RESEARCH ARTICLE

Histomorphological Changes in Testes of Broad-Snouted Caimans (*Caiman latirostris*) Associated With *In Ovo* Exposure to Endocrine-Disrupting Chemicals

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ABSTRACT

Studies regarding the effects of endocrine-disrupting chemicals (EDCs) on the reproductive functions of wild animals have raised increasing concern. Thus, here we evaluated the consequences of *in ovo* exposure to endosulfan (END) and bisphenol A (BPA) in testes from neonatal to juvenile (Juv) caimans (*Caiman latirostris*). Caiman eggs were collected from areas with low to moderate anthropogenic intervention and incubated at male-producing temperature. At stage 20 of embryonic development (previous to gonad sex determination), eggs were exposed to either END (20 ppm) or BPA (1.4 ppm) and male gonad histomorphology examined in 10-day-old, 90-day-old, and Juv caimans. The relative seminiferous tubular area (RTA) was measured in testes and the proliferation index and the expression of estrogen receptor alpha (ERα) were quantified in intratubular cells. Regardless of the treatment, all eggs resulted in male hatchlings. The testes of EDC-exposed caimans presented tortuous seminiferous tubules with empty tubular lumens. The RTA of 10-day-old caimans exposed to BPA was decreased. The percentage of cells expressing ERα was not different after *in ovo* treatment with EDCs (compared to the Control group), although caimans exposed to END showed a different ERα distribution pattern. The proliferation index was lower in 90-day-old caimans exposed to END, and higher in Juv caimans exposed to BPA. *In ovo* exposure to END or BPA modified sensitive parameters of *C. latirostris* male gonads. The alterations described here might compromise not only the sexual maturation but also the reproductive performance of adult caimans. *J. Exp. Zool. 325A:84–96, 2016.* © 2015 Wiley Periodicals, Inc.

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In the last decade, many reviews addressing the effects of endocrine-disrupting chemicals (EDCs) on the reproductive organs of wild animals have generated increasing concern (Hotchkiss et al., 2008; Hamlin and Guillette, 2011; Vandenberg et al., 2013; Bhandari et al., 2015). Exposure to EDCs during the critical periods of organogenesis, which are highly sensitive to relatively small changes in hormone levels, can lead to subtle or gross irreversible organizational effects. Several reports have

linked exposure to EDCs (especially early in life) with detrimental health effects, and even with complete sex reversal, on wild animals (Stoker et al., 2003, 2008, 2011; Beldomenico et al., 2007; Hamlin and Guillette, 2011; Hayes et al., 2011; Matsumoto et al., 2014). Bisphenol A (BPA) is a plasticizer present in everyday-used items, including food and beverage packaging, adhesives, building materials, electronic components, and paper coatings (Staples et al., '98). Although BPA has a short half-life, it is ubiquitous in the environment because of continuous release (Oehlmann et al., 2009). Thus, BPA represents a significant potential risk for humans and wildlife (Crain et al., 2007; Flint et al., 2012; Peretz et al., 2014). BPA is able to act through several physiological receptors, including estrogen receptors α and β (ERα and ERβ), membrane-bound ERs, androgen receptor, peroxisome proliferator-activated receptor γ, and thyroid hormone receptor (Richter et al., 2007).

Another well-known EDC is endosulfan (END), an organochlorine pesticide that has also shown estrogenic activity (Bisson and Hontela, 2002; Gormley and Teather, 2003; Varayoud et al., 2008). END is able to bioaccumulate and biomagnify in food chains and to persist in the environment for a long time (Naqvi and Vaishnavi, '93). It has been reported that END acts as an EDC by binding to ERs (Roy et al., 2009); in fact, in vitro END competes with 17β-estradiol (E_2) for binding to ERα and is able to transactivate ERα and induce the transcription of an estrogen response element-dependent gene (Lemaire et al., 2006). Regardless of the largely described negative effects of END, in Argentina, the use of END has been banned only recently (Resolución 511/2011, Servicio Nacional de Sanidad y Calidad Agroalimentaria [SENASA], Argentina; http://www.senasa.gov.ar/contenido.php?to=n&tin=1501&tio=17737; latest access: 10/30/14).

There are evidences of the action of both END and BPA on the reproductive axis in animals and humans (Roy et al., 2009; Silva and Gammon, 2009; Da Cuña et al., 2013). Specifically, in gonads of male rodents, END causes cell death in exposed Sertoli-germ cells due to oxidative damage (Rastogi et al., 2014). In addition, it decreases testosterone secretion and androgen receptor expression, which results in an altered function of Leydig cells (Wang et al., 2014). In aquatic animals, in vivo exposure to END causes testicular histological alterations related to spermatogenesis (such as decreased progression of differentiation of spermatogonia to spermatocytes), accompanied with fibrosis in the interstitial space, and decreased expression of testicular steroidogenic enzyme genes (Da Cuña et al., 2011, 2013; Rajakumar et al., 2012). In male caimans, exposure to END alters the histoarchitecture of the testis and decreases testosterone levels (Rey et al., 2009). In humans, there is little evidence linking END exposure and reproductive alterations. However, some authors have suggested an association between high levels of END in maternal and cord blood and the occurrence of a pre-term delivery along with oxidative stress (Pathak et al., 2010). With regard to BPA, rodents exposed to this EDC early in their life show adverse effects in the adult testis. These effects include: decreased sperm count and motility, increased apoptotic cells (inside the seminiferous tubules), and alterations in the levels of hormones and/or steroidogenic enzymes (Peretz et al., 2014). Male reptiles exposed to low doses of BPA exhibit disrupted sexual differentiation and histological changes in testes (Stoker et al., 2003; Durando et al., 2013; Jandegian et al., 2015). Evidence of the effects of BPA in humans compared to other animals is relatively lacking (and discrepant); however, strong evidence exists that BPA has a toxicant action in the ovary (Peretz et al., 2014). In addition, limited evidence has shown an association between sexual dysfunction in men occupationally exposed to BPA and impaired implantation in women undergoing in vitro fertilization (Peretz et al., 2014).

Many species of reptiles exhibit several features that facilitate their use as biomonitors for EDCs in aquatic environments. These features can be summarized as follows: a) they exhibit plasticity of the sex determination process; b) they are omnivores; c) they have strong site fidelity; d) they are a long-lived species; and e) they are poikilotherms, thus their ability to metabolize and clear contaminants can be reduced (reviewed in Boggs et al., 2011). The broad-snouted caiman (*Caiman latirostris*) is a crocodilian species that can be characterized as a biomonitor of environmental pollution. *C. latirostris* has a wide distribution in wetlands and rivers of north-eastern Argentina, southern Brazil, Paraguay, Uruguay, and Bolivia (Yanosky, '90). Like in all crocodilians, the sex of *C. latirostris* offspring is determined by the incubation temperature (temperature-dependent sex determination; TSD) (Lang and Andrews, '94), and influenced by steroid hormones (Stoker et al., 2003). The TSD pattern in caimans is assessed as female-male-female, since eggs incubated at 29°C and 31°C produce 100% females, eggs incubated at 33°C produce

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Abbreviations: BPA, Bisphenol A; E_2, 17β-estradiol; EDCs, endocrine-disrupting chemicals; END, endosulfan; ERα, estrogen receptor alpha; GAM, gonad-adrenal-mesonephros; Juv, juvenile; PCNA, proliferating cell nuclear antigen; RTA, relative tubular area; SVL, snout-vent length; TL, total length; TSD, temperature sex determination.

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100% males, and eggs incubated at a higher temperature (34.5°C) produce both sexes (Piña et al., 2003). Furthermore, female and male caiman reproductive tissues are highly sensitive to the effects of EDCs such as END, atrazine and BPA (Stoker et al., 2003, 2008; Rey et al., 2009; Durando et al., 2013). Prenatal exposure to EDC modifies ovarian follicular dynamics and hormonal steroid levels in postnatal female caimans (Stoker et al., 2008). Preliminary results revealed that early postnatal exposure to EDCs alters the temporal and spatial expression pattern of histo-functional differentiation biomarkers in the oviduct later in life (Galoppo et al., 2014). Previously, we have demonstrated that prenatal exposure to EDCs modifies several related-reproductive parameters in male caiman hatchlings. An altered gonadal histomorphology is accompanied by decreased testosterone levels and/or disrupted expressions of sex-determining genes (Rey et al., 2009; Durando et al., 2013). Since *C. latirostris* can be naturally exposed to EDCs (Stoker et al., 2011, 2013), research on this species is of particular interest both to assess the impact of EDCs on caiman populations and to better characterize *C. latirostris* as a biomonitor of ecosystem health.

In the present work, we focused on male caimans to expand our knowledge and to examine potential pathways to explain our previous results. The aims were: a) to establish the ontogeny of testicular changes at tissue, cellular and molecular levels, in neonatal to juvenile *C. latirostris*, and b) to evaluate the consequences of *in ovo* exposure to END or BPA in *C. latirostris* testes. To achieve our aims, we designed two experiments: in Experiment I, we used animals exposed to vehicle only, whereas in Experiment II, we used animals exposed to vehicle (Control group), END or BPA.

MATERIALS AND METHODS

Animals

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

In six reproductive seasons, 2004–2008 and 2011, three wild clutches/year (average clutch size: 35 eggs) were collected shortly after oviposition. Nests were located in wetlands in a wildlife refuge in a remote subtropical area of the Province of Chaco (Argentina) with low impact of human activities, localized upstream of either urbanized or farming areas to avoid exposure to sewage or agriculture and/or feedlot run-off. To establish the age of the embryos, one egg from each clutch was opened in the field. Only the nests with embryos at stages lower than 15 were transported to the laboratory. To establish developmental stages of *C. latirostris* embryos, we followed defined criteria (Stoker, 2004; Iungman et al., 2008). Prior to removal from the nest, the upper surfaces of the eggs were marked with a graphite pencil to keep the original egg orientation during transportation to the laboratory and incubation in controlled laboratory conditions. At the laboratory, the eggs of each clutch were randomly allocated into two incubators, which had different incubation temperatures (33°C and 30°C), as described by Stoker et al. (2003). The incubators were glass boxes kept isolated (using expanded polystyrene) and filled with tap water until 1/3 of depth. Relative humidity in both incubators was maintained above 90% throughout the incubation period, with a mean (±SD) of 95.2% (±2.0) in the incubator at 33°C and 92.3% (±2.2) in that at 30°C. The incubators had previously been stabilized at the male (33°C) or female generating (30°C) temperatures, using aquarium heaters with a thermostat control inside the water. Temperature was monitored by HOBO temperature loggers (Onset Computer, Pocasset, MA) and by the daily recording of incubator electronic thermometer readings. The eggs were placed on a wire mesh that prevented the contact with water. The development of opaque eggshell banding was used to check embryo viability. Within each incubation temperature group, eggs from each clutch were equally distributed among treatment groups. Only male caimans were used in this study, whereas females were assigned to other studies.

Experimental Design and Sample Collection

At stage 20 of embryonic development, eggs received vehicle (50 μL absolute ethanol; Control), 20 ppm of END (END) (Icona S.A., Argentina) or 1.4 ppm of BPA (BPA) (Aldrich, Milwaukee, WI). All treatments were applied topically in a single dose (Stoker et al., 2003, 2008; Rey et al., 2009; Durando et al., 2013). Eggs collected in the 2004–2006 seasons were destined to Experiment I (Control group only), to establish the ontogeny of testicular changes from the neonatal to the juvenile stage, whereas those collected in the 2007, 2008, and 2011 seasons were destined to Experiment II. Thus, eggs from different nests were equally distributed in each experimental group (Control, END and BPA). In Experiment II, each specific parameter was measured in animals of the same age but belonging to the Control, END or BPA groups, to evaluate whether *in ovo* exposure to END or BPA had any consequences on the testis histomorphology in caimans, compared to the Control group.

At hatching, all hatchlings were individually identified by two numbered tags (style 1005-1; National Band and Tag Co., Newport, KY) and then held in controlled conditions. Housing facilities were designed to provide consistent thermal ranges (28.0 ± 2.0°C) and the opportunity to choose diverse micro-environments from a dry area to a water feature large enough to facilitate *ad libitum* full-body soaking, swimming, and social interaction. Water temperature was stabilized by a radiant underfloor heating system at 26.0 ± 2.0°C. Other environmental factors, including dark-light cycles (lights on from 0600 to 2000 hr), humidity (70 ± 5%), and air renewal (air automatically renewed every 15 min), were controlled (Zayas et al., 2011).

Caimans were euthanized using a lethal dose of sodium pentobarbital (*ip*) at 10 days of age, 90 days of age or at about an average BM of 2,000 g (juvenile caimans [Juv]). Before euthanasia, biometric parameters such as BM, total length (TL) and snout-vent length (SVL) were recorded in all animals.

At 10 days of age, 7–9 animals/experimental group were euthanized and the remaining caimans were raised under controlled conditions and fed three times a week with an amount of food that represented 15% of their BM supplemented with 1% of a vitamin and mineral complex (Vionates-S, Novartis Laboratory, Argentina). Additionally, animals were exposed daily to a 30-min cycle of ultraviolet B light (Reptistar, Sylvania, Germany) (Zayas et al., 2011) for normal calcium and vitamin D metabolism. To warrant caiman welfare, particular attention was given not only to their nutritional requirements but also to stocking densities and the microbiological and chemical quality of the water. To monitor the health of hatchlings, qualified caiman keepers daily checked for early signs of disease such as loss of appetite, inactivity, isolation from other caimans, external appearance (wounds, fungal lesions), loss of coordination, changes in feces, vomiting, coughing, and swellings on the body or legs.

The gross anatomy of gonads and the absence of oviducts observed at the time of euthanasia strongly suggested that 100% of the individuals hatched from the eggs incubated at 33°C were males. Gonadal sex was confirmed by histological examination as previously reported (Stoker et al., 2003, 2008; Rey et al., 2009; Durando et al., 2013).

At necropsy, both gonad-adrenal-mesonephros (GAM) complexes were dissected from 10-day-old caimans, whereas gonads were dissected from the GAMs from 90-day-old and Juv caimans. The left GAM complexes or the left gonads were immediately frozen in liquid nitrogen and conserved at −80°C until used (for other studies), while the right GAM complexes 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. Serial sections (5 μm) of the right GAM complex or the right gonad were cut in transverse planes and stained with trichromic Picrosirius/hematoxylin (Stoker et al., 2003; Rey et al., 2009; Durando et al., 2013) for histological analysis or morphometric evaluation, while other tissue sections were immunostained to evaluate proliferative activity, gonadal steroid receptor expression or the co-expression pattern of estrogen receptor alpha (ERα) and desmin.

Gonadal Histology and Morphometric Analysis

Gonadal sex was determined by histological examination. This allowed confirming the sex recorded by gross anatomy. To evaluate the ontogeny of morphological changes and the impact of *in ovo* exposure to EDCs on the histoarchitecture of seminiferous tubules, digitalized images were morphometrically analyzed. Briefly, images from Picrosirius/hematoxylin-stained sections were recorded by a SPOT color video camera (Diagnostic Instruments, Inc., Arnold, MD) attached to an Olympus BH2 microscope (Olympus Optical, Tokyo, Japan). Images were analyzed using Image Pro-Plus 4.1.0.1 system (Media Cybernetics, Silver Spring, MD). The relative tubular area (RTA) was calculated as: A_{st}/A_T, where A_{st} is the area occupied by seminiferous tubules (expressed in μm^2) and A_T is the total area (including seminiferous tubules plus interstitial space, expressed in μm^2); thus, RTA is a dimensionless parameter. The volume density of the interstitial space was measured by applying an orthogonal line grid mask on each image, as previously described, with minor modifications (Kass et al., 2015). The ratio (*Vv*) between the intersections hitting the interstitial space (P_i) and the total number of intersections occurring in the whole image (P_t: seminiferous tubules plus interstitial compartment) was calculated as follows: $Vv \times 100 = (P_i/P_t) \times 100$.

Immunoperoxidase and Immunofluorescence Stains

Immunohistochemistry techniques were used to detect proliferating cells, desmin and ERα protein expression. Immunoperoxidase staining was performed as previously described (Rey et al., 2009). Briefly, after removing the paraffin, sections were dehydrated in a graded ethanol series. Microwave pretreatment was performed for antigen retrieval. Endogenous peroxidase activity and non-specific binding sites were blocked. Primary antibodies were used at the dilutions mentioned in Table 1 and incubated overnight at 4°C. After incubation with biotin-conjugated secondary antibodies (anti-mouse or anti-rabbit) for 1 hr, reactions were developed using a streptavidin-biotin peroxidase method and diaminobenzidine (Sigma–Aldrich, Buenos Aires, Argentina) as a chromogen substrate. Slides

Table 1. Primary antibodies used for immunohistochemistry.

Antibody	Animal source	Supplier	Dilution
PCNA (clone PC-10)	Monoclonal mouse	Novocastra (Newcastle upon Tyne, UK)	1:2000
ERα (LETH-ER-202y)[a]	Polyclonal rabbit	LETH-ISAL	1:200
Desmin (clone DER11)	Monoclonal mouse	Novocastra	1:50

[a]Information regarding the generation and characterization of the ERα antibody is described in Varayoud et al. (2012).

were counterstained with Mayer's hematoxylin (Biopur, Rosario, Argentina). Each run included negative controls in which the primary antibody was omitted.

To detect whether myoid peritubular cells expressed ERα, a double labeling immunofluorescence assay was conducted using ERα and desmin antibodies (Table 1). An optimized protocol was followed (Durando et al., 2011). Briefly, to minimize autofluorescence, sections were blocked with 10 mg/mL sodium borohydride (Sigma–Aldrich), and then the antigen was retrieved using a microwave pretreatment with sodium citrate buffer. The incubation with primary antibodies was performed overnight at 4°C. The secondary antibodies (Invitrogen, Buenos Aires, Argentina) anti-rabbit Alexa Fluor 488 (green) and anti-mouse Alexa Fluor 546 (red) were incubated for 1 hr and mounted in ProLong® Antifade Reagents (Life Technologies Argentina, Thermo Fisher Scientific, Buenos Aires, Argentina) with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Fluka, Sigma-Aldrich) and stored in the dark at room temperature. All immunostained slides were evaluated using an Olympus BX-51TRF microscope equipped for epifluorescence detection and with the appropriate filters (Olympus Optical Co., Ltd., Tokyo, Japan). Images were recorded using a high-resolution USB 2.0 Digital Color Camera (QImaging® Go-3, QImaging, Surrey, BC, Canada).

Quantification of Proliferation Index and ERα Protein Expression

Tissue sections were evaluated using a BH2 microscope (illumination: 12-V halogen lamp, 100 W, equipped with a stabilized light source; Olympus, Tokyo, Japan) with the Dplan ×40 objective (numerical aperture = 0.65; Olympus). Both the proliferation index and ERα expression were obtained by considering the number of intratubular positive cells respect to the total number of intratubular cells, and expressed as a percentage. In Juv animals, intratubular cells were classified as basal (if they were touching the basement membrane) or luminal (if they were not), to quantify ERα protein expression.

Data Analysis

The non-parametric Kruskal–Wallis test followed by the Mann–Whitney test were performed to find the particular differences in histological features, proliferative activity and ERα expression in testes from 10-day-old and Juv Controls (vehicle-exposed). The Mann–Whitney test was used to establish differences between Control and END-exposed caimans or Control and BPA-exposed caimans for each specific age or stage in biometric parameters and in all the variables evaluated. The Wilcoxon matched-pairs signed rank test was used to analyze whether ERα protein expression was different between 10-day-old and Juv caimans in each experimental group. The analyses were conducted using the statistical software R (R Foundation for Statistical Computing, http://www.r-project.org). Results are reported as mean ± S.E.M. Significance was defined as $P < 0.05$ in all cases.

RESULTS

As previously reported, all embryos incubated at male-producing temperature were males, regardless of the doses of END and BPA used (Stoker et al., 2003; Beldomenico et al., 2007; Rey et al., 2009; Durando et al., 2013).

Experiment I

Morphological Changes and Proliferative Activity in Testes. Temporal changes in the histoarchitecture of caiman testes are illustrated in Figure 1. In 10-day-old caimans, the testes were characterized by the presence of well-differentiated seminiferous tubules, filled by seminiferous cords containing single layers of Sertoli cells and spermatogonia, surrounded by a basement membrane and myoid peritubular cells. Leydig cells with a characteristic foamy aspect were observed in the interstitial compartment.

Testes of 90-day-old caimans exhibited morphological changes compared to those of 10-day-old caimans. While 10-day-old caimans exhibited filled seminiferous tubules, 90-day-old

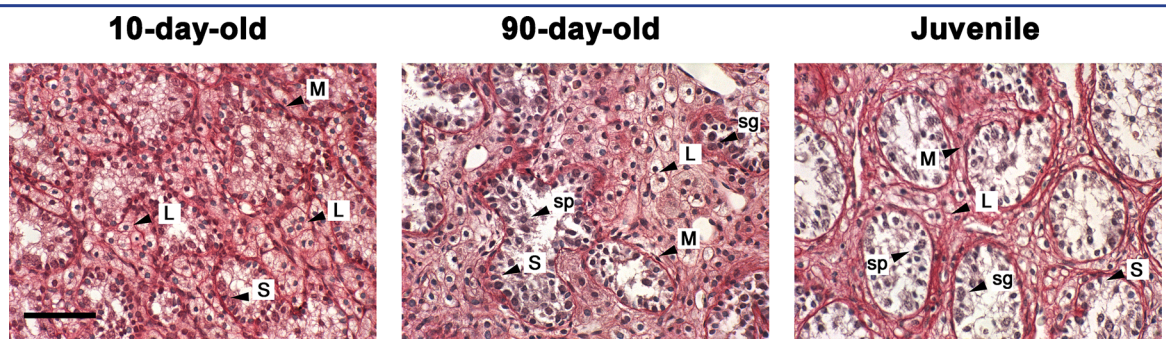

Figure 1. Temporal changes in the histoarchitecture of caiman testes. Representative images of cross sections of testes stained with Picrosirius/hematoxylin. Arrowheads show different cells: S, Sertoli cell; L, Leydig cell; M, myoid peritubular cell; sg, spermatogonia; sp, spermatocyte. The juvenile stage comprises caimans with an average BM of 2,000 g (see Materials and Methods section). Scale bar = 50 μm.

caimans presented seminiferous cords that hollowed out to broaden the luminal space. Seminiferous tubules from 90-day-old caimans contained germ cells in several differentiation stages, including primary spermatocytes; myoid peritubular cells were also visualized surrounding each tubule. At 90 days of age, the interstitial tissue had expanded until occupying a larger area than in the testes of 10-day-old caimans (Fig. 1); the *Vv* of the interstitial tissue reflects this trend (43.00 ± 1.5 vs. 48.73 ± 4.6; $P = 0.49$). In the interstitial tissue, Leydig cells were visualized with their characteristic aspect.

In Juv caimans, seminiferous tubules showed several layers of cells, including Sertoli cells and germ cells in advanced differentiation stages, and, like that observed in testes of 10-day-old and 90-day-old caimans, they were surrounded by myoid cells. The area occupied by interstitial tissue was in the same range as that measured in testes of 90-day-old animals (*Vv*: 48.73 ± 4.6 vs. 47.94 ± 1.6; $P = 0.84$).

Besides the particular features described above, the morphometric analysis showed that the RTA in 90-day-old caimans decreased as compared to that observed in 10-day-old caimans ($P = 0.03$) and exhibited a significant increase in Juv ($P = 0.0006$) caimans (Fig. 2A). The RTA between 10-day-old and Juv caimans was marginally different ($P = 0.05$).

The proliferation index of 90-day-old animals was higher than that of 10-day-old ($P = 0.005$) and Juv ($P = 0.004$) caimans (Fig. 2B) and no differences were detected between 10-day-old and Juv caimans ($P = 0.66$).

ERα Protein Expression. The ontogeny of testicular ERα protein expression was evaluated from 10 days of age to the Juv stage. ERα protein expression was mainly detected in the nuclei of intratubular cells. The percentage of cells expressing ERα between 10-day-old, 90-day-old, and Juv caimans was not different, but tended to increase from 10-day-old to Juv caimans (Fig. 3). The interstitial compartment showed few cells expressing ERα at all the time points studied. These cells exhibited strong ERα expression only in 90-day-old caimans and few myoid peritubular cells showed a clear ERα expression (Fig. 4).

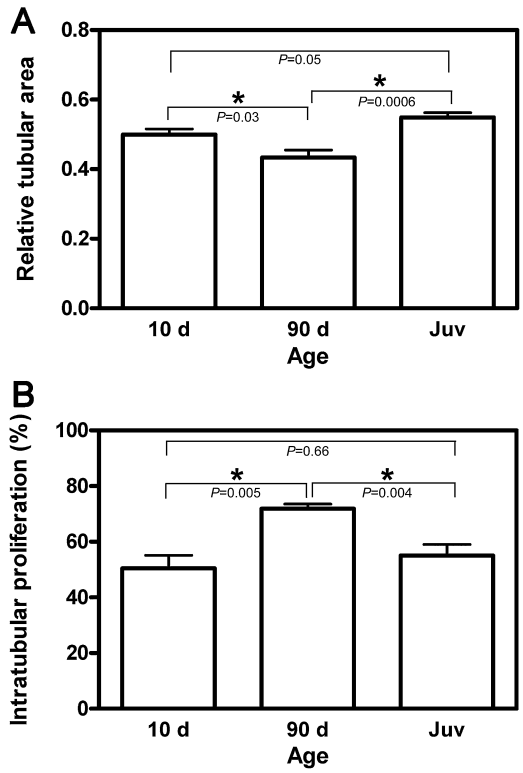

Figure 2. Ontogeny changes in relative tubular area (RTA) and intratubular proliferation. In Experiment I, 90-day-old caimans exhibited decreased RTA (**A**) and increased intratubular proliferation (**B**). Values are shown as mean ± S.E.M. (7–9 caimans/group) and significant differences are depicted with an asterisk (*$P < 0.05$). *P*-values are shown above bars. 10 d, 10-day-old; 90 d, 90-day-old; Juv, Juvenile stage.

Experiment II

Biometric parameters recorded from 10-day-old, 90-day-old, and Juv *C. latirostris* are summarized in Table 2. No differences were found between Control and EDCs-exposed animals.

Morphological Changes and Proliferative Activity in Testes. In agreement with our previous data, testes of caimans *in ovo* exposed to EDCs showed an altered histoarchitecture at all the ages evaluated (10-day-old, 90-day-old, and Juv caimans). These alterations were characterized by disrupted seminiferous tubules with empty lumens (Durando et al., 2013). Regarding END exposure, no differences were found in the RTA or the *Vv* of the interstitial compartment at any age or stage (Fig. 5A). Regarding BPA exposure, 10-day-old caimans showed a lower RTA (P=0.03) (Fig. 5B) and a higher *Vv* of the interstitial tissue (42.20 ± 1.1 vs. 53.14 ± 2.3; $P = 0.009$) than the Control group. No differences were detected in 90-day-old and Juv caimans.

In ovo exposure to END decreased the percentage of intratubular proliferating cells in 90-day-old caimans ($P = 0.015$) (Fig. 6A), whereas BPA exposure increased the proliferative activity in Juv caimans ($P = 0.004$) (Fig. 6B).

ERα protein expression. ERα protein expression was not affected by exposure to EDCs, compared to the Control group. The percentage of cells expressing ERα was similar both between Control and END-exposed caimans (Table 3) and between Control and BPA-exposed caimans (Table 3). Although the aim of Experiment II was to compare animals of the same age but belonging to the Control, END or BPA groups, there was a result that caught our attention: the individual analysis within the BPA

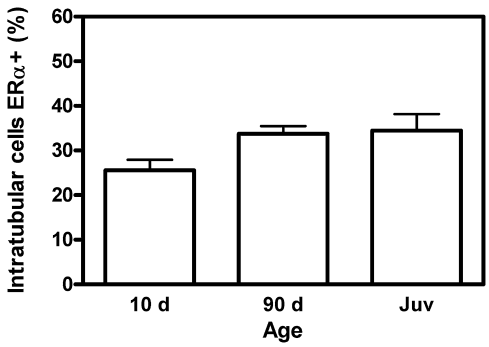

Figure 3. Protein expression of estrogen receptor α (ERα). In Experiment I, no differences were found between 10-day-old, 90-day-old, and Juv caimans in the percentage of intratubular cells expressing ERα. Values are shown as mean ± S.E.M. (7–9 caimans/group). 10 d, 10-day-old; 90 d, 90-day-old; Juv, Juvenile stage.

and the END groups showed that ERα expression exhibited a clear tendency to increase from 10 days of age to the Juv stage in BPA-exposed and END-exposed caimans. In fact, the Wilcoxon test showed that END-exposed caimans presented a significant increase in ERα expression between 10 days of age and the Juv stage (23.6 ± 2.2% vs. 41.1 ± 2.4%; $P = 0.02$) and that BPA-exposed caimans presented a marginally significant difference (23.9 ± 2.9% vs. 34.0 ± 3.0%; $P = 0.06$) between 10 days of age and the Juv stage (Table 3). In the interstitial compartment, ERα expression was weak both in BPA-exposed and END-exposed caimans.

Different from early developmental stages, testes from Juv animals exhibited seminiferous tubules lined by several layers of cells. Thus, ERα expression was evaluated in basal and luminal layers (as mentioned). END-exposed caimans showed a tendency to a lower percentage of luminal cells expressing ERα. This percentage was marginally different from that of the Control group (Control: 59.2 ± 9.1% vs. END: 43.4 ± 9.1%; $P = 0.057$).

Histological analysis allowed identifying a small subgroup of Juv caimans (n = 5) exposed to EDCs (age: 21.2 ± 2.8 months, BM: 2,765 ± 272 g) which exhibited advanced spermatogenesis, with round and elongated spermatids. No spermatids were observed in matched controls (n = 3; age: 18.8 ± 1.8 months; BM: 2,422 ± 285 g). In the testis of EDC caimans, we carefully observed cells expressing ERα and found that round and elongated spermatids were ERα negative, thus showing that the higher the differentiation stage, the lower the ERα protein expression (Fig. 7).

DISCUSSION

In the present study, we established the ontogeny of morphological features, proliferative activity and ERα protein expression in testes of *C. latirostris* from the neonatal to the juvenile stage. We also evaluated the effects of *in ovo* exposure to END or BPA on these parameters. These two EDCs are used worldwide. In caiman natural habitats, no data are available regarding the levels of BPA, but in areas with agriculture activities, END egg levels range between 20 ng/g and 50 ng/g lipids. Regarding END, we have recently reported its presence in caiman eggs collected in areas with different anthropogenic intervention (Stoker et al., 2011), with the negative impacts that could have on caiman development according to the reported results (Rey et al., 2009; Durando et al., 2013). However, in the Wildlife Refuge, Chaco Province (Argentina), the site where the nests used in this work were harvested, we found no detectable END levels in the eggs collected.

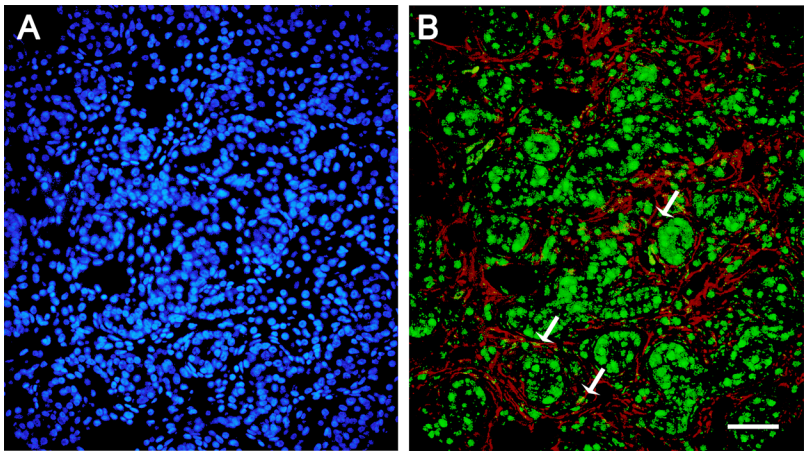

Figure 4. Co-expression pattern of ERα (green) and desmin (red) in testes of the Control group (90-day-old). (**A**) shows uniform DAPI staining of the nuclei. In (**B**), the arrows indicate the co-localization between the molecules in myoid peritubular cells. Scale bar = 50 μm.

Table 2. Biometric parameters of male *C. latirostris*.

Age	Experimental group (n)	Body mass (g)	P^a	Total length (cm)	P^a	Snout–vent length (cm)	P^a
10-day-old	Control (8)	46.8 ± 2.0	–	22.7 ± 0.8	–	11.0 ± 0.3	–
	END (8)	48.0 ± 1.9	0.46	22.3 ± 0.9	0.89	10.9 ± 0.5	0.90
	BPA (8)	49.3 ± 1.5	0.34	22.8 ± 0.8	0.99	10.9 ± 0.5	0.88
90-day-old	Control (7)	154.7 ± 7.6	–	36.7 ± 0.8	–	17.0 ± 0.3	–
	END (8)	172.6 ± 4.0	0.07	37.2 ± 0.4	0.90	17.8 ± 0.2	0.13
	BPA (8)	159.9 ± 8.8	0.22	35.6 ± 1.1	0.67	17.4 ± 0.7	0.67
Juv[b]	Control (8)	2164 ± 193	–	82.4 ± 2.9	–	40.6 ± 2.3	–
	END (8)	1601 ± 275	0.13	73.9 ± 5.2	0.25	35.2 ± 2.4	0.14
	BPA (8)	2283 ± 337	0.94	86.4 ± 4.1	0.65	41.8 ± 2.3	0.78

[a]Compared to Control group (Mann–Whitney test).
[b]Average age is 15 months.

The caimans included in this study were hatched from eggs collected shortly after oviposition (i.e., embryos at stages lower than stage 15 (Iungman et al., 2008)) from regions of low anthropogenic intervention, and were raised in controlled conditions. Thus, the contribution of the wild environment to the potential contamination of these caimans was both via maternal transfer and via the very early nest environment. Both sources contributed to the nest effect. The nest effect was avoided by including eggs from each clutch in all experimental groups (Willingham, 2005).

In both Experiments I and II, gonadal sex was assessed and, in agreement with our previous reports, all the caimans hatched from the eggs incubated at 33°C were males, regardless of the *in ovo* exposure to END or BPA (Stoker et al., 2003; Beldomenico et al., 2007; Rey et al., 2009; Durando et al., 2013). Biometric parameters were analyzed comparing animals from the Control group with animals exposed to a single dose of END or BPA. We focused on biometric parameters because we have previously reported that egg weight loss during incubation is greater in eggs treated with the same dose of END used in the present study. END also causes a reduction in hatchling fractional weight (Beldomenico et al., 2007), thus, impaired growth could be expected. Besides, in turtles and alligators, exposure to EDCs modifies body morphometric variables (de Solla et al., '98; Kitana et al., 2007; Moore et al., 2010b). Unlike that reported in turtles and alligators (de Solla et al., '98; Kitana et al., 2007; Moore et al., 2010b), we found no differences in caiman biometric parameters after experimental exposure to EDCs. In turtles, exposure to several contaminants has been found to affect precloacal length in adult males of *Chrysemys picta* (Kitana et al., 2007) and *Chelydra serpentina* (de Solla et al., '98). In juvenile *Alligator mississippiensis*, animals hatched from eggs collected from a contaminated lake had greater BM, SVL, and TL than animals of a reference lake (Moore et al., 2010b). Although in the present work we tested the effect of individual chemicals (END or BPA), our results are in agreement with those reported in 12-month-old *C. latirostris* exposed to a pesticide mixture where no differences in BM, SVL, or TL were detected (Poletta et al., 2011).

Since testicular morphological changes in *C. latirostris* had not been described in depth from the neonatal to the juvenile stage, we characterized gonads from animals exposed to vehicle (Experiment I). After this characterization, animals exposed to END or BPA were compared with animals exposed to vehicle (Experiment II). Our descriptions of the histological features of caiman testes belonging to Experiment I are similar to those reported for neonatal (7 days old) and immature alligators (up to 5 months old) (Moore et al., 2010a). We extended our description up to juvenile peri-pubertal animals. This is valuable information because we found no previous reports on this issue. Although in previous works, we described the histological features of caiman ovaries from neonatal up to 12 months of age (Stoker et al., 2008) and the histological changes in testes of caimans exposed to EDCs (Durando et al., 2013), this is the first time that ontogenic changes are described for *C. latirostris* testes. After a deep evaluation of male gonadal tissue from the caimans of Experiment I, we performed similar evaluations in animals of Experiment II. Results of Experiment II are consistent with those of our previous work on how EDCs affect the histological features in testes of 10-day-old, 90-day-old, and Juv caimans, with testes showing an altered histoarchitecture (Durando et al., 2013).

Proliferating cell nuclear antigen (PCNA) is used as a marker of cell proliferation in normal and pathological tissues (Dietrich, '93). In previous works, we examined the proliferative activity in caiman gonads by assessing in vivo bromodeoxyuridine incorporation (Stoker et al., 2008; Rey et al., 2009). Here, as larger caimans were included, large amounts of bromodeoxyuridine would be administered. To minimize handling and caiman stress, we quantified the percentage of cells expressing PCNA as a measure of proliferation. Several works have evaluated the testicular proliferation in reptiles, mainly during different phases

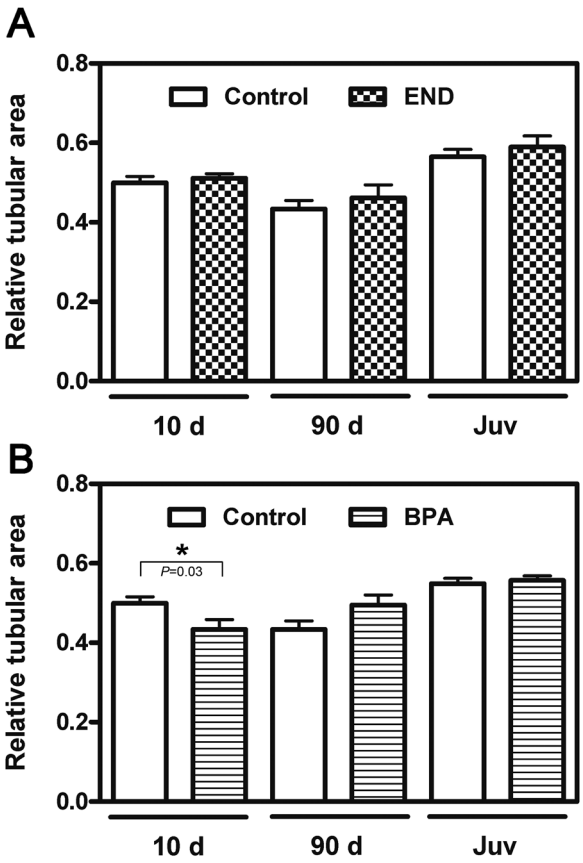

Figure 5. Effect of *in ovo* exposure to EDCs on the relative tubular area (RTA). END exposure did not modify the RTA (**A**), while BPA decreased the RTA in 10-day-old caimans (**B**). Values are shown as mean ± S.E.M. (7–9 caimans/group) and significant differences are depicted with an asterisk ($^{*}P < 0.05$) above bars. 10 d, 10-day-old; 90 d, 90-day-old; Juv, Juvenile stage.

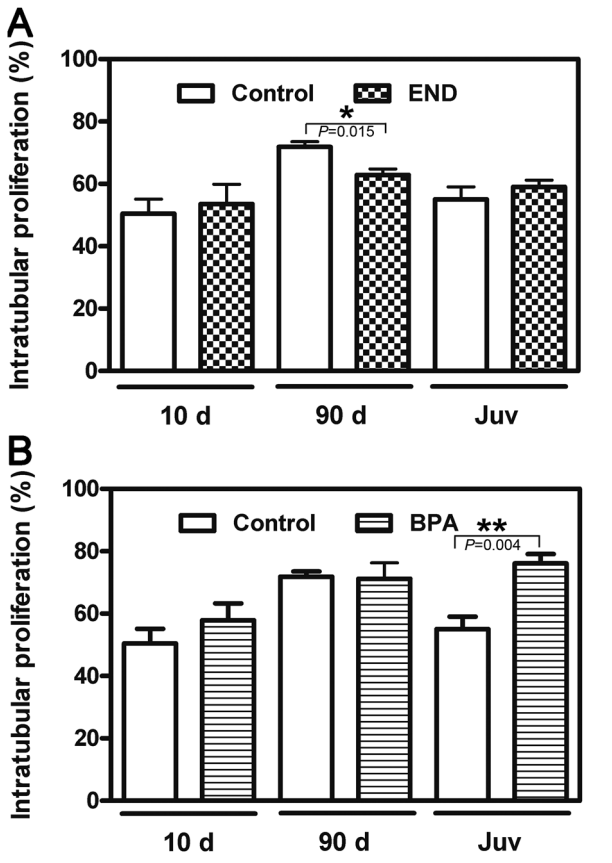

Figure 6. Effect of *in ovo* exposure to EDCs on the intratubular proliferation. The percentage of proliferating cells was decreased in 90-day-old caimans exposed to END and increased in Juv caimans exposed to BPA. Values are shown as mean ± S.E.M. (7–9 caimans/group) and significant differences are depicted with asterisks ($^{**}P < 0.01$; $^{*}P < 0.05$) above bars. 10 d, 10-day-old; 90 d, 90-day-old; Juv, Juvenile stage.

of the spermatogenic cycle. In testes of the adult Chinese soft-shelled turtle (*Pelodiscus sinensis*), PCNA expression is high in spermatogonia and spermatocytes during the active spermatogenic phase, but mostly expressed by spermatogonia during the quiescent phase (Zhang et al., 2008). The caimans studied in the present work did not display seasonal changes because they were raised in controlled environmental conditions. Furthermore, we evaluated the expression of PCNA in somatic and germ cells

Table 3. Percentage of intratubular cells expressing ERα.

Experimental group	10-day-old (n)	P^a	90-day-old (n)	P^a	Juv (n)	P^a
Control	25.6 ± 2.4 (9)	–	33.8 ± 1.7 (7)	–	34.3 ± 3.6 (6)	–
END	23.6 ± 2.2 (7)	0.52	35.3 ± 5.1 (6)	0.44	41.1 ± 2.4[b] (6)	0.24
BPA	23.9 ± 2.9 (6)	0.69	33.5 ± 2.2 (8)	0.95	34.0 ± 3.0 (6)	1.00

[a]Compared to Control group (Mann–Whitney test).
[b]Statistical difference in END-exposed caimans between 10-day-old and Juv (Wilcoxon matched-pairs signed rank test; $P < 0.05$) (See Fig. 7).

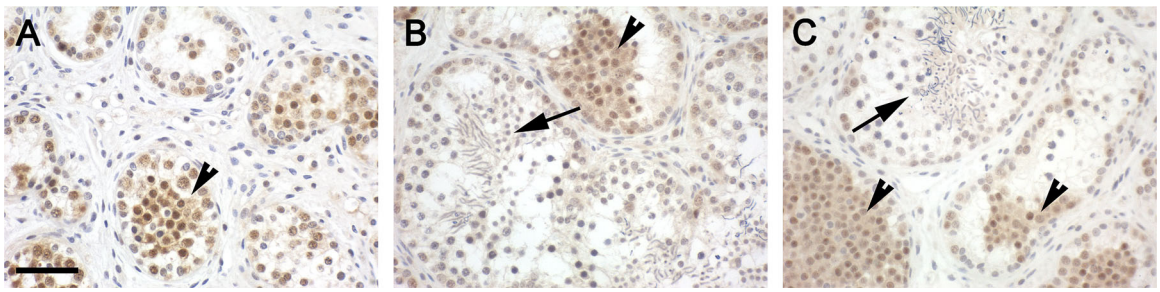

Figure 7. ERα protein expression in testes of a subset of juvenile caimans. Cross sections are representative of the Control (**A**), END (**B**) and BPA (**C**) groups. In (**B**) and (**C**), testes exhibit round and elongated spermatids ERα negative (arrows), while less differentiated germ cells are expressing ERα (arrowheads in **A–C**). Slides were counterstained with Mayer's hematoxylin. Scale bar = 50 μm.

inside the seminiferous tubules, without distinguishing between germ cell maturation stages. In animals from Experiment I, we detected the highest intratubular proliferation in 90-day-old caimans. In reptiles, testicular cell proliferation is frequently described in adult animals, when the seasonal spermatogenic cycle is described (Gribbins et al., 2006; Zhang et al., 2008). Since testicular proliferative activity has also been studied as a target of the endocrine-disrupting effect (Kitana et al., 2007; Rey et al., 2009), we first established the ontogeny of the intratubular proliferation (Experiment I), and then evaluated whether *in ovo* exposure to EDCs affected this parameter (Experiment II). Our results of Experiment II demonstrate changes in intratubular proliferation, both in animals exposed to END and in those exposed to BPA. These changes included a decrease in 90-day-old caimans exposed to END and an increase in Juv caimans exposed to BPA. In turtles, Kitana et al. (2007) found no differences in germ cell proliferation in adult males of *C. picta* when comparing turtles collected from a reference site with those from a contaminated site. In *C. latirostris*, we have previously reported increased proliferative activity in testes of neonatal caimans after *in ovo* exposure to END (2 and 20 ppm) (Rey et al., 2009).

In vertebrates, the testis can be both a source and a target for estrogen hormones (O'Donnell et al., 2001; Akingbemi, 2005). Like other hormones, testicular estrogens are able to act on distant target tissues but can also remain in the testis and act in a paracrine manner. Estrogen receptors (ERs) play an essential role in mediating estrogen action. In testis, ERs can have different localization depending on several factors (i.e. studied species, developmental stage of the cell and type of receptor) (Abney, '99; O'Donnell et al., 2001; Hess and Carnes, 2004). In mammals, the localization and expression levels of ERα and ERβ have been previously described in detail (O'Donnell et al., 2001), but are less known in reptiles. In *A. mississippiensis*, ERα mRNA expression was measured in gonads of juvenile animals (Kohno et al., 2008; Moore et al., 2010b) and no sexual dimorphism was found, since males and females exhibited similar levels of ERα mRNA. In testes of juvenile alligators hatched from eggs collected from a contaminated lake, ERα mRNA expression was not different from that of alligators hatched from eggs collected from a reference lake (Moore et al., 2010b). Furthermore, ERα protein has been detected in Leydig cells but not in spermatogonia and Sertoli cells of quiescent testes of adult *Trachemys scripta* (Gist et al., 2007); however, in adult males of *Chelonia mydas*, ERα protein expression is modified according to the developmental stage of seminiferous tubules, although Leydig and Sertoli cells are always ERα positive while germ cells are always ERα negative (Otsuka et al., 2008). Here, we first quantified the percentage of intratubular cells expressing ERα protein from the neonatal up to the peri-pubertal juvenile stage in vehicle-exposed caimans. No age-dependent differences were found in the testicular expression of ERα. In the interstitial compartment, ERα expression was not differentially quantified; however, the presence of ERα in peritubular myoid cells in 90-day-old animals caught our attention. In mammals, peritubular myoid cells and Sertoli cells are proposed as key players in the longitudinal growth of seminiferous cords in the postnatal testis (Nurmio et al., 2012). In the testis of stallions, the localization of ERα protein is age-dependent: only post-pubertal animals have peritubular myoid cells expressing ERα (Pearl et al., 2011). In our study, the presence of ERα in myoid cells coincides with a higher intratubular proliferation. Once the ontogeny of ERα expression was established, we evaluated how the exposure to END or BPA modified its expression. Neither END nor BPA modified ERα protein expression (compared to the Control group); however, it is worth mentioning that juvenile caimans exposed to END showed a tendency to a lower expression of ERα in luminal cells. In mammals, the higher the differentiation of spermatogenic cells, the lower the expression of ERα (O'Donnell et al., 2001; Mutembei et al., 2005) and at the light of the results illustrated in Figure 7, we may suggest that exposure to END accelerates spermatogenesis. However, this needs to be deeply explored.

In conclusion, our results suggest that the alterations described could compromise not only the sexual maturation but also the reproductive life of caimans. These results highlight the importance of preserving the wild environments from potential pollution with EDCs.

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LITERATURE CITED

Abney TO. 1999. The potential roles of estrogens in regulating Leydig cell development and function: a review. Steroids 64:610–617.

Akingbemi BT. 2005. Estrogen regulation of testicular function. Reprod Biol Endocrinol 3:51.

American Society of Icthyologists and Herpetologists (ASIH). 2004. Guidelines for use of live amphibians and reptiles in field and laboratory research. Revised by the Herpetological Animal Care and Use Committee, second ed. Available from: http://www.asih.org/publications.

Bhandari RK, Deem SL, Holliday DK, et al. 2015. Effects of the environmental estrogenic contaminants bisphenol A and 17a-ethinyl estradiol on sexual development and adult behaviors in aquatic wildlife species. Gen Comp Endocrinol 214:195–219.

Beldomenico PM, Rey F, Prado WS, et al. 2007. In ovum exposure to pesticides increases the egg weight loss and decreases hatchlings weight of *Caiman latirostris* (Crocodylia: Alligatoridae). Ecotoxicol Environ Saf 68:246–251.

Bisson M, Hontela A. 2002. Cytotoxic and endocrine-disrupting potential of atrazine, diazinon, endosulfan, and mancozeb in adrenocortical steroidogenic cells of rainbow trout exposed in vitro. Toxicol Appl Pharm Acol 180:110–117.

Boggs ASP, Botteri NL, Hamlin HJ, Guillette LJ Jr. 2011. Endocrine disruption of reproduction in reptiles. In: Norris DO, Lopez KH, editors. Hormones and reproduction of vertebrates. Vol 3. San Diego, CA: Elsevier Inc. p 373–396.

Crain DA, Eriksen M, Iguchi T, et al. 2007. An ecological assessment of bisphenol-A: evidence from comparative biology. Reprod Toxicol 24:225–239.

Da Cuña RH, Rey Vázquez G, Piol MN, et al. 2011. Assessment of the acute toxicity of the organochlorine pesticide endosulfan in *Cichlasoma dimerus* (Teleostei, Perciformes). Ecotoxicol Environ Saf 74:1065–1073.

Da Cuña RH, Pandolfi M, Genovese G, et al. 2013. Endocrine disruptive potential of endosulfan on the reproductive axis of *Cichlasoma dimerus* (Perciformes, Cichlidae). Aquat Toxicol 126:299–305.

de Solla SR, Bishop CA, Van der Kraak G, Brooks RJ. 1998. Impact of organochlorine contamination on levels of sex hormones and external morphology of common snapping turtles (*Chelydra serpentina*) in Ontario, Canadá. Environ Health Perspect 106: 253–260.

Dietrich DR. 1993. Toxicological and pathological applications of proliferating cell nuclear antigen (PCNA), a novel endogenous marker for cell proliferation. Crit Rev Toxicol 23:77–109.

Durando M, Kass L, Perdomo V, et al. 2011. Prenatal exposure to bisphenol A promotes angiogenesis and alters steroid-mediated responses in the mammary glands of cycling rats. J Steroid Biochem Mol Biol 127:35–43.

Durando M, Cocito L, Rodríguez HA, et al. 2013. Neonatal expression of *amh*, *sox9* and *sf-1* mRNA in *Caiman latirostris* and effects of in ovo exposure to endocrine disrupting chemicals. Gen Comp Endocrinol 191:31–38.

Flint S, Markle T, Thompson S, Wallace E. 2012. Bisphenol A exposure, effects, and policy: a wildlife perspective. J Environ Manage 104:19–34.

Galoppo GH, Canesini G, Vigezzi L, et al. 2014. Postnatal exposure to xenoestrogens alters wnt7a expression and oviductal radial pattern in *Caiman latirostris*. Biocell 38(Suppl 2):A120.

Gist DH, Bradshaw S, Morrow CM, Congdon JD, Hess RA. 2007. Estrogen response system in the reproductive tract of the male turtle: an immunocytochemical study. Gen Comp Endocrinol 151:27–33.

Gormley KL, Teather KL. 2003. Developmental, behavioral, and reproductive effects experienced by Japanese medaka (*Oryzias latipes*) in response to short term exposure to endosulfan. Ecotoxicol Environ Saf 54:330–338.

Gribbins KM, Elsey RM, Gist DH. 2006. Cytological evaluation of the germ cell development strategy within the testis of the American alligator, *Alligator mississippiensis*. Acta Zoologica 87:59–69.

Hamlin HJ, Guillette LJ Jr. 2011. Embryos as targets of endocrine disrupting contaminants in wildlife. Birth Defects Res C Embryo Today 93:19–33.

Hayes TB, Anderson LL, Beasley VR, et al. 2011. Demasculinization and feminization of male gonads by atrazine: consistent effects across vertebrate classes. J Steroid Biochem Mol Biol 127:64–73.

Hess RA, Carnes K. 2004. The role of estrogen in testis and the male reproductive tract: a review and species comparison. Anim Reprod 1:5–30.

Hotchkiss AK, Rider CV, Blystone CR, et al. 2008. Fifteen years after "Wingspread"-environmental endocrine disrupters and human and wildlife health: where we are today and where we need to go. Toxicol Sci 105:235–259.

Iungman J, Piña CI, Siroski P. 2008. Embryological development of *Caiman latirostris* (Crocodylia:Alligatoridae). Genesis 46:401–417.

Jandegian CM, Deem SL, Bhandari RK, et al. 2015. Developmental exposure to bisphenol A (BPA) alters sexual differentiation in painted turtles (*Chrysemys picta*). Gen Comp Endocrinol 216:77–85.

Kass L, Durando M, Altamirano GA, et al. 2015. Prenatal Bisphenol A exposure delays the development of the male rat mammary gland. Reprod Toxicol 54:37–46.

Kitana N, Won SJ, Callard IP. 2007. Reproductive deficits in male freshwater turtle *Chrysemys picta* from cape cod. Massachusetts. Biol Reprod 76:346–352.

Kohno S, Bermudez DS, Katsu Y, Iguchi T, Guillette LJ Jr. 2008. Gene expression patterns in juvenile American alligators (*Alligator mississippiensis*) exposed to environmental contaminants. Aquat Toxicol 88:95–101.

Lang JW, Andrews HV. 1994. Temperature-dependent sex determination in crocodilians. J Exp Zool 270:28–44.

Lemaire G, Mnif W, Mauvais P, Balaguer P, Rahmani R. 2006. Activation of a- and b-estrogen receptors by persistent pesticides in reporter cell lines. Life Sci 79:1160–1169.

Matsumoto Y, Hannigan B, Crews D. 2014. Embryonic PCB exposure alters phenotypic, genetic, and epigenetic profiles in turtle sex determination, a biomarker of environmental contamination. Endocrinology 155:4168–4177.

Moore BC, Hamlin HJ, Botteri NL, et al. 2010a. Posthatching development of *Alligator mississippiensis* ovary and testis. J Morphol 271:580–595.

Moore BC, Milnes MR, Kohno S, et al. 2010b. Influences of sex, incubation temperature, and environmental quality on gonadal estrogen and androgen receptor messenger RNA expression in juvenile American alligators (*Alligator mississippiensis*). Biol Reprod 82:194–201.

Mutembei HM, Pesch S, Schuler G, Hoffmann B. 2005. Expression of oestrogen receptors alpha and beta and of aromatase in the testis of immature and mature boars. Reprod Domest Anim 40:228–236.

Naqvi SM, Vaishnavi C. 1993. Bioaccumulative potential and toxicity of endosulfan insecticide to non-target animals. Comp Biochem Physiol 105:347–361.

Nurmio M, Kallio J, Adam M, et al. 2012. Peritubular myoid cells have a role in postnatal testicular growth. Spermatogenesis 2:79–87.

O'Donnell L, Robertson KM, Jones ME, Simpson ER. 2001. Estrogen and spermatogenesis. Endocr Rev 22:289–318.

Oehlmann J, Schulte-Oehlmann U, Kloas W, et al. 2009. A critical analysis of the biological impacts of plasticizers on wildlife. Philos Trans R Soc Lond B Biol Sci 364:2047–2062.

Otsuka S, Suzuki M, Kamezaki N, et al. 2008. Growth-related changes in histology and immunolocalization of steroid hormone receptors in gonads of the immature male green turtle (*Chelonia mydas*). J Exp Zool A Ecol Genet Physiol 309:166–174.

Pathak R, Suke SG, Ahmed T, et al. 2010. Organochlorine pesticide residue levels and oxidative stress in preterm delivery cases. Hum Exp Toxicol 29:351–358.

Pearl CA, Mason H, Roser JF. 2011. Immunolocalization of estrogen receptor alpha, estrogen receptor beta and androgen receptor in the pre-, peri- and post-pubertal stallion testis. Anim Reprod Sci 125:103–111.

Peretz J, Vrooman L, Ricke WA, et al. 2014. Bisphenol A and reproductive health: update of experimental and human evidence, 2007–2013. Environ Health Perspect 122:775–786.

Piña CI, Larriera A, Cabrera M. 2003. Effect of incubation temperature on incubation period, sex ratio, hatching success, and survivorship in *Caiman latirostris* (Crocodylia, Alligatoridae). J Herpetol 37:199–207.

Poletta GL, Kleinsorge E, Paonessa A, et al. 2011. Genetic, enzymatic and developmental alterations observed in *Caiman latirostris* exposed in ovo to pesticide formulations and mixtures in an experiment simulating environmental exposure. Ecotoxicol Environ Saf 74:852–859.

Rajakumar A, Singh R, Chakrabarty S, et al. 2012. Endosulfan and flutamide impair testicular development in the juvenile Asian catfish, *Clarias batrachus*. Aquat Toxicol 110–111:123–132.

Rastogi D, Narayan R, Saxena DK, Chowdhuri DK. 2014. Endosulfan induced cell death in Sertoli-germ cells of male Wistar rat follows intrinsic mode of cell death. Chemosphere 94:104–115.

Rey F, González M, Zayas MA, et al. 2009. Prenatal exposure to pesticides disrupts testicular histoarchitecture and alters testosterone levels in male *Caiman latirostris*. Gen Comp Endocrinol 162:286–292.

Richter CA, Birnbaum LS, Farabollini F, et al. 2007. In vivo effects of bisphenol A in laboratory rodent studies. Reprod Toxicol 24:199–224.

Roy JR, Chakraborty S, Chakraborty TR. 2009. Estrogen-like endocrine disrupting chemicals affecting puberty in humans—a review. Med Sci Monit 15:RA137–RA145.

Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA) Argentina. http://www.senasa.gov.ar/contenido.php?to=n&tin=1501&tio=17737

Silva MH, Gammon D. 2009. An assessment of the developmental, reproductive and neurotoxicity of endosulfan. Birth Defects Res 86:1–28.

Staples CA, Dorn PB, Klecka GM, O'Block ST, Harris LR. 1998. A review of the environmental fate, effects, and exposures of bisphenol A. Chemosphere 36:2149–2173.

Stoker C. 2004. *Caiman latirostris* as sentinel specie of environmental pollution by xenoestrogens. PhD Thesis; Laboratorio de Endocrinología y Tumores Hormonodependientes, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral.

Stoker C, Rey F, Rodríguez H, et al. 2003. Sex reversal effects on Caiman latirostris exposed to environmentally relevant doses of the xenoestrogen bisphenol A. Gen Comp Endocrinol 133:287–296.

Stoker C, Beldoménico PM, Bosquiazzo VL, et al. 2008. Developmental exposure to endocrine disruptor chemicals alters follicular dynamics and steroid levels in *Caiman latirostris*. Gen Comp Endocrinol 156:603–612.

Stoker C, Repetti MR, García SR, et al. 2011. Organochlorine compound residues in the eggs of broad-snouted caimans (*Caiman latirostris*) and correlation with measures of reproductive performance. Chemosphere 84:311–317.

Stoker C, Zayas MA, Ferreira MA, et al. 2013. The eggshell features and clutch viability of the broad-snouted caiman (*Caiman latirostris*) are associated with the egg burden of organochlorine compounds. Ecotoxicol Environ Saf 98:191–195.

Vandenberg LN, Colborn T, Hayes TB, et al. 2013. Regulatory decisions on endocrine disrupting chemicals should be based on the principles of endocrinology. Reprod Toxicol 38:1–15.

Varayoud J, Monje L, Bernhardt T, et al. 2008. Endosulfan modulates estrogen-dependent genes like a non-uterotrophic dose of 17beta-estradiol. Reprod Toxicol 26:138–145.

Varayoud J, Monje L, Moreno-Piovano GS, et al. 2012. Sexually dimorphic expression of receptor-alpha in the cerebral cortex of neonatal *Caiman latirostris* (Crocodylia: Alligatoridae). Gen Comp Endocrinol 179:205–213.

Wang N, Xu Y, Zhou XQ, et al. 2014. Protective effects of testosterone propionate on reproductive toxicity caused by Endosulfan in male mice. Environ Toxicol DOI: 10.1002/tox.22029

Willingham EJ. 2005. The effects of atrazine and temperature on turtle hatchling size and sex ratios. Front Ecol Environ 3: 309–313.

Yanosky AA. 1990. Histoire naturelle du Caiman à museau large (*Caiman latirostris*), un Alligatorinè mal connu. Rev Fr Aquariol Herpetol 17:119–131.

Zayas MA, Rodríguez HA, Galoppo GH, et al. 2011. Hematology and blood biochemistry of young healthy broad snouted caimans (*Caiman latirostris*). J Herpetol 45:516–524.

Zhang L, Han XK, Qi YY, Liu Y, Chen QS. 2008. Seasonal effects on apoptosis and proliferation of germ cells in the testes of the Chinese soft-shelled turtle, *Pelodiscus sinensis*. Theriogenology 69:1148–1158.