

8. ABSTRACT

Exposure to environmental contaminants, known as endocrine disruptors (EDs), alter development and functions of the endocrine system, affecting varied taxa as amphibians, fishes, reptilians, rodents, and human beings. Endocrine disruptors include a wide variety of chemical compounds of different structure, which include synthetic and natural hormones, pesticides, and compounds used in the plastic and pharmaceutical industries, among others. Several EDs mimic the actions of estrogen, and for that reason are called xenoestrogens. Among the reproductive alterations caused by xenoestrogen exposure, we can emphasize the reduction of fertility, lower birth rate, reduction in hatchling survival, alterations in hormonal profiles and in sexual behaviour.

Bisphenol A (BPA), endosulfan (END) and atrazine (ATZ) are EDs to which animals and human beings are exposed on a daily basis. BPA is a compound widely used in the plastic polycarbonate production and epoxy resins. It was demonstrated that such products may release BPA molecules in normal conditions of use by rupture of the ester bond between monomers. Endosulfan is an organochloride insecticide widely used in Argentina since monocrotophos was forbidden, and nowadays, is one of the insecticides of preference in crops. Atrazine is a herbicide that inhibits photosynthesis. It is used in weed control in agriculture and as a non-selective herbicide in lands that are not used for crops. Effects on the endocrine system and reproductive function have been shown after environmental or experimental exposure to BPA, END or ATZ acting mainly as xenoestrogens. Moreover, they were found in superficial water, sediments, human and animal fluids and tissues, for those reasons they represent a highly significant potential risk for humans and wildlife. The encroachment of natural habitats for their use as croplands is on the rise in Argentina. Growing agriculture brings growing pesticide use, thus increasing the exposure of wildlife to hormonally active compounds. For environmental pollution screening and to evaluate pollutant effects, an adequate selection of sentinel species and biomarkers is very important. *Caiman latirostris* (broad-snouted caiman) are widely distributed in northeastern Argentina, and are being used by sustainable ranching programs. Caimans have ecological and physiological characteristics that make it a possible sentinel of xenoestrogen exposure. Among caiman's main characteristics, we could mention that: it is an oviparous species, with temperature sex determination, with a top position in

the food web, and a lifespan similar to that of humans allowing bioaccumulation of contaminants, it is a species with land and aquatic habits that can be exposed to a high variety of contaminants at all life stages. The above-mentioned characteristics could allow identifying and selecting suitable biomarkers of exposure to contaminants with estrogenic activity. Xenoestrogens could alter development and histoarchitecture of gonads, and hormonal profiles, providing potential biomarkers to evaluate in caimans. Vitellogenin (Vtg) is a yolk precursor protein synthesized by the liver of non-mammalian vertebrates and induced in response to estrogen. Detection of plasma Vtg in male caimans might be a valuable tool in biomonitoring xenoestrogen exposure in a polluted environment, becoming a useful biomarker of EDs exposure.

General hypothesis: *Caiman latirostris* is a potential sentinel species for the presence of EDCs in the environment and a direct indicator that readily alert of ecosystem health problems.

Aim of this thesis: to study the effects of exposure to E2, BPA, END and ATZ on putative targets of estrogen action in order to identify biomarkers and characterize *Caiman latirostris* as sentinel of xenoestrogen pollution. To achieve this, the subsequent specific aims were followed:

8. To verify whether E2 induces Vtg in *Caiman latirostris* as it does in other oviparous species. In case of an affirmative answer,
 - a. To characterize caiman Vtg.
 - b. To generate specific antibodies against caiman Vtg to quantify plasma levels and determine cell site and pattern of Vtg expression.
 - c. To characterize the anti-Vtg antibodies, looking for cross reactivity with Vtg from other wild reptiles.
 - d. To optimize immunoassays such as dot blot, western blot, ELISA, and immunohistochemistry in order to screen and quantify Vtg tissue and serum levels.
9. To identify target organs and biomarkers of estrogen action in caiman reproductive tract.
10. To describe biomarker alterations after *in ovo* exposure to environmentally relevant doses of BPA, END and ATZ.
11. To assess the effect of *in ovo* exposure to ATZ or END on sex determination.

12. To assess any change in caiman growth and development due to prenatal exposure to BPA, ATZ or END.

13. To determine E2 and T profiles to complement the investigation on sex determination and/or to alert about possible alterations related to environmental estrogens.

14. To establish associations between results obtained in order to explain possible consequences of environmental pollution on the growth, development and reproduction of *C. latirostris*, and to contribute with a better knowledge of this species and our environment.

Materials and Methods. Juvenile caimans were used to induce Vtg synthesis by estrogenic treatment (7 consecutive injections with 1 μ g/g of E2). Vtg was identified and purified from the plasma by saline precipitation followed by ionic exchange chromatography. Then, with the purified Vtg, rabbits were inoculated to obtain serum with antibodies anti-Vtg. It was purified by 2 consecutive affinity chromatography, obtaining specific antibodies against Vtg. Techniques were optimized: dot blot for screening, western blot to detect the protein qualitatively and specifically, not only in caiman but also in turtle, and an ELISA was designed to quantify Vtg content in different caiman serum samples. These optimized methodologies were applied: to detect Vtg induced by estrogenic treatment in juvenile caiman males (1 and 2 doses of E2 1 μ g/g) and in neonatal animals (1, 2 and 7 doses of E2 1 μ g/g), and to determine Vtg in wild turtle in reproductive season. In E2-treated caiman hatchlings, we evaluate oviductal epithelial proliferation by immunohistochemistry and the epithelial height of the oviduct by image analyses.

Effects of *in ovo* treatment with END, ATZ and BPA were evaluated. *Caiman latirostris* eggs were collected from nests of wetlands located in regions of low to moderate anthropic intervention. Eggs were transported to laboratory where they were weighed and incubated in controlled conditions of humidity and temperature (33°C to obtain males and 30°C to obtain females). Once at stage 20 of embryonic development, eggs were topically treated. In different experiments the following compounds were used: BPA was applied at concentrations of 1.4 and 140 ppm, E2 at 0.014 and 1.4 ppm, END at concentrations of 0.02, 2 and 20 ppm, and ATZ at 0.2 ppm. In every case,

negative control (treated with vehicle –ethanol) and positive control (with E2 1.4 ppm) groups were used. Incubation continued after treatment, we assisted the births and at 10 days of age, hatchlings were sacrificed and sex was determined by macroscopic and histological evaluation of the gonads. In males, the perimeter of seminiferous tubules was determined by image analysis of histological slides. Expression of α -actin and desmin proteins were characterized in myoid cells in animals of 10 days and 12 months of age, by immunohistochemistry. Desmin expression was quantified related to tubular perimeter in the different experimental groups of 10 day-old caimans. Moreover, intratubular proliferation was evaluated assessing BrdU incorporation by immunohistochemistry and apoptosis was determined by TUNEL technique.

On the other hand, E2 and T profiles were determined by RIA in animals of different ages in normal conditions, and then to evaluate xenoestrogen effects on those profiles at short term (animals of 10 days of age) and long term (12 month-old caimans).

Results. Vtg induction after E2 treatment was confirmed in male and female juvenile caimans. Caiman Vtg was characterized as two proteins of 205 and 225 kDa by SDS-PAGE, and 395 and 415 kDa in native PAGE, with phosphorylated characteristics. Proteins were purified and used to generate antibodies. The antibody obtained was used successfully in the development and optimization of dot blot, western blot, ELISA and immunohistochemistry showing high specificity and sensitivity. Moreover, the new generated antibody recognized Vtg in E2 treated and adult female fresh water turtles. We induced and quantified Vtg in juvenile males treated with E2 and in hatchlings subjected to different treatment protocols. In males, we observed an increase in protein synthesis due mainly to Vtg, and we determined that not only a first E2 exposure induced Vtg, but also a new dose caused an induction significantly larger related to the first one (priming effect). In hatchlings, we observed that E2 induced Vtg synthesis in a dose-dependent manner, while no priming effect was observed. In females, besides Vtg induction, we observed that in the oviduct, the epithelia height and proliferative activity epithelia were proper biomarkers of postnatal exposure to estrogenic agents, showing a significant increase in their response after E2 treatment.

Prenatal exposure to END 2 and 20 ppm or ATZ 0.2 induced a fractional egg weight loss significantly higher than in controls, moreover, in these groups smaller hatchlings were born.

In contrast with the results previously reported for BPA 140 ppm and E2 1.4 ppm, all hatchling from eggs incubated at 33°C were males, showing absence of sex reversion caused by ATZ and END in the applied doses.

Ten days old male testis from caiman prenatal exposed to END 20 ppm and ATZ exhibited altered histoarchitecture with a significant increase in the perimeter of seminiferous tubules. In order to determine the cause of tubule disruption, the expression of α -actin and desmin proteins in peritubular myoid cells was studied at 10 days and 12 months of age. At both ages, myoid cells expressed desmin while α -actin expression was absent, probably due to functional immaturity. Disrupted tubules presented lower percentage of desmin expression relative to the tubular perimeter. To determine whether the loss of continuity observed in the distribution of myoid cells and the increase in tubular perimeter was caused by an increase in the number of intratubular cells, proliferative activity was evaluated. No differences in the percentage of proliferating cells inside the seminiferous tubules were observed among different experimental groups, either an alteration in programmed cell death, suggesting the existence of an adequate balance in the cellular turnover.

Female and male E2 and T levels were evaluated at different ages in control animals raised in captivity. As expected, male and female exhibited different steroid hormones profiles. In neonatal caimans exposed *in ovo* to pesticides or BPA, a significant decrease in T levels was observed, while no changes in E2 levels were detected. Females obtained by sex reversion (BPA and E2 treatments) had similar steroid hormonal levels to control females either at 10 days or at 12 month of age.

Conclusions. Results obtained in this thesis allowed us to conclude that:

1. *Caiman latirostris* males and females, as other oviparous species, are able to synthesize Vtg in response to estrogenic stimulus; this was confirmed in neonatal and juvenile animals.
2. The methodologies optimized in the present study allowed characterizing caiman Vtg as a biomarker of postnatal exposure to agents with estrogenic activity.

3. The new generated antibody and optimized techniques, allowed measuring plasma levels and identify hepatic Vtg synthesis, not only in caimans but also in other reptilian species of our region such as the fresh-water turtles *Phrynops hilarii* and *Trachemys scripta*.

4. The oviduct and the testis were identified as target organs of E2 action in females and males, respectively.

5. Histoarchitecture changes in the oviduct such as epithelial height alterations and cell proliferation, can be used as a biomarker of estrogenic action.

6. Changes in the seminiferous tubule perimeter and alterations in myoid cells distribution pattern were identified as biomarkers of *in ovo* exposure to xenoestrogens.

7. *In ovo* exposure to END and ATZ (currently used pesticides), at a stage immediately before the window for sexual determination, generated larger loss in fractional egg weight during the incubation and smaller hatchlings. Smaller hatchlings would have less chance of survival during the first weeks after birth. These might have a significant impact on caiman population dynamics.

8. Prenatal exposure to ATZ or END caused perturbations in male gonadal histoarchitecture, but did not override temperature effect on sex determination.

9. Normal circulating E2 and T levels exhibited characteristic profiles for female and male caimans (evaluated in the summer season, from neonate to juvenile, 5-6 year old caimans).

10. T and E2 levels were not suitable to distinguish between temperature sex determined females and females obtained by sex reversion since no differences were found between both groups of females.

11. Caimans exposed *in ovo* to xenoestrogens exhibited lower T levels even a year after exposure finished, therefore T serum concentration could be used as biomarker of environmental estrogen exposure.

Finally, we can state that the biomarkers evaluated resulted useful tools to determine estrogen activity *in vivo*. The studies performed in this thesis allow improving the knowledge of caiman's reproductive biology and generating biomarkers to screen exposure to xenoestrogen contamination at different life stages of caimans. Moreover, evidence obtained support the usefulness of *Caiman latirostris* as a sentinel of xenoestrogen contamination.