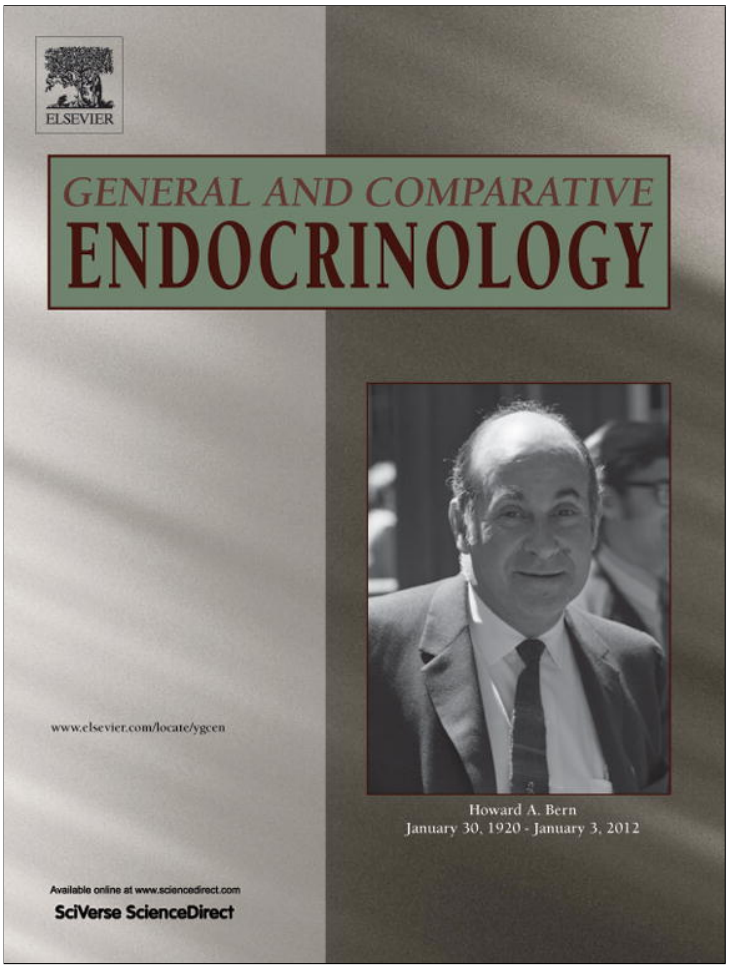

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Neonatal expression of *amh*, *sox9* and *sf-1* mRNA in *Caiman latirostris* and effects of *in ovo* exposure to endocrine disrupting chemicals

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ABSTRACT

Caiman latirostris is a reptilian species that exhibits temperature-dependent sex determination (TSD). Male-to-female sex reversal can be achieved after *in ovo* estrogen/xenoestrogen exposure. This is known as hormone-dependent sex determination (HSD). The *amh*, *sox9* and *sf-1* genes are involved in sex determination, sex differentiation, and steroidogenesis. The aims of this study were: (a) to establish the expression patterns of *amh*, *sox9* and *sf-1* mRNA in the gonad-adrenal-mesonephros (GAM) complexes of neonatal TSD-male and TSD-female caimans, (b) to compare the expression of these genes between TSD-females and HSD-females (born from E_2-exposed eggs incubated at the male-producing temperature) and (c) to evaluate whether *in ovo* exposure to a low dose of E_2 or bisphenol A (BPA) or to a high dose of endosulfan (END) modifies *amh*, *sox9* or *sf-1* mRNA expressions in neonatal males. The mRNA expressions of *amh*, *sox9* and *sf-1* in GAM complexes from TSD-males and TSD-females and from HSD-females were quantitatively compared by RT-PCR. A sexually dimorphic pattern of *amh* and *sox9* mRNA expression was found, with a higher expression in TSD-males than in TSD-females. *sf-1* mRNA did not differ between TSD-males and TSD-females. HSD-females exhibited a higher expression of *sox9* than TSD-females. In males, increased mRNA expression of sex-determining genes was observed after *in ovo* exposure to END. E_2 decreased *sox9* but increased *sf-1* mRNA expression. Changes induced by BPA were evident although not significant. These results provide new insights into the potential mechanisms that lead to the gonadal histo-functional alterations observed in caimans exposed to contaminated environments.

1. Introduction

The sex of most turtles and all species of crocodilians is determined by the environment after fertilization. In these reptiles, the temperature of the eggs during a certain period of development is the deciding factor in determining sex (Gilbert, 2000). In these species with temperature-dependent sex determination (TSD), the incubation temperature of the egg during the middle third of development (called the thermosensitive-period (TSP)) establishes sex, as it occurs in all crocodilians, many turtles and some lizards (Lang and Andrews, 1994). Previously, we have demonstrated that *Caiman latirostris* is one of the reptilian species that exhibits TSD, since we confirmed that sex determination is temperature-dependent: females were born from all eggs incubated at 30 °C (TSD-females), whereas males were born from those eggs incubated at 33 °C (TSD-males) (Rey et al., 2009; Stoker et al., 2003, 2008).

Estrogen can override temperature and induce ovarian differentiation even at masculinizing temperatures (Gilbert, 2000). In fact, experimental *in ovo* exposure to 17β-estradiol (E_2) overrides the effects of male incubation temperature, producing phenotypic females (defined as hormone-dependent sex determination; HSD-females) in the turtle *Trachemys scripta*, in *Alligator mississippiensis* and in *C. latirostris* (Crain et al., 1997; Crews et al., 1996; Milnes et al., 2002; Stoker et al., 2003, 2008).

In mammals, the sex-determining region of the Y chromosome (*SRY*), the main sex-determining gene, acts as a switch that initiates the testis-determining pathway (Koopman et al., 1991; Sinclair et al., 1990). However, a master sex-determining switch has not been identified in reptiles. In contrast, many temperature-sensitive genes have been involved in gonadal differentiation in TSD species (Warner, 2011). In alligators, the expression of *sox9* (*SRY*-like HMG box) is upregulated in gonads of embryos exposed to male-producing temperatures, although it occurs after the initiation of male gonadal differentiation (Western et al., 1999a). *sox9*

Abbreviations: TSD, temperature-dependent sex determination; TSP, thermosensitive-period; E_2, 17β-estradiol; HSD, hormone-dependent sex determination; *SRY*, sex-determining region of the Y chromosome; *sox9*, *SRY*-like HMG box; AMH, anti-Müllerian hormone; *sf-1*, steroidogenic factor-1; EDCs, endocrine-disrupting chemicals; BPA, bisphenol A; END, endosulfan; GAM, gonad-adrenal-mesonephros; M-MLV, Moloney Murine Leukemia Virus.

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has been associated with testis development in all vertebrates examined to date (Morais da Silva et al., 1996; Morrish and Sinclair, 2002) and it is known that estrogen represses *sox9*. However, the mechanism of this repression is different in each species (Barske and Capel, 2010; Mork and Capel, 2010).

Anti-Müllerian hormone (AMH) induces regression of the Müllerian ducts during male development and is one of the genes earliest expressed by Sertoli cells during testis development in the mouse (Münsterberg and Lovell-Badge, 1991). The relationship between *amh* and *sox9* has been hypothesized in mammals, where *SRY* acts with *sox9* early during testis determination to activate male-specific genes such as *amh* (Kent et al., 1996; Morais da Silva et al., 1996). Interestingly, this up-regulation of *amh* has not been found in alligators, where *amh* expression precedes *sox9* expression, suggesting that other genes may regulate *amh* (Western et al., 1999b).

Steroidogenic factor-1 (*sf-1*) is an important regulator of steroidogenesis and has been shown to play a developmental role in gonadogenesis (Schimmer and White, 2010). In mammals, after the initial stages of gonadal differentiation, *sf-1* is strongly expressed in the testis and down-regulated in the ovary (Schimmer and White, 2010); in contrast, *sf-1* appears to be expressed at higher level in the developing ovary than in the testis of chickens and alligators (Western et al., 2000).

Chemical contamination of the environment is a global concern. Human and wildlife are exposed to numerous hormonally active pollutants commonly known as endocrine-disrupting chemicals (EDCs) (National Research Council, 1999). Exposure to EDCs can alter development, reproduction and endocrine physiology, and convincing data link these EDCs to serious health effects on wildlife and experimental animals (Beldomenico et al., 2007; Cooke et al., 2010; Hamlin and Guillette, 2011; Hayes et al., 2011; Johns et al., 2011; Stoker et al., 2011). Bisphenol A (BPA), a chemical present in everyday-used items, has shown estrogenic and antiandrogenic properties (Richter et al., 2007; Wetherill et al., 2007). Since BPA has been found in food, water and sediments (Fromme et al., 2002), it poses a significant potential risk for human and wildlife (Flint et al., 2012). Endosulfan (END), a manufactured organochlorine pesticide classified as an EDC, has shown estrogenic activity (Bisson and Hontela, 2002; Gormley and Teather, 2003; Varayoud et al., 2008). Previously, we have observed that *in ovo* exposure to E_2, BPA or END affects the ovarian follicular dynamics, and the proliferation/apoptosis balance of testicular cells of hatchlings and steroid hormone circulating levels in *C. latirostris* (Rey et al., 2009; Stoker et al., 2008).

Temporal and spatial expression patterns of genes associated with sex determination and/or sex differentiation can differ according to the species (Morrish and Sinclair, 2002) and could be modulated by developmental exposure to EDCs (Fan et al., 2007; Kohno et al., 2008; Varayoud et al., 2008). However, no data on the expression of genes associated with sex determination and/or sex differentiation have been described in *C. latirostris*. Thus, the aims of this study were: (a) to characterize the expression pattern of *amh*, *sox9* and *sf-1* mRNA in the gonad–adrenal–mesonephros (GAM) complex, by evaluating whether the expression of these genes is sexually dimorphic in neonatal *C. latirostris*; (b) to compare the expression of *amh*, *sox9* and *sf-1* mRNA between TSD-females and HSD-females; and (c) to establish whether *in ovo* exposure to a low dose of E_2 or BPA or to a high dose of END modifies *amh*, *sox9* or *sf-1* mRNA expressions in neonatal TSD-males.

2. Materials and methods

2.1. Animals

All laboratory and field experiments were conducted according to the published guidelines for use of live amphibians and reptiles in field and laboratory research (ASIH, 2004), and in full compliance with the Institutional Animal Care and Use Committee of Universidad Nacional del Litoral (Santa Fe, Argentina).

In 2008 and 2011, four wild clutches/year (n = 148 and 136 eggs, respectively) were collected within the first two weeks after oviposition. The nesting area, localized in the protected area Reserva Natural "El Cachapé", Chaco Province, in northeastern Argentina, is characterized by low anthropogenic intervention. Eggs collected in the 2008 season were destined to Experiment I and those collected in the 2011 season were destined to Experiment II.

All eggs were transported to the laboratory maintaining the original position (upside eggshell was marked with graphite). At the laboratory, the eggs of each clutch were randomly allocated into two incubators set at 30 °C and 33 °C (Beldomenico et al., 2007; Rey et al., 2009; Stoker et al., 2003). Eggs from each clutch were assigned to each experimental group and the remaining eggs were assigned to other experiments. All treatments (Table 1) were applied topically in a single dose to the eggshell at stage 20 of embryonic development, as previously reported (Rey et al., 2009; Stoker et al., 2003, 2008).

2.2. Assessment of developmental stage

The criteria defined by Ferguson (1985) were followed to define developmental age of *C. latirostris* embryos. At the moment of collection, one egg from each clutch was opened in the field in order to establish the age of the embryo. Only the nests with embryos in stages lower than 15 were transported to the lab for this study. Two days after the eggs arrived at the lab, one egg from each clutch was opened in order to check for synchronization. The procedure was repeated at both incubation temperatures every 7 days up to the point when embryos showed the features characteristic of stage 20 (Stoker et al., 2003).

2.3. Experimental design and sample collection

Experiment I was designed to characterize the *amh*, *sox9* and *sf-1* mRNA expression in TSD-males *versus* TSD-females, and to establish similarities or differences in expression patterns of these genes between TSD-females and HSD-females. For these purposes, seven eggs per group received the treatments detailed in Table 1.

Experiment II was designed to evaluate whether the expression of *amh*, *sox9* and *sf-1* mRNA is affected in TSD-males by *in ovo* exposure to E_2, BPA or END that were selected based on previous results, which showed that 0.014 ppm of E_2, 1.4 ppm of BPA and 20 ppm of END do not cause sex reversal (Rey et al., 2009; Stoker et al., 2003). Eggs (n = 14/group) were incubated at 33 °C and allocated to the groups detailed in Table 1. Six animals/group were sacrificed at 10 days of age, while 4 animals/group were sacrificed at 90 days of age and juvenile.

In Experiment I, caimans were euthanized by sodium pentobarbital at 10 days of age (body mass: 48.5 ± 0.45 g). In Experiment II, all animals were euthanized by sodium pentobarbital (10 days old, body mass: 47.1 ± 1.23 g; 90 days old, body mass: 166.3 ± 9.75 g; or older than 12 months of age (juvenile), body mass: 1888.5 ± 450.0 g). These caimans were raised under controlled conditions and fed three times a week with an amount of food that represented 15% of their mass, until sacrificed. For more details regarding housing facilities, see Zayas et al. (2011).

At sacrifice, both GAM complexes were dissected. Unlike that of turtles (Ramsey and Crews, 2007), the GAM complex of neonatal caimans and alligators is a structure with morphological features that prevent a clean dissection (Kohno et al., 2010). Thus, at 10 days of age the GAM complexes were processed while at 90 days of age and in juveniles the gonads were dissected from the GAMs. The left GAM complexes or the left gonads were

Table 1
Experimental groups.

Experiment	Treatment[a] (*n*)	Incubation temperature	Gonadal sex[b]
Experiment I	Control/vehicle[c] (7)	30 °C	Female
	Control/vehicle[c] (7)	33 °C	Male
	E_2: 1.4 ppm[d] (7) (Sigma)	33 °C	Female
Experiment II	Control/vehicle[c] (14)	33 °C	Male
	E_2: 0.014 ppm (14) (Sigma)	33 °C	Male
	BPA: 1.4 ppm (14) (Aldrich, Milwaukee, WI)	33 °C	Male
	END: 20 ppm (14) (Icona S.A. Argentina)	33 °C	Male

(*n*) Number of eggs. In each experiment, *Caiman latirostris* eggs came from four wild clutches.
[a] All treatment were applied topically to the eggshell on stage 20 of embryonic development.
[b] Gonadal sex was determined by histological examination.
[c] Vehicle, 50 μl ethanol.
[d] E_2 dose that overrides temperature effect and provokes 100% females at hatching (HSD-females) (Stoker et al., 2003).

immediately frozen in liquid nitrogen and then conserved at −80 °C until used (for the present study or future studies), while samples from the right were fixed by immersion in 10% phosphate-buffered formalin (pH 7.4) for 6 h at room temperature. Fixed tissues were dehydrated in ascending series of ethanol, cleared in xylene, and embedded in paraffin.

2.4. Gonadal histology

Gonadal sex was determined by histological examination. Serial sections (5 μm) of the right GAM complex or the right gonad were cut in transverse planes and stained with trichromic picrosirius/hematoxylin staining, as previously described (Rey et al., 2009; Stoker et al., 2003, 2008).

2.5. Real-time PCR analysis

An optimized reverse transcription-PCR protocol was used to analyze the relative expression levels of *amh*, *sox9* and *sf-1* in the left GAM, as previously described (Kass et al., 2012; Varayoud et al., 2008). In brief, an individual GAM from each animal was homogenized in TRIzol (Invitrogen, Carlsbad, CA), and RNA was prepared according to the manufacturer's protocol. The concentration and purity of total RNA was determined by measuring the optical density at 260 and 280 nm, and the samples were stored at −80 °C until needed. Equal quantities (4 μg) of total RNA were reverse-transcribed into cDNA with 300 U Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega Corp., Madison, WI, USA) using 200 pmol of random primers (Promega). Twenty units of ribonuclease inhibitor (RNase OUT™) (Invitrogen Argentina, Buenos Aires, Argentina) and 100 nmol of deoxynucleotide triphosphate mixture were added to each reaction tube in a final volume of 30 μl of 1× M-MLV buffer. Reverse transcription was performed at 37 °C for 90 min. Each reverse-transcribed product was diluted with RNAse-free water to a final volume of 60 μl. The primer pairs used for amplification of *amh*, *sox9*, *sf-1* and 18SRNA (the reference gene) are shown in Table 2. cDNA levels were detected using real-time PCR (q-PCR) with the Rotor-Gene Q Series Software 1.7 (Qiagen Instruments AG, Switzerland) and EvaGreen® dye (Biotium, Inc., Hayward, CA). For cDNA amplification, 5 μl of cDNA was combined with a commercial pre-mix HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Estonia) following the manufacturer's protocol.

After initial denaturation at 95 °C for 15 min, the reaction mixture was subjected to successive cycles of denaturation at 96 °C for 15 s, annealing at 55 °C (*sf-1* and 18SRNA) or 61 °C (*amh* and *sox9*) for 15 s, and extension at 72 °C for 15 s. Product purity was confirmed by dissociation curves and random agarose gel electrophoresis. Controls containing no template DNA were included in all assays, yielding no consistent amplification. For each analysis, a standard curve was prepared from eight serial dilutions of a standard sample containing equal amounts of cDNA from the different experimental groups. All standards and samples of each independent experiment were assayed in triplicate.

2.6. Data analysis

Statistical analyses were performed using the Mann–Whitney *U* test. Results are reported as mean ± S.E.M. $p < 0.05$ was accepted as significant.

3. Results

3.1. Experiment I

Experiment I was designed to characterize *amh*, *sox9* and *sf-1* mRNA expressions in TSD-males *versus* TSD-females and to establish similarities and/or differences in the expression patterns of these genes between TSD-females and HSD-females. As expected, all caimans incubated at 33 °C developed their gonads to testes (TSD-males), whereas all those incubated at 30 °C did so to ovaries (TSD-females). One hundred percent male-to-female sex reversal was obtained after E_2 (1.4 ppm) treatment when eggs were incubated at 33 °C (the male-determining temperature). These animals were called HSD-females.

3.1.1. amh and sox9 genes are sexually dimorphic in neonatal C. latirostris

As shown in Fig. 1A, *amh* and *sox9* mRNA expression in the TSD-males was higher than in the TSD-females, exhibiting a sexually dimorphic expression, whereas, relative expression of *sf-1* mRNA did not differ between TSD-males and TSD-females. Fig. 1B shows

Table 2
Primer accession numbers (GeneBank ID) and primer sequences for targeted *Caiman latirostris* gonadal genes.

Gene	Sense primer	Antisense primer	Size (bp)	GenBank ID
amh	TCCACCCGTGCCGACTACTA	CAGAGTATTGGACGGGCACG	106	AF180294
sf-1	GGCTCCATCCTGAACAACCT	TTGAGGCAGACGAACTCCTG	95	AF180296
sox9	GGCTCGGAGCAAACCCACAT	TGCCAGGCTGGACGTCTGTT	172	AF106572
18 SRNA	TAAGTCCCTGCCCTTTGTACACA	GATCCGAGGGCCTCACTAAAC	71	M11188

a representative agarose gel with the qPCR products. A single product with the expected length was obtained in each reaction (*amh*: 106 bp; *sox9*: 172 bp; *sf-1*: 95 bp and 18SRNA: 71 bp).

3.1.2. Gene expression in TSD-females versus HSD-females

We assessed whether the expression of the developmental genes differs between TSD-females and HSD-females. HSD-females showed a higher level of *sox9* mRNA expression and no differences between *amh* and *sf-1* mRNA levels (Fig. 2).

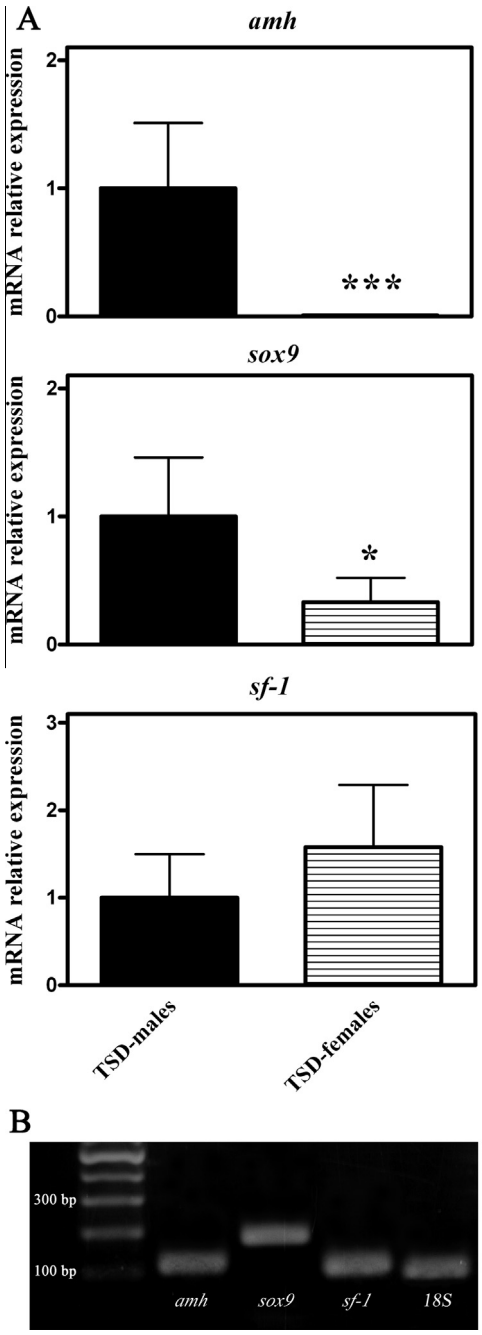

Fig. 1. Expression of *amh*, *sox9* and *sf-1* on TSD-male and TSD-female caimans. (A) A dimorphic pattern of relative mRNA expression of *amh* and *sox9* is shown. The vertical axis corresponds to the relative mRNA level of each target gene normalized to 18SRNA expression. The mRNA level of the TSD-male group is expressed as 1. Values are shown as mean ± S.E.M. 7 caimans/group) and significant differences are depicted with asterisks ($^{*}p < 0.05$; $^{***}p < 0.0001$). (B) Representative 2% (w/v) agarose gel stained with Gel-Red® showing qPCR products from GAM complexes. A single and specific band on the expected molecular weight from each gene studied (*amh*, *sox9*, *sf-1* and 18SRNA) was observed. On the left, the molecular weight marker is shown.

3.2. Experiment II

Experiment II was performed to evaluate whether *in ovo* exposure to EDCs affects the expression of genes associated with sex determination.

As described in M&M, the treatments were topically applied prior to the window for *C. latirostris* sex determination. All the animals hatched from eggs incubated at 33 °C and treated with E_2 (0.014 ppm), BPA (1.4 ppm) or END (20 ppm) were males. Fig. 3 shows temporal changes in the histoarchitecture of caiman testes from each experimental group. In control males, the testes were characterized by the presence of well-differentiated seminiferous tubules, filled by germ cells and Sertoli cells lining the basement membrane. Testes of animals *in ovo* exposed to EDCs showed an altered histoarchitecture characterized by disrupted seminiferous

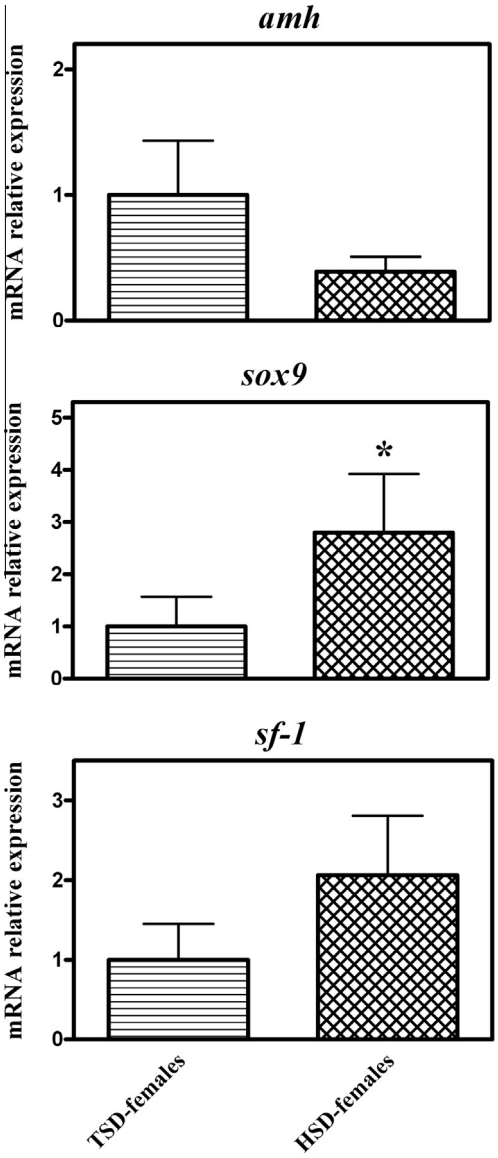

Fig. 2. mRNA relative expression of *amh*, *sox9* and *sf-1* in TSD-female and HSD-female caimans. The vertical axis corresponds to the relative mRNA level of each target gene normalized to 18SRNA expression. The mRNA level of the TSD-female group is expressed as 1. Values are shown as mean ± S.E.M. (7 caimans/group) and significant differences are depicted with asterisks ($^{*}p < 0.05$).

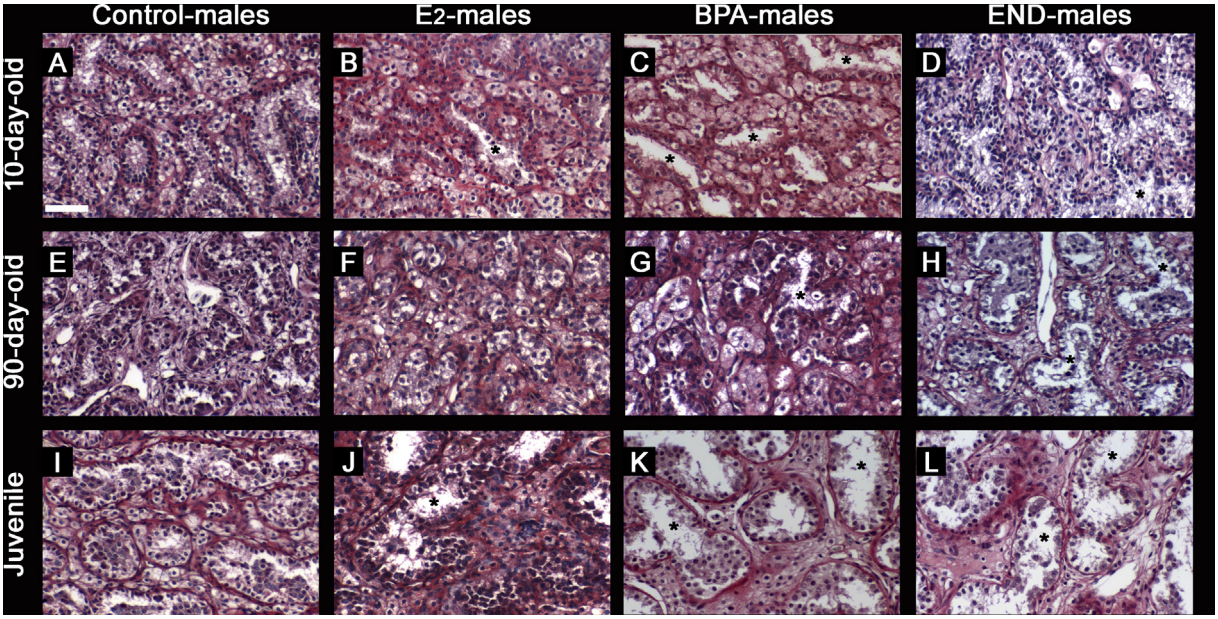

Fig. 3. Gonadal histoarchitecture in male caimans. Representative photomicrographs of testis cross-sections obtained from 10-day-old (A–D), 90-day-old (E–H) and juvenile (I–L) caimans *in ovo* exposed to vehicle (A, E, I), 17β-estradiol (B, F, J), bisphenol A (C, G, K) or endosulfan (D, H, L). Testes from caimans exposed to EDCs exhibited disorganized seminiferous tubules (asterisks) and a thickening of interstitial spaces. Scale bar = 50 μm.

tubules with empty lumens. Moreover a higher proportion of interstitial tissue, rich in extracellular matrix, was evident in testis samples from 90-day-old and juvenile EDCs-exposed caimans.

3.2.1. Effects of prenatal exposure to EDCs on mRNA expression of amh, sox9 and sf-1 genes

Caiman embryos were *in ovo* exposed to E_2, BPA and END to determine the effects on the expression of the selected genes on post-natal day 10. Males treated with E_2 exhibited lower *sox9* and higher *sf-1* mRNA expression than unexposed control-males. *In ovo* exposure to END caused an increased expression of all the genes evaluated (Fig. 4). Thus, *sf-1* transcription was up-regulated by exposure to either E_2 or END. However, after exposure to BPA, the changes in *amh* and *sf-1* expressions were not significant and followed the same direction as those observed in END-exposed males.

4. Discussion

Here, we demonstrated a dimorphic pattern of *amh* and *sox9* expression in TSD-caimans. The expression of *sox9* in HSD-females was higher than that in TSD-females. In neonatal males, alterations in *sox9* and *sf-1* after exposure to E_2 were observed, and END exposure disrupted the expression of all the genes evaluated.

In *C. latirostris* GAM complexes, *amh* and *sox9* mRNA expressions were higher in TSD-males than in TSD-females. Our results are in line with previous observations that established a dimorphic pattern of *amh* and *sox9* expression in neonatal alligators (Kohno et al., 2010; Urushitani et al., 2011). In the American alligator, the expression of *amh* mRNA increased in the GAM incubated at the male-producing temperature, starting on embryonic stage 21. During stages 21–23, supporting cells (presumptive Sertoli cells) proliferate within the medulla of the gonad (Smith and Joss, 1993). Sertoli cells produce AMH during differentiation and development of the male gonad (Rey et al., 2003). The regulation of *amh* expression has been studied in the chick, the pejerrey and the alligator (Fernandino et al., 2008; Oréal et al., 1998; Western et al., 1999b). Interestingly, *sox9* does not seem to be necessary for the initiation of *amh* transcription, since *amh* is detected in the fetal gonad before *sox9* (Oréal et al., 1998; Western et al., 1999b). Our results show that *sox9* mRNA expression is still dimorphic in neonatal caimans. In the alligator, it has been suggested that *sox9* could activate the *amh* promoter region (Urushitani et al., 2011). Thus, a closer relationship between *amh* and *sox9* could not be excluded. We also examined *sf-1* expression in 10-day-old caimans and found no differences between TSD-males and TSD-females. *sf-1* expression in the developing embryonic gonads of TSD turtles and the American alligator is down-regulated in female turtles and male alligators during the TSP (Morrish and Sinclair, 2002).

In ovo administration of estrogens induces ovarian differentiation in reptile embryos incubated at the male-producing temperature (Nakamura, 2010). Accordingly, we have previously described that sex determination in *C. latirostris* is also hormone-dependent, since *in ovo* administration of xenoestrogens induces ovarian development in embryos incubated at the male-producing temperature (Stoker et al., 2003, 2008). In the present study, we confirmed that a high dose of estradiol causes sex reversal in *C. latirostris* and that *sox9* expression remained at male levels. Thus, HSD-females showed a higher *sox9* expression than TSD-females. *amh* and *sox9* expressions have been described in the GAM of neonatal TSD- and HSD-female alligators (<48 h-old) (Urushitani et al., 2011). Our results are in line with those about *amh* expression but not with those about *sox9* expression. *sox9* has been associated with testis development in all vertebrates examined to date (Morrish and Sinclair, 2002) and it is known that it is down-regulated in the developing ovary (Kobayashi et al., 2005). As discussed below, in the present study, we observed that up- or down-regulation of *sox9* expression depends on the E_2 doses. The mechanism(s) by which estrogen diverts the bipotential gonad to the female fate has not been definitively elucidated. In the red-eared slider turtle *T. scripta*, estrogen acts to suppress testis differentiation at the female-producing temperature by repressing SOX9 protein expression in the medulla (Barske and Capel, 2010). This result aligns with studies in mice that detected a similar antagonistic relationship between estrogen signaling and SOX9, in which mutations in aromatase or estrogen receptors induced up-regulation of *sox9* and trans-differentiation of adult granulosa cells into Sertoli-like cells (Couse et al., 1999; Dupont et al., 2003).

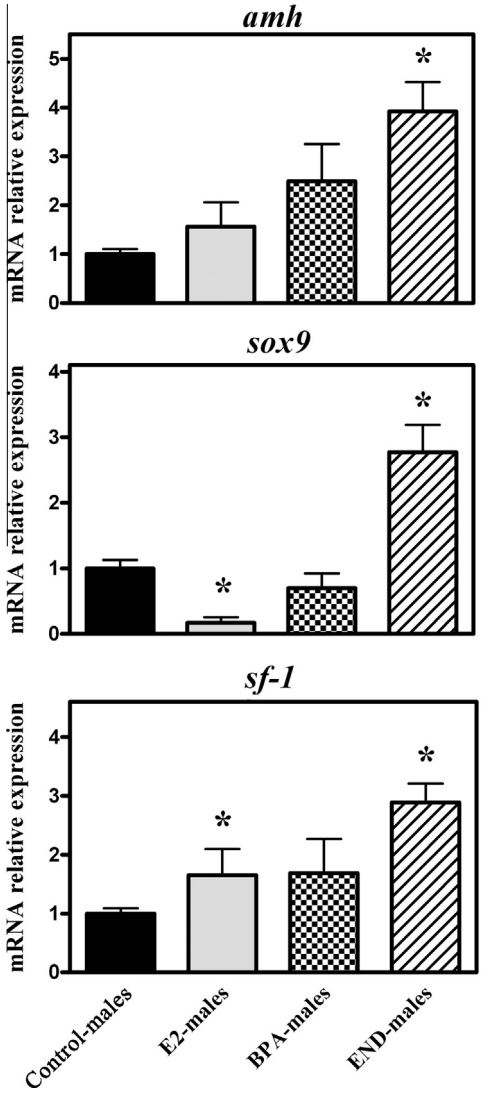

Fig. 4. mRNA relative expression of *amh*, *sox9* and *sf-1* in male caimans exposed to vehicle (control-males), 17β-estradiol (E_2), bisphenol A or endosulfan (END). Exposure to END induced a high expression of all the genes evaluated. E_2 decreased *sox9* but increased *sf-1* expression. The vertical axis corresponds to the relative mRNA level of each target gene normalized to 18SRNA expression. The mRNA level of the control-male group is expressed as 1. Values are shows as mean ± S.E.M. (6 caimans/group) and significant differences are depicted with asterisks (*$p < 0.05$).

Previously, we have described that neonatal HSD-females show delayed follicle development, lacking type III follicles and that juvenile HSD-females exhibit higher incidence of multioocyte follicles (Stoker et al., 2008). Here, we showed that HSD-females exhibited higher expression of *sox9* than TSD-females. Therefore, having in mind the well-characterized functional importance of SOX9 in mouse gonadal differentiation, we could hypothesize that HSD-females have impaired ovarian functions. New experiments are needed to assess this topic deeply.

Many recent studies in wildlife have generated increasing concern regarding the effects of EDCs on reproductive functions (Guillette and Edwards, 2008). EDCs can disrupt the endocrine system through several mechanisms including up- or down-regulation of hormonal synthesis (Boggs et al., 2011; Durando et al., 2011; Kass et al., 2012; Ramos et al., 2003; Rey et al., 2009; Stoker et al., 2008). Hormone production can be altered by modifying the enzymes and other molecules involved. As an example, atrazine (a widely used pesticide) up-regulates aromatase in frogs (Hayes et al., 2002) and cultured human cells in an *sf-1*-dependent manner (Fan et al., 2007). In the present study, the *in ovo* exposure of *C. latirostris* embryos (incubated at the male-producing temperature) to low doses of E_2 or BPA or to a high dose of END did not override the temperature effect. Thus, these animals were all males, in agreement with our previous reports (Beldomenico et al., 2007; Rey et al., 2009; Stoker et al., 2003).

The chronological changes observed in the histoarchitecture of testes from control males were similar to those described in *A. mississippiensis* (Moore et al., 2010). Moreover, the present results demonstrate that embryonic exposure to E_2, BPA or END during the critical period of sex determination altered the histoarchitecture of the testis not only in 10-day-old caimans as already reported (Rey et al., 2009), but also in 90-day-old and juvenile caimans. In samples from caimans exposed to EDCs, disorganized seminiferous tubules exhibited a loss of intratubular intercellular connections and either emptied tubular lumens or luminal cellular detritus. It is known that intercellular connections play a critical role in the control of cell proliferation and differentiation during spermatogenesis (de Montgolfier et al., 2007; Sridharan et al., 2007). Besides, the observed thickening of the peritubular connective tissue could interfere with the paracrine signaling of the testis, leading to an impaired testicular function. The changes observed in caiman testes long after exposure ended highlight the vulnerability of the embryo to a single dose of EDCs.

C. latirostris could be naturally exposed to END (Stoker et al., 2011). END residues have been detected in caiman eggs harvested in areas with high agricultural activity but not in eggs collected in the same area where the eggs used in these experiments come from Stoker et al. (2011). We have demonstrated that experimental embryonic exposure to END during the critical period of sex determination alters the balance between proliferation and apoptosis of testicular cells of hatchlings, demonstrating that testes are sensitive to END action (Rey et al., 2009). Here, we observed that *in ovo* exposure to END increased mRNA expression of *amh*, *sox9* and *sf-1* in GAM complexes of 10-day-old males and induced a significant disruption of the histoarchitecture of the seminiferous tubules not only in neonates but also in 90-day-old and juvenile caimans. We found no reports regarding the effects of exposure to END on the GAM of other neonatal reptilian species with TSD. Results from field work describe that juvenile American alligators caught in Apopka Lake, Florida (a population that is chronically exposed to a complex mixture of environmental contaminants), exhibit an increased *sf-1* mRNA expression compared to alligators from a non-contaminated lake (Kohno et al., 2008). Our results regarding the changes observed in caiman males experimentally exposed to END are in agreement with those of Kohno and co-workers (Kohno et al., 2008). Moreover, it has been suggested that, in males, *sf-1* regulates the transport of cholesterol. Thus, the higher expression of *sf-1* in caiman males exposed to END could modify the steroidogenic pathway, since these animals show decreased levels of testosterone, compared to untreated males (Rey et al., 2009). Because the adrenal gland is also a steroidogenic tissue with high expression of *sf-1* (Schimmer and White, 2010), and in this study, the GAM complex was evaluated as a whole, the testis and/or the adrenal could be the source of *sf-1*. Ongoing experiments using laser capture microdissection will allow establishing the individual contribution of the gonad, adrenal and mesonephros to the expression of the genes studied.

In E_2-male caimans, we showed a reduced *sox9* and an increased *sf-1* mRNA expression. Other studies have shown the role of E_2 in *sox9* and *sf-1* regulation, but in sex reversal events in turtles (Barske and Capel, 2010; Fleming and Crews, 2001). However, in Experiment II, the low dose of E_2 was unable to induce sex reversion, and E_2-males are estrogenized males, which are relevant points of our work.

Although a significant disruption of gonadal histoarchitecture was observed after *in ovo* exposure to BPA the changes in *amh* and *sf-1* were not significant. These discrepancies could be explained by the small sample size and the high heterogeneity of wild animals.

The results presented here provide new tools for future studies designed to understand the mechanisms that lead to the abnormalities observed in testicular and/or ovarian biology in caimans exposed to EDCs (Rey et al., 2009; Stoker et al., 2008). Although the present study was restricted to the evaluation of the expression of genes that regulate sexual determination and/or differentiation in neonatal caimans, we showed the gonadal disruption in EDC-exposed groups long after exposure had ended. Thus, adverse consequences over the reproductive life of caimans could be expected.

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