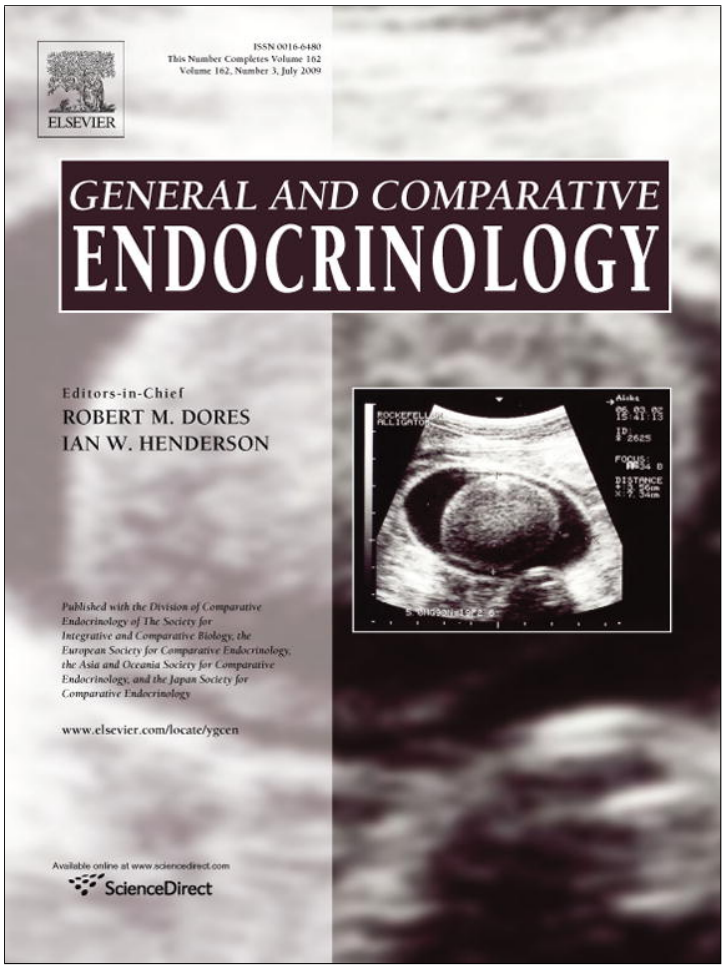

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Prenatal exposure to pesticides disrupts testicular histoarchitecture and alters testosterone levels in male *Caiman latirostris* ☆

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ABSTRACT

The increased use of agrochemical pesticides, such as atrazine (ATZ) and endosulfan (END), may have a significant impact on ecosystem health and biodiversity. The aim of this study was to investigate the consequences of *in ovum* exposure to ATZ and END on *Caiman latirostris* gonadal histo-functional features. Caiman eggs were collected from environmentally pristine areas and incubated in controlled conditions at male producing temperature (33 °C). At stage 20 of embryonic development, the sensitive stage for gonadal sex determination, eggs were exposed to one dose of either END or ATZ. Gonadal histo-morphology was examined in caiman hatchlings and serum levels of testosterone were measured. Regardless of treatment condition, all eggs incubated at 33 °C resulted in male hatchlings. Tortuous seminiferous tubules with increased perimeter, disrupted distribution of peritubular myoid cells (desmin positive), and emptied tubular lumens characterized the testes of pesticide-exposed caiman. An imbalance between proliferative activity and cell death was observed in the testes of caiman exposed to the higher doses of END, mainly due to a high frequency of apoptosis in intratubular cells. This altered cell turnover was associated with decreased testosterone levels. Prenatal exposure to only one dose of END and ATZ disrupted neonatal male gonadal histo-functional features. Alterations described here could have detrimental effects on the sexual maturation of the caiman and, ultimately, on the success of male caiman reproduction.

1. Introduction

There is a substantial body of research regarding endocrine disruption due to the prevalence of man-made chemicals in the environment. Numerous wildlife species have been affected by exposure to endocrine disruptor compounds (EDCs) showing impaired reproduction with reduced hatchability and/or viability of offspring, decreased androgen plasma levels, alteration in spermatogenesis and penis/gonopodium development, and aberrant sexual behavior (Oberdörster and Cheek, 2001; IPCS, 2002; Jobling et al., 2006; Milnes et al., 2006; Guillette and Edwards, 2008). Furthermore, it has been demonstrated that exposure to EDCs affect sex determination, differentiation of the gonads and timing of sexual maturation (Crain et al., 2007; Orlando and Guillette, 2007; Stoker et al., 2003, 2008).

Exposure to EDCs during critical periods of organogenesis with high sensitivity to relatively small changes in hormone levels can lead to subtle irreversible organizational effects. Supporting this hypothesis, we have reported sex reversal effects and/or gonadal histoarchitecture disruption on *Caiman latirostris* hatchlings and juveniles exposed *in ovum* to low doses of estrogen or to various EDCs, such as bisphenol A (BPA), atrazine (ATZ), or endosulfan (END) (Beldomenico et al., 2007; Stoker et al., 2003, 2008). Since the effects of prenatal exposure to EDCs may be evident long after exposure has ended, controlled experiments on wildlife are needed for a better understanding of the developmental consequences of EDC exposure.

ATZ and END are widely used pesticides that have been classified as EDCs (Bisson and Hontela, 2002; Hayes et al., 2002; Gormley and Teather, 2003). While END has been found to be estrogenic (Soto et al., 1994; Hodges et al., 2000; Varayoud et al., 2008), research data show that ATZ has no estrogen receptor-mediated effects (Sanderson et al., 2001; Legler et al., 2002; Wilhelms et al., 2006; Oka et al., 2008). Despite this, evidence on endocrine disruption produced by ATZ is growing and concerning (Hayes et al., 2002, 2006; Fan et al., 2007; Stoker et al., 2008).

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The present study was carried out to further elucidate gonadal histo-functional alterations in *C. latirostris* males exposed *in ovum* to EDCs. Testosterone levels and testicular histo-morphological characteristics, with a particular focus on cellular turnover, were evaluated in 10-day-old hatchlings prenatally exposed to either END or ATZ.

2. 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 Universidad Nacional del Litoral Institutional Animal Care and Use Committee.

Eggs were collected shortly after oviposition from six nests randomly selected from regions of Chaco and Entre Ríos provinces in Argentina. These sites are far upstream of urbanized, industrial and farming areas, thus minimizing exposure to sewage or agriculture and/or feedlot run-off. Prior to removal from the nest, the upper surfaces of the eggs were marked with a graphite pencil to keep the original orientation during their incubation in controlled laboratory conditions. Eggs (n = 209) were transported to the laboratory and randomly distributed into two groups such that half of the eggs from one clutch were incubated at a constant temperature of 30 °C (100% female producing temperature) and the other half at a constant temperature of 33 °C (100% male producing temperature) (Stoker et al., 2003). Opaque eggshell banding development was used to check embryo viability. Within each incubation temperature group, eggs from each clutch were equally distributed among treatment groups. All treatments were applied topically to the eggshell at stage 20 of embryonic development, the period that occurs prior to the window for *C. latirostris* sex determination (Stoker et al., 2003). Fifty-five caimans born from eggs incubated at 33 °C were included in this study.

2.2. Treatments

Control eggs received vehicle (50 μl absolute ethanol) and treated groups received 0.02 ppm (END0.02), 2 ppm (END2) or 20 ppm (END20) of END (Icona S.A., Argentina) or 0.2 ppm ATZ (Icona S.A.). END at 0.02 ppm is a similar dose to the concentration found in the environment (Laabs et al., 2002). Treatments with higher doses of END were conducted to evaluate dose–response effects. The ATZ dose was considered ecologically relevant and has been associated with hermaphroditism and gonadal disruption in amphibians (Hayes et al., 2002).

At ten days of age, caiman were euthanized with sodium pentobarbital (*ip*). Two hours before sacrifice they were injected with bromodeoxyuridine (BrdU; Sigma Chemical, St. Louis, MO; 6 mg/100 g body weight/0.25 ml PBS, *ip*) in order to evaluate BrdU incorporation as a proliferation marker. Animals were decapitated, trunk blood was collected, and serum was stored at −20 °C until used for hormone assays. Gonadal–Adrenal–Mesonephros (GAM) complexes were dissected and immediately fixed for histological studies.

2.3. Gonadal histoarchitecture and morphometric analysis

Gonad sex determination and morphometric studies were done by histological examination. After dissection, GAM complexes were fixed by immersion in 4% phosphate-buffered formalin (pH 7.4) for 6 h at room temperature. Fixed tissues were dehydrated, cleared in xylene, and embedded in paraffin. Serial transverse sections (5 μm) were stained with the trichromic Picrosirius solution and counterstained with Harris hematoxylin (Biopur, Rosario, Argentina). Sex was determined by histology as we described previously (Stoker et al., 2003). To evaluate the impact of *in ovo* exposure to pesticides on seminiferous tubules histoarchitecture, we manually delimited and measured the perimeter of the seminiferous tubules using an image analysis system as has been previously described (Stoker et al., 2003). Briefly, images were recorded by a SPOT color video camera (Diagnostic Instruments Inc., USA) attached to an Olympus BH2 microscope (Olympus Optical, Tokyo, Japan). Image analysis was carried out using the Image Pro-Plus 4.1.0.1 system (Media Cybernetics, Silver Spring, MD, USA).

2.4. Immunolocalization of desmin and smooth muscle actin

Expression of desmin and smooth muscle actin (α-SMA) was used to establish the phenotype of peritubular cells surrounding the seminiferous tubules. After microwave pre-treatment, tissue samples were incubated overnight at 4 °C with primary antibodies (anti-desmin 1:50, clone DER11 or anti-SMA 1:50, clone α-SM1; Novocastra Laboratories Ltd., Newcastle upon Tyne, UK). Antibodies' cross-reactivity was tested. Anti-SMA cross-reactivity was also verified using blood vessel muscle cells as an intra sample positive control. Each immunohistochemical run included positive and negative controls. For negative controls the primary antibody was replaced with non-immune mouse serum (Sigma Chemical, St. Louis, MO). Reactions were developed using the avidin–biotin peroxidase method and diaminobenzidine (DAB, Sigma) as a cromogen substrate. Samples were counterstained with Mayer hematoxylin (Biopur). At least three sections with a minimal separation of 150 μm were evaluated in each gonad. Manual delimitation of seminiferous tubules perimeter was conducted and the length occupied by immunostained peritubular cells was measured. Calibration with reference rulers was performed at the beginning of each measurement. Results were expressed as the percentage of the tubular perimeter occupied by immunostained cells.

2.5. Intratubular cell turnover

Since in the pesticide exposed groups, somatic and germ cells could not be reliably distinguished from one another, to establish the balance between proliferative activity and cell death all cells from the seminiferous epithelium were included. Cellular turnover was calculated as the ratio between proliferation and apoptosis percentages.

2.5.1. Proliferative activity

As already mentioned, BrdU was administered 2 h before sacrifice. BrdU incorporation was evaluated by immunohistochemistry (Kass et al., 2000). After microwave pre-treatment for antigen retrieval and acid hydrolysis for DNA denaturation, the primary antibody was incubated overnight at 4 °C (anti-BrdU 1:100, clone 85-2C8; Novocastra Laboratories Ltd., Newcastle upon Tyne, UK). The reaction was developed using the avidin–biotin peroxidase method and diaminobenzidine (DAB). Samples were counterstained with Harris hematoxylin and mounted with a permanent mounting medium (PMyR, Buenos Aires, Argentina). Each immunohistochemical run included positive and negative controls. For negative controls the primary antibody was replaced by non-immune mouse serum (Sigma). Results were expressed as percentage of proliferating cells.

2.5.2. Apoptosis assay

Apoptosis was evaluated by the TUNEL (terminal deoxynucleotidyl transferase-mediated sUTP nick end-labelling) method using the ApopTag kit (Chemicon International Inc., Temecula, CA, USA)

(Muñoz-de-Toro et al., 1998). Apoptotic cells were identified using the cromogen substrate DAB. Tissue samples were counterstained with Mayer hematoxylin. Results were expressed as percentage of apoptotic cells.

2.6. Testosterone assay

Serum levels of testosterone (T) were determined by radioimmunoassay (RIA) using T, [1,2,6,7-^{3}H (N)] (PerkinElmer Life And Analytical Sciences, Inc., Boston, MA, USA) and a specific antibody provided by Dr. G.D. Niswender. Steroids were extracted from 200 μl of serum with 2 ml of ethyl ether (Merck, Buenos Aires, Argentina). The extraction procedure was repeated three times. The percent recovery of extraction was calculated by the addition of a fixed amount of tracer to dextran coated charcoal stripped caiman serum and was over 85%. A sensitivity 0.4 pg/ml for T assays was achieved. Quality control standards consisting of a pool of caiman serum and pooled human serum of known hormone concentrations were run in every assay. The interassay coefficient of variation was 10.44% and the intra-assay variation coefficient was 6.96%.

2.7. Statistical analysis (Siegel, 1956)

All data are reported as the means ± SEM. Kruskal–Wallis analyses were performed to obtain the overall significance followed by the Mann–Whitney *U* post-test to establish differences between control and experimental groups. $p < 0.05$ was accepted as significant.

3. Results

3.1. Gonadal histoarchitecture

At 10 days of age, the male GAM complex was characterized by the presence of the testis associated with adrenal tissue and mesonephros. Hatchling males either completely lacked or showed vestiges of Müllerian ducts. Testes were characterized by the presence of well-differentiated seminiferous tubules, filled by germ cells and Sertoli cells lining on the basement membrane (Fig. 1A and C). Seminiferous tubules were surrounded by a monolayer of peritubular cells, interstitial Leydig cells, and extra cellular matrix.

As recently reported (Beldomenico et al., 2007), neither ATZ nor END were able to override the temperature's effect on sex determination; therefore, all the eggs incubated at 33 °C resulted in male hatchlings. However, *in ovum* exposure to END or ATZ disrupted the testicular histoarchitecture, resulting in disorganized and tortuous seminiferous tubules (Fig. 1B). Moreover, a loss of intratubular intercellular connections and either emptied tubular lumens or luminal cellular detritus were observed (Fig. 1D and E). A significant increase in the seminiferous tubular perimeter was demonstrated in caiman prenatally exposed to 0.2 ppm ATZ, END0.02 or END20. No differences in seminiferous tubular perimeter were found between controls and END2 (Fig. 2).

3.2. Peritubular cell distribution pattern

Following the observation of an altered testicular histoarchitecture, characterized by an increase in the perimeter of the seminiferous tubules, the immunophenotype of the peritubular cells was established and their distribution pattern was determined. Peritubular cells of the seminiferous tubules of hatchling caiman expressed desmin but not α-SMA. As shown in Fig. 3A, the testis of control caiman exhibited a monolayer of desmin positive cells surrounding the seminiferous tubular perimeter. A significant reduction in the percentage of tubular perimeter length occupied by desmin positive cells was observed in the testis of pesticide-exposed caiman (Fig. 3).

3.3. Intratubular cell turnover

Altered organization of the peritubular myoid cells and/or the evident lost of intratubular intercellular connections could modify

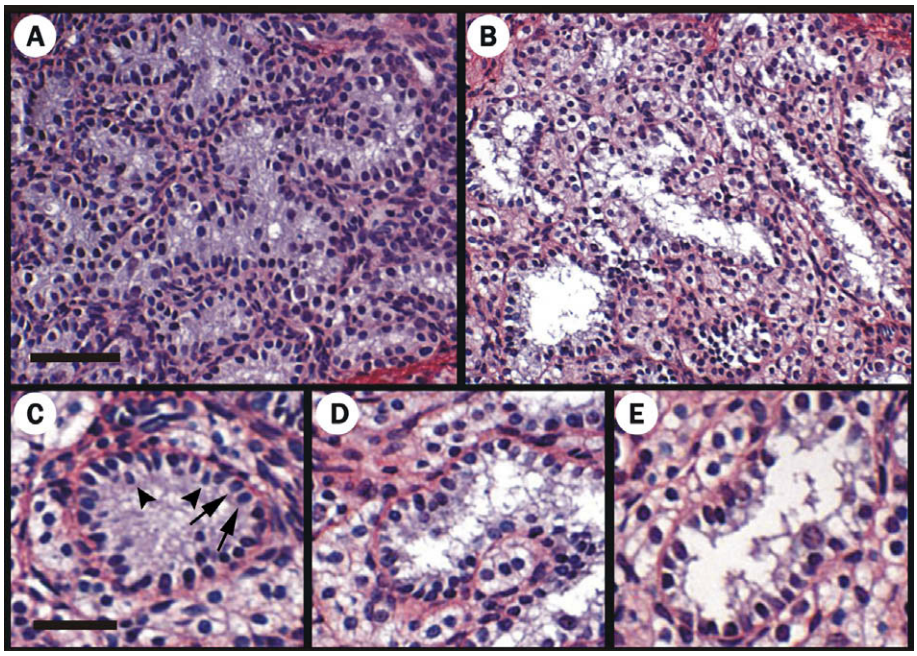

Fig. 1. Gonadal histoarchitecture in male caiman hatchlings. Upper panel, (A) low magnification photomicrographs of testis cross-sections obtained from 10 day old caimans exposed *in ovum* to vehicle or (B) to ATZ at a dose of 0.2 ppm. Testes from pesticide-exposed caimans exhibited disorganized and tortuous seminiferous tubules compared to control. Lower panel, representative photomicrographs of seminiferous tubule cross sections, (C) Vehicle, (D) END20, (E) ATZ. Arrows, germ cells; arrow heads, Sertoli cells. Tissue sections were stained with Picrosirius solution and counterstained with Harris hematoxylin. Scale bars = 50 μm (upper panel) and 30 μm (lower panel).

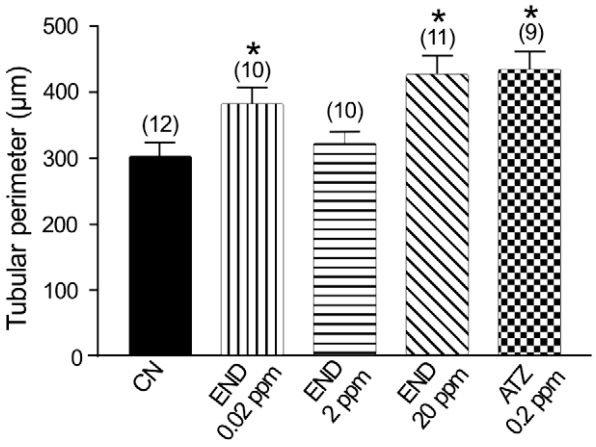

Fig. 2. Effect of *in ovum* exposure to pesticides on the perimeter of seminiferous tubules in hatchling caimans. Values are means ± SEM, between brackets: number of hatchlings by group, asterisks denote differences between treatment groups and control (Kruskal-Wallis, Mann–Whitney *U* post test, $p < 0.05$). CN: vehicle. END: endosulfan, ATZ: atrazine.

signals involved in the control of cell proliferation and survival. Therefore, intratubular cell proliferative activity and apoptosis were evaluated. A mild increase in proliferative activity (Fig. 4A), in parallel with a drastic increase in apoptosis (Fig. 4B) was observed in END2- and END20-treated caiman. As a result of both events, a marked tendency to a decreased cellular turnover was observed (control versus END20, $p = 0.057$ Mann–Whitney *U*) (Fig. 4C).

3.4. Testosterone levels

A significant decrease in testosterone levels was found in caimans prenatally exposed to END2 and END20 whereas testosterone levels in END0.02- or ATZ-exposed caiman did not differ from untreated male controls (Fig. 5).

4. Discussion

In this study, the effects of *in ovo* exposure to EDC pesticides on the testicular histomorphology and circulating levels of testosterone in *C. latirostris* were assessed. Results demonstrated that embryonic exposure to ATZ and END during the critical period of sex determination altered histoarchitecture of the testis as well as the balance between proliferation and apoptosis of hatchlings' testicular cells. On the other hand, decreased testosterone levels were displayed by END2 and END20 exposed males. The present study was restricted to neonatal caiman and therefore did not address whether the adverse testicular changes in pesticide exposed groups resulted in consequences over the lifespan.

Many recent studies in wildlife have generated increasing concern regarding endocrine disrupting chemicals effects on reproductive functions (Guillette and Edwards, 2008). In non-mammalian vertebrates, studies have focused mainly on fish, but rarely on amphibians or reptiles (Milnes et al., 2006). In fish, delayed spermatogenesis, decreased sperm counts, altered sperm motility and a higher incidence of intersex were demonstrated (Jobling et al., 2002; Aravindakshan et al., 2004; de Montgolfier et al., 2008). Both END and ATZ are classified as EDCs. While it is known that ATZ dis-

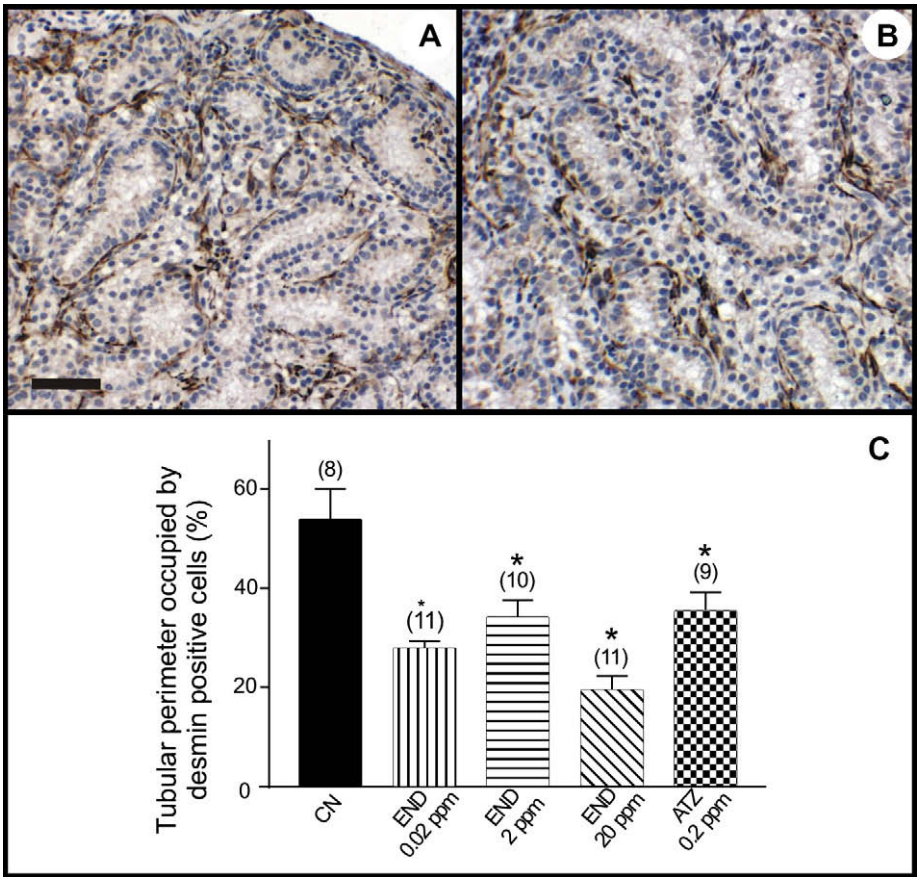

Fig. 3. Distribution pattern of peritubular myoid cells in hatchling testis. (A) A monolayer of desmin positive cells enveloped the seminiferous tubules' perimeter (control). (B) A significantly reduced proportion of tubular perimeter delimited by desmin positive cells in pesticide-exposed caimans. (C) Graphic representation of the percentage of tubular perimeter occupied by cells expressing desmin. Values are means ± SEM, between brackets: number of hatchlings by group, asterisks denote differences between treatment groups and control (Kruskal-Wallis, Mann–Whitney *U* post test, $p < 0.05$). Scale bar: 50 µm.

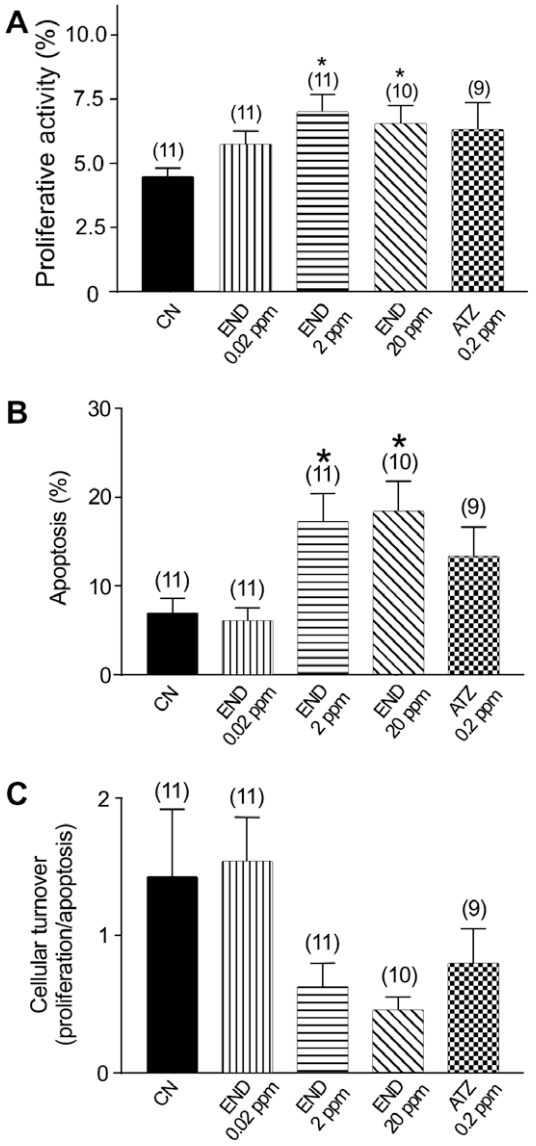

Fig. 4. Effect of *in ovum* exposure to pesticides on cellular turnover in the seminiferous tubules of hatchling caimans. (A) Proliferative activity was evaluated by BrdU incorporation, (B) Apoptosis was evaluated by TUNEL, (A and B), expressed as percentages, (C) Cellular turnover was expressed as the ratio proliferation/apoptosis. Values are means ± SEM, between brackets: number of hatchlings by group, asterisks denote differences between treatment groups and control (Kruskal-Wallis, Mann–Whitney *U* post test, $p < 0.05$).

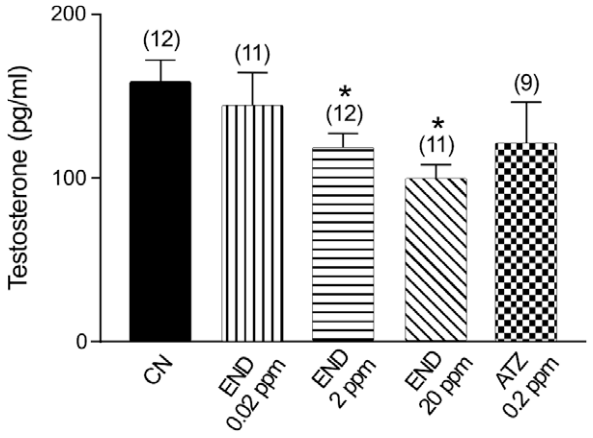

Fig. 5. Effect of *in ovum* exposure to pesticides on the testosterone levels of hatchling caimans. Values are means ± SEM, between brackets: number of hatchlings by group, asterisks denote differences between treatment groups and control (Kruskal-Wallis, Mann–Whitney *U* post test, $p < 0.05$).

rupts the metabolism of steroid hormones in non-mammalian vertebrates (Bisson and Hontela, 2002; Hayes et al., 2002) its estrogenic potential has not been undoubtly established (Storrs and Semlitsch, 2008). Eventhough, ATZ was able to bind *in vitro* to estrogen receptors in the oviductal tissue of the female alligator (Vonier et al., 1996); in many *in vivo* systems from other species, no estrogenic activity was exhibited (Wilhelms et al., 2006; Oka et al., 2008; Kloas et al., 2009). END has behaved like a xenoestrogen *in vitro* (Soto et al., 1994; Hodges et al., 2000). Moreover, using an *in vivo* model, it has been demonstrated that END modulates the expression of estrogen-dependent genes in the rat uterus (Varayoud et al., 2008). In *C. latirostris*, 17β-estradiol (E2)- and BPA-induced sex reversion were noted (Stoker et al., 2003); however, neither END nor ATZ were able to induce sex reversion (Beldomenico et al., 2007). The present results confirm our previous findings. In American alligators, E2 and *p,p′*-DDE induced sex reversion have been found (Milnes et al., 2005). Conversely, ATZ was unable to override male temperature effect (Crain et al., 1999). In this study, we used only incubation temperatures which are known to produce all female or all male caiman hatchlings (Stoker et al., 2003); however, developing caimans could be more sensitive to EDCs at intermediate temperatures that produce both sexes (Milnes et al., 2005). Studies concerning the ability of environmental contaminants to alter sex determination and reproductive tract development and function, should take into account not only incubation temperature but also pollutant concentration. In this study, doses evaluated were selected with consideration of ecological relevance. The lowest dose of END and the dose of ATZ represent concentrations similar to those found in the environment (Menone et al., 2000; Wu et al., 2000; Hayes et al., 2002; Laabs et al., 2002).

In the present study, *in ovum* exposure to END or ATZ disrupted testicular histoarchitecture in hatchlings. Disorganized and tortuous seminiferous tubules exhibited a loss of intratubular intercellular connections and either emptied tubular lumens or luminal cellular detritus. 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). END and many other chemical pollutants such as DDT, dieldrin, HCB, and PCBs have the ability to inhibit gap junctional intercellular communication altering signal traffic from adjacent cells (Kang et al., 1996, 2002). Furthermore, it has been shown that the expression of conexins represents a sensitive endpoint in response to cellular stress induced by environmental contaminants in the testis (Fiorini et al., 2004; de Montgolfier et al., 2008). Since spermatogenesis is a complex process involving highly coordinated intercellular communication between Sertoli cells and developing germ cells, adverse effects of END and ATZ exposure on male caiman reproduction could be expected. Evidences of END action on male gonads were recently reported by Dutta et al. (2006). High levels of disorganization in the testicular structure of the bluegill fish were observed, with significant variations in the connective tissue, increases in seminiferous tubule diameter and loss of the adequate delimitation of tubular walls (Dutta et al., 2006). Peritubular myoid cells provide structural integrity to the seminiferous tubules, and their interactions with Sertoli cells are important for the maintenance and control of testicular function (Anthony and Skinner, 1989; Maekawa et al., 1996). Therefore, we evaluated the peritubular myoid cells as possible targets of endocrine disruptor effects in caimans. Testis peritubular cells of hatchling caimans expressed desmin but not α-SMA. A significant reduction in the percentage of perimeter length occupied by desmin positive cells was observed in the pesticide-ex-

posed caiman. This provides evidences that endocrine disruptor effects on the caiman testis may be mediated in part through alterations in mesenchymal–epithelial cell interactions. It is important to remember that alterations seen in hatchling testes are the result of a unique pesticide dose applied during the developmental stage most sensitive for gonadal sex determination.

Altered organization of the peritubular myoid cells and/or the evident lost of intratubular intercellular connections could modify signals involved in the control of cell proliferation and survival as well as the coordinated relationship between proliferative activity and cell death necessary to maintain tissue histoarchitecture and functions. The number of cells in the seminiferous tubules is determined by a dynamic balance between cell proliferation and apoptotic cell death (Williams and Smith, 1993). An imbalance between proliferative activity and cell death was observed in the testis of caiman exposed to the higher doses of END, mainly due to a high frequency of apoptosis in intratubular cells. Taking this into consideration, prenatal exposure to pesticides could reprogram the genome and modify gene expression; likewise, inappropriate signals from neighboring cells and/or altered intracellular signaling could be expected. Recent results showing that prenatal exposure to androgen induces a failure of Sertoli cell functions that lead to increased germ cell apoptosis later in life support this hypothesis (Boekelheide et al., 2000; Benbrahim-Tallaa et al., 2008).

Since the role of steroid hormones in the adequate development of reproductive tract and the regulation of spermatogenesis is well established in vertebrates, the effect of *in ovo* exposure to environmental pollutants on T levels was evaluated. We found that males exposed to ENDs exhibited a significant decrease in T levels (versus control males); their T levels being similar to those recently reported for control females (Stoker et al., 2008). Another interesting finding is that T levels in male hatchlings exposed to ATZ did not differ either from control males or control females. The overlapping pattern of T levels for males (both treatments) and females is consistent with the typical pattern found in alligators inhabiting contaminated wetlands (Matter et al., 1998).

Overall, our data reveal neonatal gonadal disruption at the tissue organization level due to *in ovo* pesticide exposure. Modified testicular histoarchitecture, apoptosis/proliferation imbalance and altered steroidogenesis could have detrimental effect on sexual maturation and, ultimately, on the success of male caiman reproduction.

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