen Argentina, Buenos Aires, Argentina) and 100 nmol of a deoxynucleotide triphosphate (dNTP) mixture. The final volume of each reverse transcription reaction tube was adjusted to 30 µL with 1X reverse transcriptase buffer. Reverse transcription PCR was performed at 37° C for 90 min and at 42° C for 15 min. Finally, the reaction was stopped by heating at 80° C for 5 min followed by rapid cooling on ice bath.

Once the cDNA was obtained, the mRNA expression of four selected genes (*amh*, *sox-9*, *sf-1* and *cyp19-a1*) was quantified by real-time PCR. For this purpose, each reverse-transcribed product was diluted with RNAse-free water to a final volume of 60 µL and amplified by the Real-Time DNA Step One Cycler (Applied Biosystems Inc., Foster City, CA, USA) using previously designed primers (Durando et al., 2013). L8 (60S ribosomal protein L8) from *Alligator mississippiensis*, a species related to *C. latirostris*, was used as housekeeping gene. The characteristic of the primers used are shown in Table 2. For cDNA amplification, 5 µL of cDNA was combined with HOT FIREPol Eva Green qPCR Mix Plus (Solis BioDyne; Estonia) and 10 pmol of each primer (Invitrogen, Carlsbad, CA, USA) to a final volume of 20 µL using RNAse-free water. Negative DNA template controls were included in all the assays using 5 µL of sterile RNAse-free water instead of cDNA. After initial denaturation at 95°C for 15 min, the reaction mixture was subjected to successive cycles of denaturation at 95°C for 15 s, and annealing and extension at specific temperatures for each gene for 15 s. The relative expression levels of each target were calculated based on the cycle threshold (CT), which was calculated for each sample using the StepOne Software (Applied Biosystems Inc. Foster City, CA, USA) with an automatic threshold setting. Standard curves for each target gene as well as for the housekeeping gene were performed using eight serial dilutions of a pool cDNA containing equal amounts of cDNA from samples of different experimental groups. The optimal dilution of work for each gene tested was fixed based on the results obtained from the standard curves. Results are expressed as fold change in the expression of each target gene in relation to the housekeeping gene. Product purity was confirmed by dissociation curves provided by the StepOne Software and random samples were subjected to agarose gel electrophoresis.

Table 2
Primers and PCR conditions

Gene	GenBank ID	Sense Primer (5´-3´)	Anti-sense Primer (5´-3´)	Size (bp)
amh	AF180294	TCCACCCGTGCCGACTACTA	CAGAGTATTGGACGGGCACG	106
Sox-9	AF106572	GGCTCGGAGCAAACCCACAT	TGCCAGGCTGGACGTCTGTT	172
Sf-1	AF180296	GGCTCCATCCTGAACAACCT	TTGAGGCAGACGAACTCCTG	95
Cyp19-a1	AY029233.1	CTGGAGATGATGATCGCTGC	TGGCATGTCATCGCTCTGTA	152
L8	ES316580.1	CACGACCAGCCTTTAAGATA	CTCACAATCCTGAAACCAAG	141

2.6. Statistical analysis

Data are reported as the median and range or mean ± SEM. To determine normality, Shapiro-Wilk normality test followed by a Levene variance homogeneity test were performed. Statistical analyses were performed using Mann−Whitney U test and Kruskal−Wallis analysis of variance followed by Dunn's test as a *post hoc* test to determine significant differences (Siegel, 1956). The Q-test was performed to evaluate the presence of outliers that characterize the "clutch effect". In all cases, differences were considered significant at $p < 0.05$. For all the analyses, the IBM SPSS Statistics 19 software (IBM Inc.) was used.

3. Results

3.1. Temperature sex determination and embryonic gonadal differentiation

3.1.1. Gonadal histology and germ cell distribution

As previously reported, all embryos from eggs incubated at 33°C were males (TSD-Males), whereas those from eggs incubated at 30°C were females (TSD-Females). At DS 22, the gonads of both TSD-Females and TSD-Males showed a well-defined compartmentalization characterized by a cortex and a medulla. The cortex is defined by an external cuboidal-to-flat epithelium composed of cells with homogeneously and intensely blue-stained nuclei. The medulla underlies the cortex and is formed by medullary cells arranged in groups or cords surrounded by loose connective tissue. The medullary cords are, in turn, formed by two morphologically different types of cells: cells with homogeneously dark blue-stained nuclei and few cytoplasm and cells with heterogeneously violet-stained nuclei with decreased nuclei/cytoplasm ratio. The latter type of cells may be isolated or grouped in clusters dispersed in the underlying stroma or at the coelomic border. The loose connective tissue shows zones with empty spaces (Fig. 1). No signs of gonadal sex differentiation were observed at this DS.

In the present work, we confirmed the gonadal histological features previously described for TSD-Females at DSs 24 and 27 (Canesini et al., 2018). At DS 24, as shown in Fig. 1, the medulla of TSD-Males shows a more compact appearance with fewer empty spaces than that of TSD-Females. Both sexes showed an increased number of cells with heterogeneously violet-stained nuclei and a decreased nuclei/cytoplasm ratio either isolated among interstitial tissue or forming clusters. Besides, in the gonads of TSD-Males, the medullary cords acquire a tubular-like appearance with an empty lumen.

At DS 27, the gonads of the TSD-Male embryos showed the presence of tubular structures, which were formed by an epithelium and a central lumen occupied by a substance of foamy appearance. The epithelium is composed of both types of cells previously described (cells with heterogeneously violet-stained nuclei with decreased nuclei/cytoplasm ratio and cells with homogeneously dark blue-stained nuclei and little cytoplasm). The tubular structures are, in turn, surrounded by interstitial tissue containing enlarged cells together with cells with rounded nuclei in the proximities of the tubules (Fig. 2).

VASA-expressing cells were present at all the DSs studied both in TSD-Females and TSD-Males. At stage 22, immune-stained cells were observed both in the cortex and as clusters in the medulla. As development took place, both in TSD-Males and Females, the VASA-positive cells moved away from the external cortical epithelium and occupied more central positions in the developing gonad (Fig. 2).

3.1.2. Expression of hormonal receptors

ERα expression levels in males and females were significantly different at all DSs studied. In TSD-Females, ERα expression showed no changes throughout the DSs evaluated, whereas in TSD-Males, ERα expression was high at DSs 22 and 24 and then decreased significantly at DS 27 (Table 3), thus exhibiting a sexually dimorphic pattern.

Table 3
Expression of biomarkers of gonadal histofunctional differentiation

	DS 22		DS 24		DS 27	
	TSD-Females	TSD-Males	TSD-Females	TSD-Males	TSD-Females	TSD-Males
ERα	8.357 ± 1.048[a,α]	15.94 ± 3.450[b,α]	6.729 ± 0.957[a,α]	17.87 ± 2.315[b,α,]	6.799 ± 0.609[a,α]	2.563 ± 0.528[b,β]
PR	0.402 ± 0.146[a,α]	0.069 ± 0.035[b,α]	0.511 ± 0.255[a,α]	0.351 ± 0.162[a,αβ]	0.864 ± 0.169[a,α]	1.965 ± 0.785[b,β]
Aromatase	15.24 ± 1.321[a,α]	2.428 ± 0.281[b,α]	9.911 ± 1.012[a,αβ]	2.209 ± 0.077[b,αβ]	5.740 ± 0.673[a,β]	1.466 ± 0.243[b,β]
PCNA	0.044 ± 0.005[a,α]	2.239 ± 0.608[b,α]	0.334 ± 0.112[a,β]	0.541 ± 0.053[a,α]	0.142 ± 0.056[a,αβ]	1.787 ± 0.602[b,α]

The values represent the mean ± SEM. The different Latin letters indicate significant differences ($p < 0.05$) between TSD-Females and TSD-Males at each DS. Different Greek letters indicate significant differences ($p < 0.05$) between males and females along the three DSs studied.

PR expression levels were low. Similar to that observed for ERα, in TSD-Females, PR expression remained at a relatively constant level throughout the DSs studied (Table 3), whereas in TSD-Males, PR expression showed a sustained increase as development took place (Table 3).

3.1.3. Aromatase expression

A multi-dotted cytoplasmic pattern of immunostaining compatible with aromatase mitochondrial location was observed in the gonads of *C. latirostris* embryos at all the DSs studied. Both TSD-Females and TSD-Males exhibited a gradually decreasing pattern of aromatase expression as development advanced. However, when compared with TSD-Females, TSD-Males showed significantly lower levels of aromatase expression throughout all the DSs studied (Table 3).

3.1.4. Proliferative activity

As shown in Table 3, at DSs 22 and 27, proliferative activity, evaluated by PCNA expression, was significantly higher in male gonads than in female gonads. TSD-Female gonads showed an increase in the proliferative activity from DS 22 to 24, followed by a decrease at DS 27, whereas male gonads showed no differences in proliferative activity from DS 22 to DS 27.

3.1.5. Isolation of the embryonic gonad from the GAM complex and expression of key developmental genes

The incorporation of the microdissection technique to isolate the gonad from the GAM complex allowed obtaining enough amount of gonad RNA from embryos even at DS 22. Additionally, the use of this

technique prevents the presence of adrenal and mesonephric tissues that could mask differences between males and females at early DSs.

Regarding the expression of the key developmental genes studied, results were as follows:

The relative expression of *amh* in TSD-Females remained low and showed no changes throughout the DSs studied, whereas that in TSD-Males increased significantly from DS 22 to DS 24. In addition, the relative expression of *amh* in TSD-Males was higher than that in TSD-Females throughout the whole period studied (Table 4).

Table 4
Expression of key developmental genes in the gonad of *Caiman latirostris* embryos.

	DS 22		DS 24		DS 27	
	TSD-Females	TSD-Males	TSD-Females	TSD-Males	TSD-Females	TSD-Males
amh	$2.578 \pm 0.525^{a,\alpha}$	$22.04 \pm 3.918^{b,\alpha}$	$1.650 \pm 0.322^{a,\alpha}$	$291.5 \pm 46.32^{b,\beta}$	$1.387 \pm 0.15^{a,\alpha}$	$335.7 \pm 52.55^{b,\beta}$
Cyp19-a1	$0.046 \pm 0.007^{a,\alpha}$	$0.0003 \pm 6e\text{-}005^{b,\alpha}$	$0.012 \pm 0.001^{a,\alpha}$	$0.001 \pm 0.0002^{b,\alpha}$	$1.373 \pm 0.0004^{a,\beta}$	$0.0009 \pm 4e\text{-}005^{b,\alpha}$
Sf-1	$9.230 \pm 1.505^{a,\alpha}$	$1.596 \pm 0.170^{b,\alpha}$	$2.334 \pm 0.385^{a,\beta}$	$3.137 \pm 0.546^{a,\alpha}$	$4.663 \pm 0.676^{a,\alpha\beta}$	$8.752 \pm 1.118^{b,\beta}$
Sox-9	$1.928 \pm 0.088^{a,\alpha\beta}$	$2.191 \pm 0.338^{a,\alpha}$	$1.495 \pm 0.479^{a,\alpha}$	$10.52 \pm 1.489^{b,\beta}$	$2.528 \pm 0.341^{a,\beta}$	$17.52 \pm 1.971^{b,\beta}$

The values represent the mean ± SEM. The different Latin letters indicate significant differences ($p < 0.05$) between TSD-Females and TSD-Males at each stage of embryonic development. Different Greek letters indicate significant differences (< 0.05) between males and females along the three stages studied.

The relative expression of *cyp19-a1* in TSD-Females was higher than that in TSD-Males throughout the three stages studied (Table 4). In addition, in TSD-Females, a significant increase in *cyp19-a1* relative expression was observed from DS 24 to DS27.

The relative expression of *sf-1* in TSD-Females exhibited a U-shaped pattern with its highest level at DS 22 and the lowest at DS 24. Conversely, in TSD-Males, the expression of *sf-1* exhibited a steady increase from DS 22 to DS 27. *sf-1* expression in both TSD-Females and TSD-Males at DS 22 was significantly different from that at DS 27 (Table 4).

Finally, the relative expression of *sox-9* in TSD-Females remained unchanged throughout the stages studied, whereas that in TSD-Males *sox-9* increased significantly as development progressed (Table 4).

3.2. Effects of ATZ on the development and differentiation of the male gonad

3.2.1. Gonadal histology and germ cell distribution

In agreement with that previously reported (Beldomenico et al., 2007), all embryos from eggs incubated at 33°C were males both in the control group (TSD-Males) and in the ATZ-exposed group (ATZ-Males). At DSs 22 and 24, no differences at histological level were observed between ATZ-Males and TSD-Males, whereas at DS 27, the gonads of ATZ-Males exhibited a less organized histoarchitecture. This relative disorganization included poorly defined limits between the epithelium and the surrounding stroma, increased tortuosity of epithelium cords, and wider fluid-filled lumen. The germinal cell distribution in the gonad of ATZ-Males did not differ from that observed in TSD-Males.

3.2.2 Estrogen and progesterone receptors

Exposure to ATZ induced a significant decrease in the expression of both ER and PR at DS 24. No differences were observed at DS 22 or DS 27 (Fig. 2).

3.2.3 Aromatase

ATZ exposure dramatically increased the expression of aromatase in the ATZ-Males gonads. When compared with TSD-Males, ATZ-Males showed significantly higher levels of gonadal aromatase expression throughout the whole period studied (Fig. 4).

3.2.4 Proliferative activity

Exposure to ATZ significantly induced cell proliferation in ATZ-Male gonads at DS 22 (Fig. 5).

3.2.5 Expression of key developmental genes

Regarding the expression of the key developmental genes studied, results were as follows: ATZ exposure induced an up-regulation of *amh* expression at DSs 22 and 24. No significant differences in the expression of *cyp19-a1* were observed between TSD-Males and ATZ-Males throughout the different DSs studied. However, ATZ-Males tended to show a slightly increased expression of *cyp19-a1*.

ATZ exposure did not alter the pattern of *sf-1* expression found in TSD-Male gonads and no differences in *sf-1* expression levels were found between ATZ-Males and TSD-Males throughout the period studied.

While the relative expression of *sox-9* in TSD-Males significantly increased as development progressed, that in ATZ-Males showed a significant increase at DS 24, which differed from that found in TSD-Males at the same DS but was similar to that found at DS 27 (Fig. 6).

4. Discussion

Our findings demonstrate that, in *C. latirostris* embryos, sexually dimorphic expression of histofunctional biomarkers and key developmental genes precedes gonad histomorphological differences. They also show that *in ovo* exposure to ATZ at developmental stage of temperature sex determination induces changes in the expression of histofunctional biomarkers and genes. Although no changes were observed in testicular differentiation after *in ovo* ATZ exposure, disruption of testicular histoarchitecture was observed.

4.1. Temperature-induced gonadal development and differentiation

The embryonic gonad of *C. latirostris* shows the same compartmentalization pattern and general histological characteristics as those described for *Alligator mississippiensis* (Smith and Joss, 1994). Although no differences were observed in the histoarchitecture of TSD-Males and TSD-Females at DS 22, at this early developmental stage most of the genes and proteins evaluated here showed sexually dimorphic expression. This suggests that, at DS 22, the gonads of *C. latirostris* overrode the bisexual period in which the developing gonad shows both characteristics of males and females. As caiman embryo development takes place, the histoarchitecture acquires different features in male and female gonads. At DS 24, TSD-Male gonads present tubular structures -seminiferous tubules precursors-. As demonstrated in Canesini et al. (2018), the VASA-expressing cells, present in tubular structures, correspond with medullar cells morphologically described as cells with heterogeneously violet-stained nuclei and decreased nuclei/cytoplasm ratio. Although this type of cells shows many similarities to those described as pre-Sertoli cells for *A. mississippiensis* (Smith and Joss, 1993), in *C. latirostris* the expression of VASA denotes their germinal lineage. At DS 27, the presence of seminiferous tubules that exhibit a characteristic lumen and the absence of the Müller ducts confirm the gonadal male phenotype.

In TSD species, temperature-induced changes include changes in the expression of steroid hormone receptors. Thus, here we studied the effect of the incubation temperature on the expression of ERα and PR. In TSD-Female gonads, ERα expression remained unchanged throughout the DSs studied. In contrast, TSD-Male gonads showed a period of high ERα expression that spread over DSs 22 and 24, followed by a period of low ERα expression at DS 27. It has been reported that both increased and depleted levels of estrogen can upregulate ER levels (Hagenfeldt and Eriksson, 1988; Prins and Birch, 1997; Tran et al., 2016). Sex steroid levels in the developing embryo are unknown. The levels of steroid hormones in the chorioallantois fluid were reported to be a useful tool for estimating the circulating steroid levels in turtles (Gross et al., 1995). However, this approach was not useful to assess embryonic hormonal environment in alligators (Crain et al. 1997) and probably would not be for *C. latirostris.* As far as we know, no steroid synthesis has been reported in crocodilian gonads at morphologically undifferentiated developmental stages. Thus, we could speculate that, in *C. latirostris*, at the earliest DS, low estrogen levels rather than high estrogen levels may be involved in the regulation of gonadal ERα expression. Increased ERα expression could be a strategy to enhance tissue sensitivity to low estrogen levels. In mice, it has been found that estrogens, acting through highly expressed ERα, might promote normal development and

function of the male reproductive tract (Hess and Cooke, 2018). Consequently, in male *C. latirostris*, the high ERα expression period here observed over DSs 22 and 24 would be critical for normal gonadal development and function. Additionally, our findings confirm that the earlier DSs of *C. latirostris* are highly sensitive to subtle changes in the levels of estrogens or xenoestrogens (Stoker et al., 2003). Conversely, during DS 27, when the gonad is morphologically differentiated, ERα expression decreased significantly. Male gonad development requires of both ERα and androgen receptors (AR), and the balance between androgen and estrogen (acting through their respective receptors) is of central importance in male reproductive development and function (Hess and Cooke, 2018). Thus, different DSs may be characterized by a different estrogen:androgen balance and a different ERα:AR expression ratio. At DS 27, androgen synthesis by the male-differentiated gonad may modify the estrogen:androgen balance and ERα expression.

Regarding PR, this receptor is considered a target molecule of estrogenic action and its regulation differs between species (Boyd-Leinen et al., 1984; Giannoukos and Callard, 1996; Hora et al., 1986; Schultz et al., 2003). Our results showed that, as expected, in TSD-Females, PR expression follows the same pattern as ERα. Conversely, in TSD-Males, increased PR expression corresponds with the low ERα expression when estrogenic action is supposed to be decreased. This controversy could be explained by the complex PR regulation in reptiles. As it has been previously reported, PR levels could be regulated by other factors besides estrogen action (Gist, 2011; Jones, 2011).

In TSD species, steroidogenic enzymes have been suggested to be temperature-regulated. The differential expression of aromatase between TSD-Females and TSD-Males confirmed this for *C. latirostris*. The high aromatase levels in TSD-Females were observed throughout the whole period studied. In *Crocodylus porosus* and *A. mississippiensis*, the aromatase levels observed in female embryos remain low up to the end of the thermosensitive period and, then, dramatically increases (Gabriel et al., 2001; Smith and Joss, 1994), suggesting a role in gonadal differentiation but not necessarily in sex determination. In *C. latirostris*, this role is supported by the female-related high aromatase expression levels observed during the DS characterized by a morphologically differentiated gonad. However, these female-related high levels of aromatase expression were observed even during the thermosensitive period. This result suggests that, in *C. latirostris*, aromatase expression could be a key factor not only for gonadal female determination but also for sex differentiation. Additionally, our findings suggest that, in *C. latirostris*, the levels of aromatase expression would be a reliable sign of gonadal determination towards the female phenotype.

Developmental processes involve cell proliferation and differentiation. Regarding this, Zhu et al. (2009) stated that cell proliferation and differentiation often act as two mutually exclusive partners that are related but cannot coexist. In this context, the gonadal increased proliferative activity here observed in TSD-Females at DS 24 suggests that the gonad is in a phase more related to growth than to differentiation. Noteworthy, no differences in proliferative activity were found for TSD-Males during the DSs studied, suggesting that differentiation rather than proliferation govern this developmental period in males.

In the present work, we also studied four key genes involved in sexual determination and gonad differentiation, *amh*, *sox-9*, *sf-1* and *cyp19-a1*. To this aim, gonadal tissue was isolated from the GAM complex using the microdissection technique previously described by Lazzarino et al. (2019). The microdissection technique allows working with gonadal tissue, avoiding the use of adrenal or mesonephric tissue. Thus, results are representative of the gonad and differences between males and females, when present, are clearer. Noteworthy, *amh* showed clear sexual dimorphism throughout the whole developmental period studied, being several orders of magnitude higher in TSD-Males than in TSD-Females. The *amh* gene encodes for the anti-Müllerian hormone (AMH), which is involved in male gonad differentiation by causing degeneration of Müllerian ducts in the embryo bipotential gonad. This mechanism of gonadal differentiation highly conserved among vertebrates is also present in *C. latirostris* and seems to be the hallmark of sexual male determination. In addition, *amh* expression significantly increased from DS 22 (when the gonad is morphologically classified as bisexual) towards DS 24 (when sexual differentiation has begun). Noteworthy, at DS 24, we observed a significant increase in the *sox-9* mRNA level in TSD-Male gonads. *Sox9* is a gene that encodes for the transcription factor SOX9, which in turn, activates the transcription of *amh* and, consequently, promotes the development of testes in all vertebrates examined to date (Morrish and Sinclair, 2002). Additionally, in alligators, the increase in *amh* levels precedes that in *sox-9* (Warner, 2011), suggesting that other genes besides *sox-9* may regulate the increase in *amh*. In *C. latirostris*, we confirmed this finding. We propose that although *sox-9* is not necessary for the increase in *amh* levels during the bisexual gonadal stage, it is involved in the further upregulation of *amh* during the gonadal differentiation into testis. Moreover, molecular cloning studies of *amh* in *A. mississippiensis* have confirmed that *sox-9* up-regulates transcriptional activity through the *amh* promoter region (Urushitani et al., 2011). A similar mechanism could be operating in *C. latirostris*.

In addition to *sox-9*, *sf-1* has been described as a positive regulator of *amh* (Warner, 2011). Our results support this role for *sf-1*. We hypothesize that, during DS 27, *sf-1*, together with *sox-9*, upregulates *amh* expression, maintaining high levels of AMH, necessary for gonadal male differentiation. Nevertheless, *sf-1* expression was also found increased in TSD-Female gonads at DS 22, suggesting that *sf-1* could be related to other functions. In fact, *sf-1* regulates the expression of gonadal steroidogenic enzymes, including aromatase (Parker and Schimmer, 1997). In TSD-Females, the highest level of *sf-1* found coincided with increased *cyp19-a1* expression levels and the highest aromatase expression levels. Taken together, our results support the idea that *sf-1* has different functions at different DSs of embryos developed at the MPT or FPT. While, at the earlier DSs of embryos developed at the FPT, *sf-1* is involved in the upregulation of aromatase expression, at the latest DSs of embryos developed at the MPT, *sf-1* is involved in the upregulation of *amh* expression.

4.2 Effects of ATZ exposure on gonadal development and differentiation

The histomorphological alterations observed in ATZ-Male gonads at DS 27 are similar to those previously reported in 10-day-old caimans with the same protocol of exposure (Rey et al., 2009). This suggests that

the altered testicular histoarchitecture observed postnatally is a consequence of the gonadal disruptive effect of ATZ initiated during embryo development.

The dramatic increase in aromatase expression here observed in ATZ-Males throughout the DSs studied is in concordance with the reported role of ATZ as a xenoestrogen in different species, acting through increased aromatase induction (Hayes et al., 2011, 2006). In alligator male hatchlings, for example, ATZ has been found to induce GAM aromatase activity that was characteristic neither of males nor of females, but did not alter testicular differentiation (Crain et al., 1997). In our model, increased aromatase expression would increase androgen-to-estrogen conversion, creating an estrogenic gonadal microenvironment in ATZ-Males that would be responsible for downregulating ERα expression, as observed at DS 24. The reduced period of high ERα expression, previously defined as critical for normal gonadal development and function in male *C. latirostris*, could affect estrogen-depending developmental processes involved in the sexual differentiation of the gonad. The decreased PR expression levels here observed at DS 24 in ATZ-Males may be interpreted as a consequence of the altered estrogen signaling pathway. Thus, changes in PR could suggest that other estrogen-induced molecules which have a role in proper gonad differentiation could be affected.

ATZ exposure also increased the expression of *amh* (at DSs 22 and 24) and *sox-9* (at DS 24). These genes are part of a highly conserved pattern of gonadal development in vertebrate species known as the male pathway (Hirst et al., 2018). Surprisingly, regarding the expression of *amh* and *sox*-9, ATZ exposure seems to support the male phenotypic pathway rather than the female one. Although these findings may lead to hypothesize that the increased expression of *amh* and *sox-9* could result in a protective response against the feminizing effect of ATZ, different studies on developmental processes highlight the importance of the temporal and spatial expression pattern of key molecules. In chicken, for example, AMH overexpression has been found to lead to alterations in the number of Sertoli cells and steroidogenesis (Lambeth et al., 2016). In this sense, for developmental processes, it is critical that "the right molecule is expressed at the right level at the right time" (Ferraro et al., 2016). Thus, subtle alterations in the developmental programming could lead to impaired reproductive function. In the present work, ATZ exposure altered the temporal pattern of expression of both *amh* and *sox-9*. Thus, histofunctional alterations as a consequence of ATZ-induced changes in the levels of expression of *amh* in ATZ-Male caimans cannot be ruled out.

Finally, it is important to highlight that ATZ could cause greater endocrine disruption in caimans hatched in the wild than in those hatched in controlled laboratory conditions. This is because, in the wild, eggs are also incubated at intermediate temperatures that produce both males and females (Parachú Marcó et al., 2017; Simoncini et al., 2019). Thus, our results highlight the vulnerability of developing organisms and alert about ATZ-mediated developmental disruption in wildlife.

5. Conclusions

Exposure of non-target organisms to substances classified as EDCs used in productive processes -such as ATZ in weed control- during critical periods of development might cause subtle changes at gene and/or protein expression level that might lead to impairments of physiological processes in later stages of life. Studying those physiological processes and the effects of EDCs exposure in organisms that behave as sentinel species, like *C. latirostris*, allow us to identify potential early targets of endocrine disruption and to alert about the deleterious effects that developmental exposure to EDCs might induce both in wildlife and humans.

Declarations

Acknowledgments

Ethical Approval

The protocols used in this work were previously authorized by the Institutional Committee of Bioethics in Animal Care and Use of the Universidad Nacional del Litoral, Santa Fe, Argentina.

Consent to participate

Not applicable.

Consent to publish

Not applicable.

Author´s contribution statement

All authors contributed to the study conception and design. Conceptualization: Guillermina Canesini, Germán H. Galoppo, Jorge G. Ramos and Mónica Muñoz-de-Toro; Methodology:[Guillermina Canesini, Gisela P. Lazzarino and Cora Stoker; Formal analysis and investigation: Guillermina Canesini, and Germán H. Galoppo; Writing - original draft preparation: Guillermina Canesini, Germán H. Galoppo, Yamil E. Tavalieri; Visualization: Guillermina Canesini and Yamil E. Tavalieri; Writing - review and editing: Germán H. Galoppo, Yamil E. Tavalieri, Mónica Muñoz-de-Toro; Funding acquisition: Enrique H. Luque and Mónica Muñoz-de-Toro; Resources: Mónica Muñoz-de-Toro; Supervision: Cora Stoker, Enrique H. Hugo, Jorge G. Ramos and Mónica Muñoz-de-Toro. All authors read and approved the final manuscript.

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Availability of data and materials

The datasets compiled and analyzed in the present study are available from the corresponding author on reasonable request.

Compliance with ethical standards

All procedures and techniques used for egg collection, embryo handling and sampling were performed according to the guidelines of the American Society of Ichthyologists and Herpetologists (ASIH, 2004). All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Research Council (USA) (IPCS, 2002).

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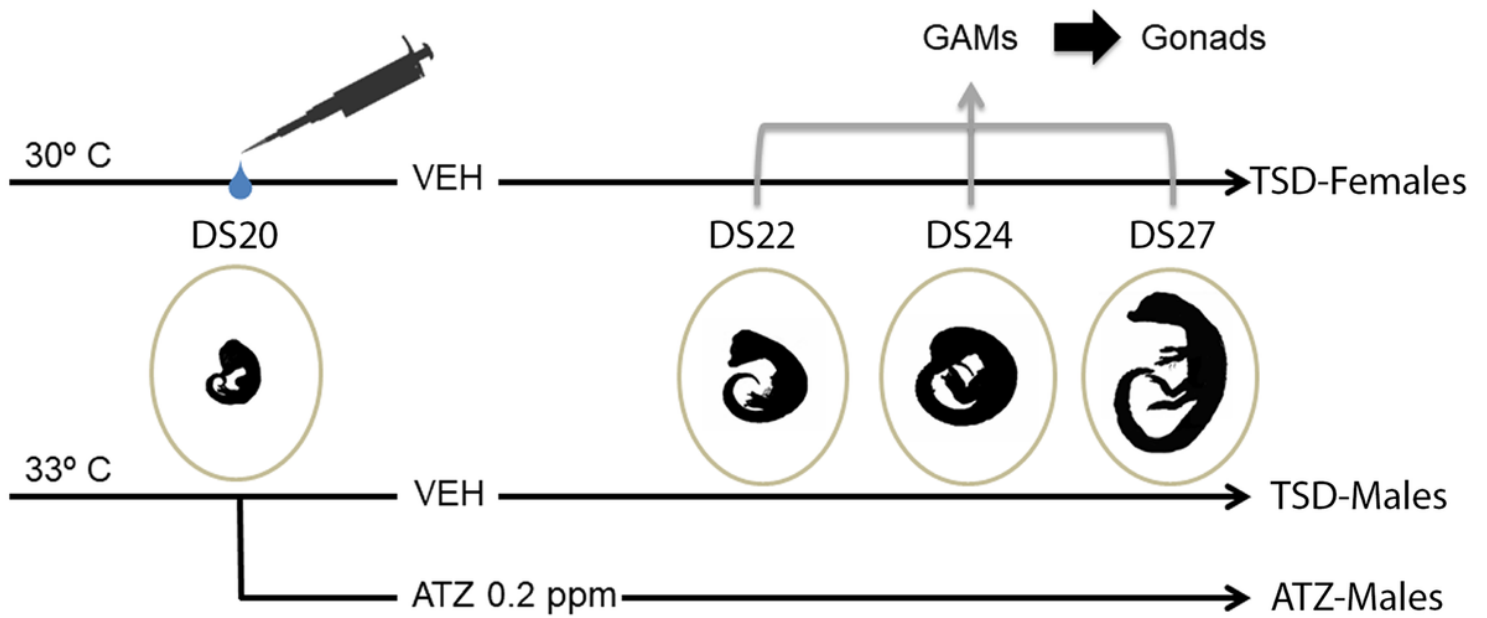
Figures

Figure 1
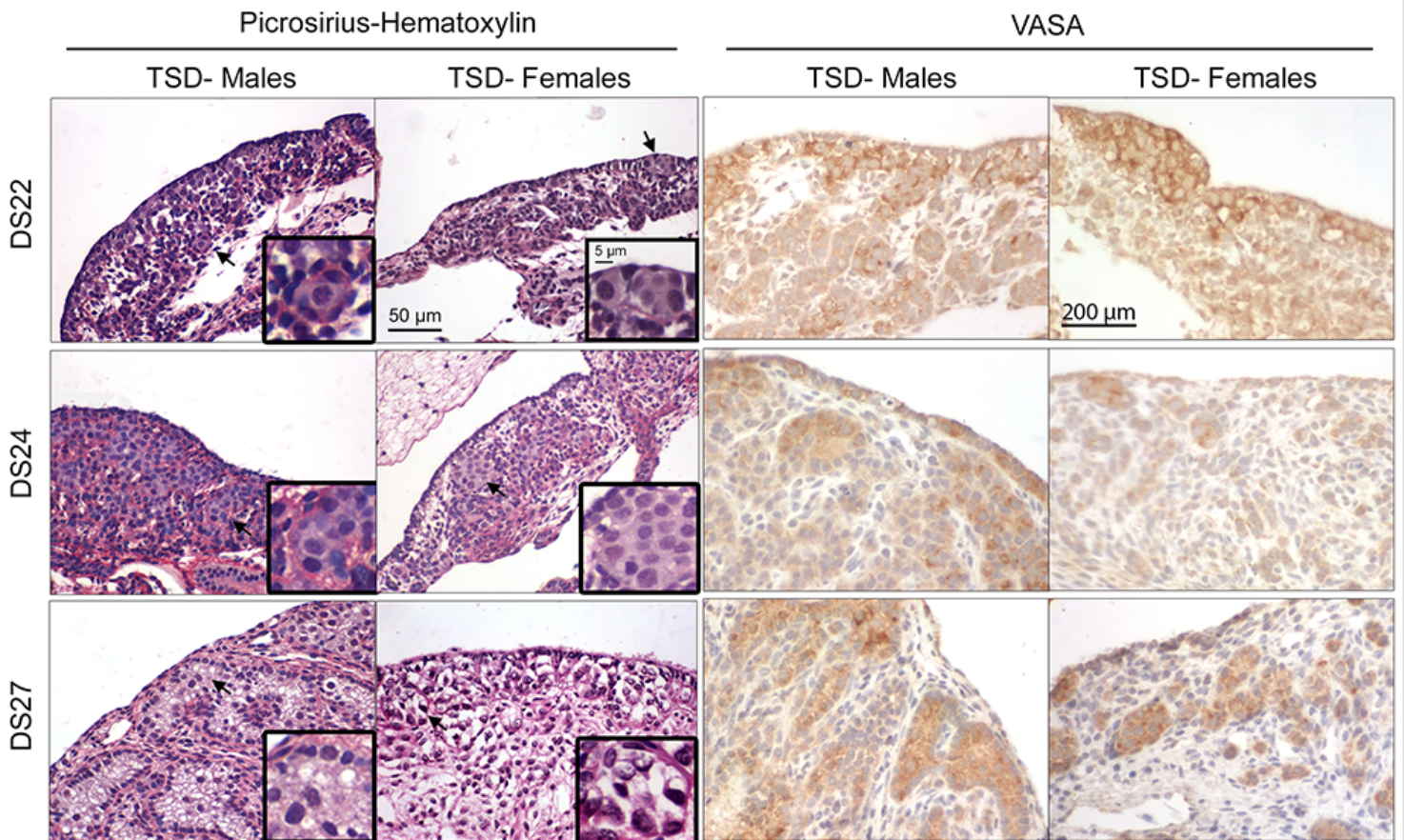
Scheme of experimental design and treatments.

Figure 2

Histological features of the caiman of the developing caiman (Left panel) and germ cell distribution (VASA-positive) (Right panel).
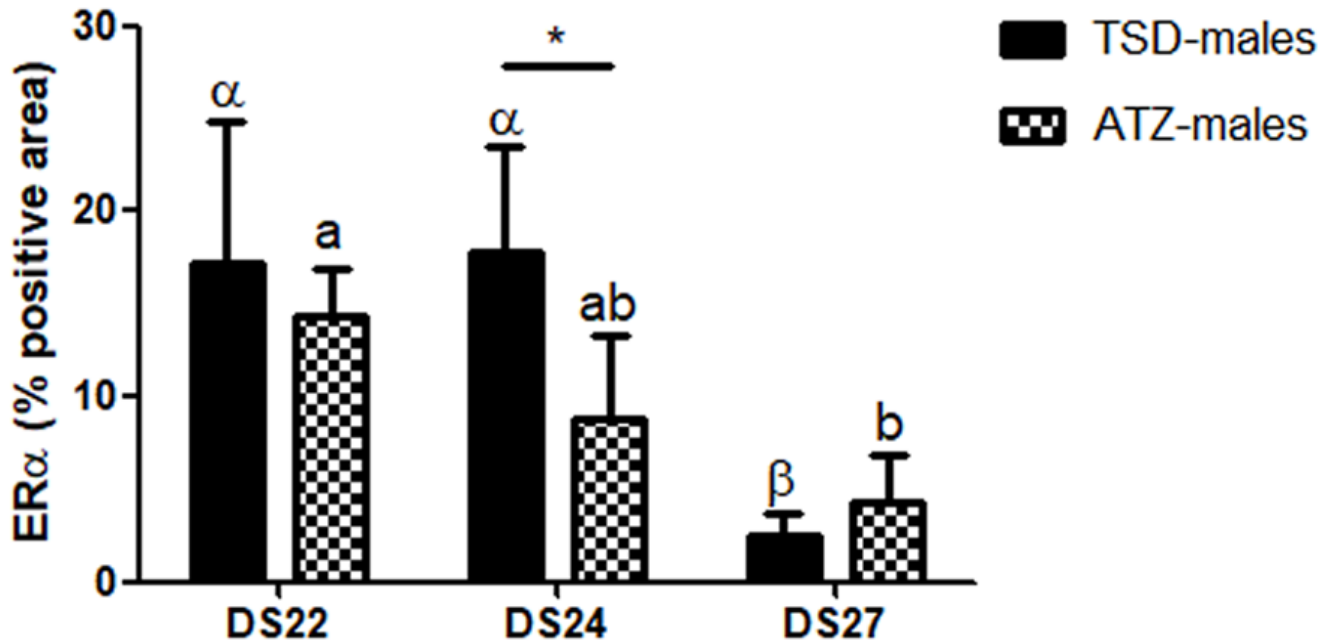

Figure 3

Effect of ATZ exposure on the expression of ERα and PR in the embryonic male gonads of *C. latirostris.* Bars represent mean ± SEM. Greek letters show statistical differences (p<0.05) between TSD-Males. Latin letters show statistical differences between ATZ-Males. Asterisks above the brackets represent statistical differences between TSD-Males and ATZ-Males at the same developmental stage.
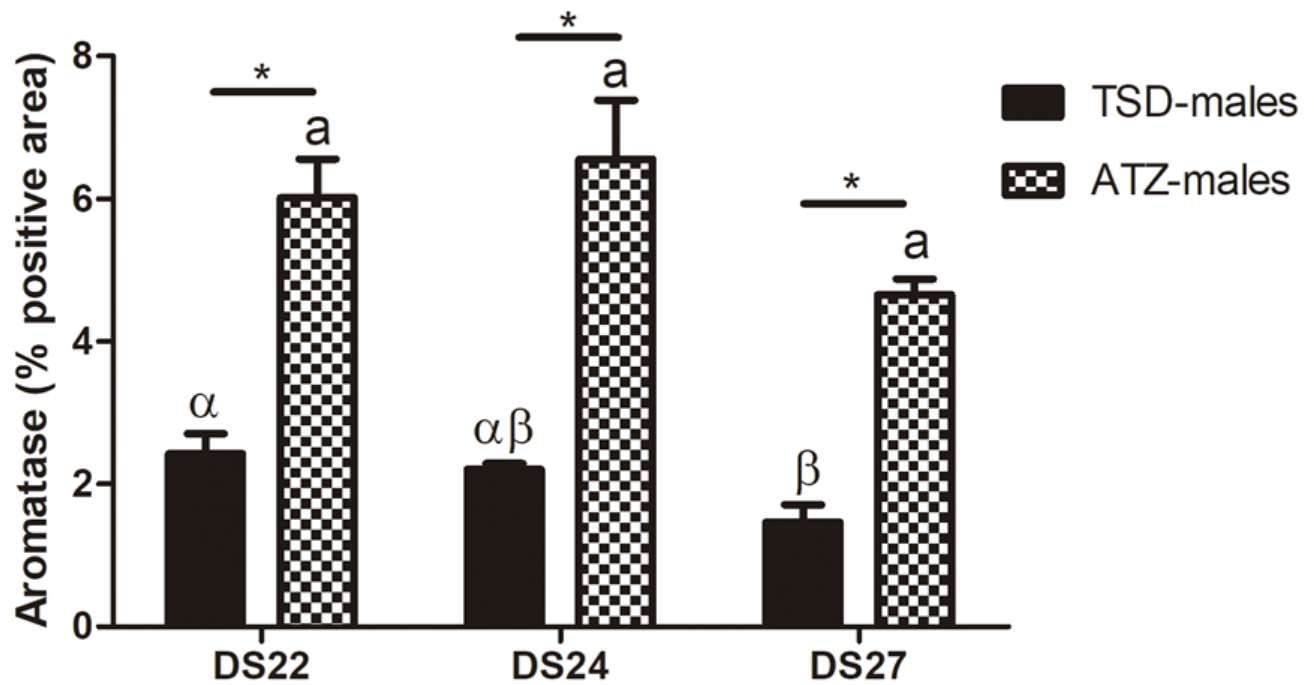
Figure 4

Effect of ATZ exposure on aromatase expression in the developing gonads of *C. latirostris*. The bars represent mean ± SEM. The letters above the bars represent statistical differences (p<0.05) between animals belonging to the same experimental group at different developmental stages. Greek letters show statistical differences between TSD-Males. Latin letters show statistical differences between ATZ-Males. Asterisks above the brackets represent statistical differences between TSD-Males and ATZ-Males.
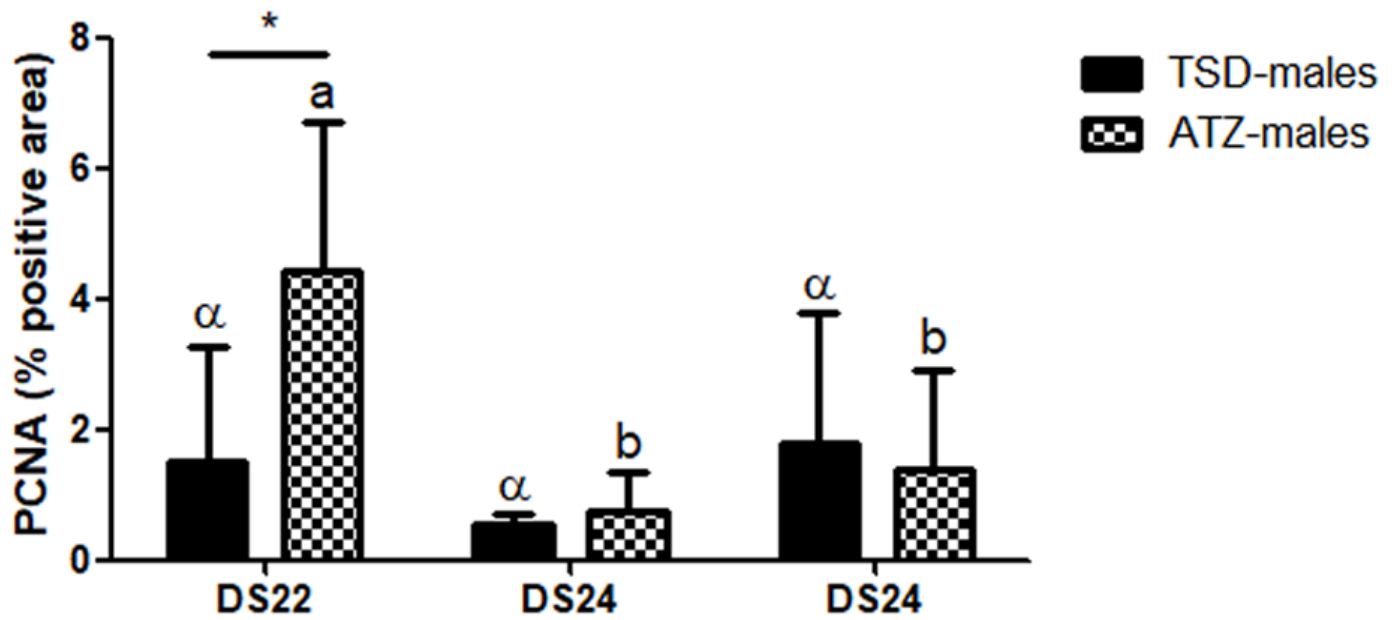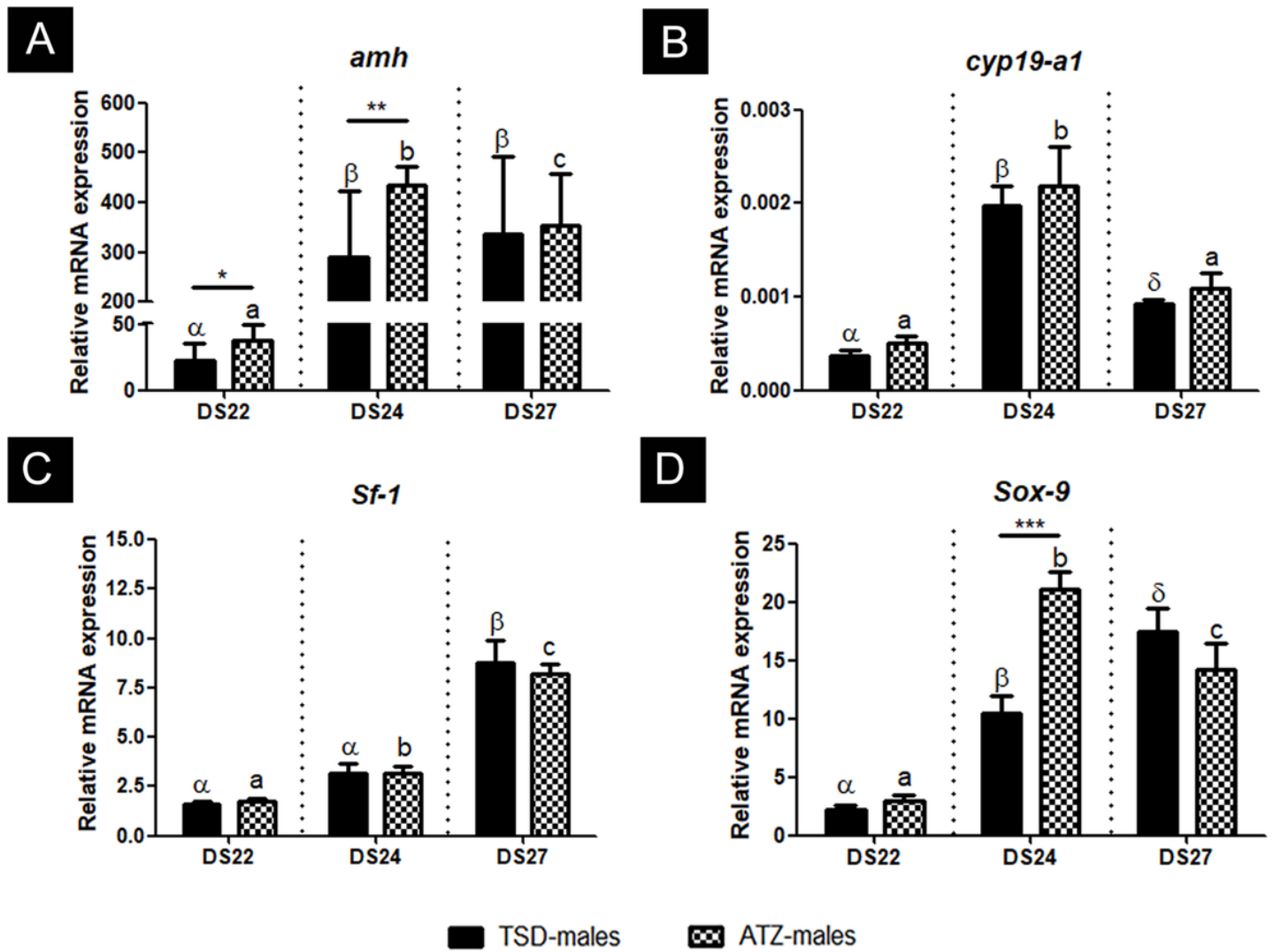
Figure 5

Effects of ATZ exposure on proliferative activity in the developing gonads of *C. latirostris*. Bars represent the mean ± SEM. Different Greek letters above the bars represent statistical differences (p<0.05) between TSD-Males at different developmental stages. Different Latin letters above the bars show statistical differences between ATZ-Males at different developmental stages. The asterisk above the brackets represents significant differences between DST-Males and ATZ-Males at the same stage of development.

Figure 6

Effect of ATZ exposure on the expression of key genes involved in *C. latirostris* embryo sex differentiation. **A:** *amh*, **B:** *Cyp19-a1* **C:** *Sf-1* **D:** *Sox-9*. Values represent the mean ± SEM. Asterisks indicate significant differences (p<0.05) between TSD-males and ATZ-males at each stage of embryonic development. Greek letters show differences between TSD-Males. Latin letters show differences between ATZ-Males.