# Development and Implementation of Endocrine Biomarkers of Exposure and Effects in American Alligators (*Alligator mississippiensis*)

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## 1. Introduction

Dioxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) elicits a number of toxic and biochemical effects in a variety of species[1]. These effects include induction of phase I and II enzymes, cancer, dermal lesions, teratogenesis, immunotoxicity, and alterations of endocrine function. TCDD inhibits the 17β-estradiol-induced increases in uterine wet weight, estrogen and progesterone receptor binding, epidermal growth factor receptor binding, and uterine peroxidase activity in Sprague-Dawley rats[2]. Moreover, gestational exposure to TCDD results in both functional and behavioral reproductive impairment in the rat[3-9]. Many of these effects have been well characterized in laboratory animals but are less well understood in wildlife species. In this study, alterations in a number of endocrine endpoints were measured in the American alligator (*Alligator mississippiensis*), following TCDD, ethinyl estradiol (EE), o,p'-DDE, p,p'-DDE, coumestrol (Coum) or indole-3-carbinol (I-3-C) exposure. Endpoints selected were gonad weight and histology; plasma estradiol and testosterone concentrations, vitellogenin determinations; and sex ratio. The rationale for selection of endpoints was their ability to indicate an alteration of reproductive function. These endpoints were evaluated for sensitivity and reproducibility among this species.

This report summarizes the dose-response relationships for endocrine effects listed above in alligators following *in ovo* exposure to TCDD. This species appears to be exquisitely sensitive to the effects of xenobiotic exposure, perhaps as a result of its temperature-dependent sex determination. TCDD, p,p'-DDE and ethinyl estradiol had marked effects on the endocrine system in this species.

## 2. Materials and Methods

**Chemicals.** Test substances used in this study were: 1) TCDD obtained from Cambridge Isotopes (Boston, MA), 2) o,p'-DDE was purchased from ChemService (West Chester, PA), 3) p,p'-DDE and 4) coumestrol were purchased from Acros (Pittsburgh, PA), 5) indole-3-carbinol and 6) ethinyl estradiol were obtained from Sigma (St. Louis, MO). Rabbit vitellogenin antibody was kindly donated by Dr. Brent D. Palmer, Ohio University. The composition of all dosing solutions was confirmed by gas chromatography-mass spectroscopy by Dr. George Cobb at TIWET.

**Animal Treatment.** Alligator eggs were collected at the Bear Island Wildlife Management Area, South Carolina, USA. Approximately 250 eggs were collected from eight nests and randomly assigned to treatment groups. Alligator eggs were dosed by applying DMSO-dissolved chemicals to filter paper pieces taped to eggs. Eggs were incubated at a male-producing temperature of 33°C until hatched. Hatchlings were reared in a brooder with an radiant heat source (for behavioural thermoregulation) and fed commercial turtle chow *ad libitum* until 21 days of age. At this time, they were euthanized by injection with sodium pentobarbital and necropsied. Tissues were collected.

**Vitellogenin Assay**. Plasma vitellogenin determinations were made by a modification of the method developed by Palmer and Palmer[10]. Briefly, plasma was solubilized in buffer (1:4 volumes), and loaded onto one-way sodium dodecylsulfate polyacrylamide electrophoretic (SDS-PAGE) gels (5% final acrylamide concentration). Samples were run adjacent to molecular weight markers at 200 V for 45 min. Gels were stained with Coomassie blue, dried, and quantified by scanning densitometry. Vitellogenin was confirmed by performing a Western transfer of a number of samples from each species, and probing the blot with an universal antibody to vitellogenin. Rabbit vitellogenin antibody was visualized by reacting it with goat anti-rabbit IgG conjugated to alkaline phosphatase.

**Gonad Histopathology.** Gonads were fixed in Bouin's solution immediately following removal, fixed for 3-5 days, washed in water and stored in 70% ethanol. Samples were dehydrated in an ethanol series, embedded in paraffin, and cut into 6-8 μm serial sections using a rotary microtome. Sections were stained with Harris' haematoxylin, biebrich scarlet/orange II and fast green (modified Schorr's stain). Clitoro-penis (CP) length was measured using a dissecting microscope equipped with a calibrated graticule.

**Statistics.** Statistical significance of treatment group means was determined by ANOVA using Statview (Abacus Concepts, Inc., Berkeley, CA), followed by Bonferroni's *post hoc* test (morphological measures) at an $\alpha = 0.05$.

## 3. Results

Overall hatching success was relatively low (56% of all eggs treated) and treatment groups were collapsed into low (0.1 and 0.3 mg/kg and μg/kg) and high (1, 3 and 10 mg/kg and μg/kg) dose groups. TCDD, p,p'-DDE and ethinyl estradiol caused dose-dependent alterations in sex ratios in American alligators incubated at male producing temperatures (Fig. 1). Dose-dependent changes were also observed in clitoro-penis length (Fig. 2) and depth (Fig. 3). Ovarian structure in female hatchlings treated with EE and Coum exhibited an increase in medullary vacuole size and numbers whereas TCDD reduced both the size and density of these vacuoles (data not shown). Testes from TCDD-treated alligators exhibited masses of abherent cells in the lumen of seminiferous tubules (data not shown).

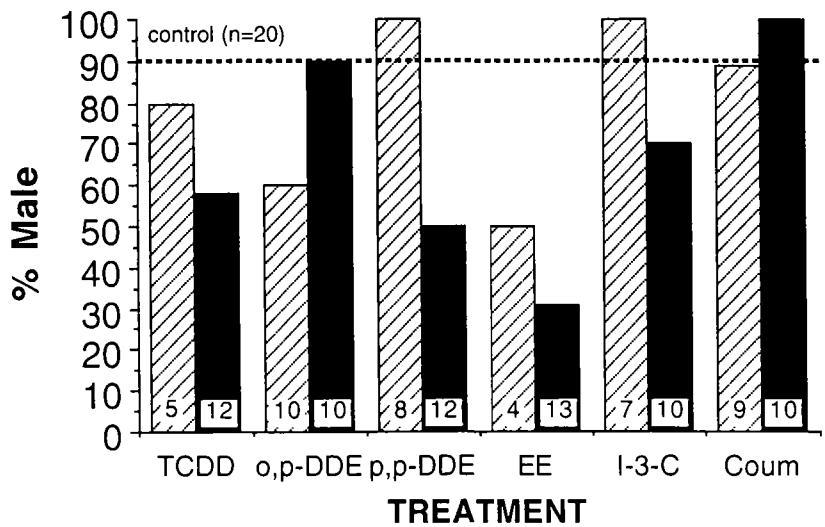


Fig. 1. Gender-altering effects of various xenobiotic substances in *Alligator mississippiensis* exposed during embryonic development. Data are presented as percent males (determined histologically) resulting from treatments. Cross-hatched bars are low-dose treatment groups; shaded bars are high-

dose treatment groups. Dashed line represents percent males resulting from incubation of control (DMSO-treated) eggs at 33 C. Numbers in bars are samples sizes.

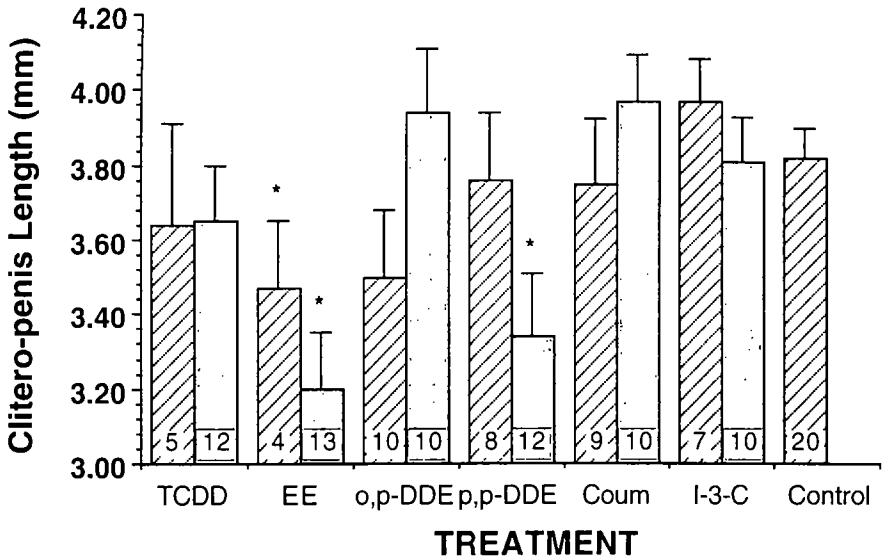


Fig. 2. Effects of *in ovo* xenobiotic exposure on clitero-penis length (mm) in hatchling *Alligator mississippiensis*. Asterisks indicate groups that are significantly different from controls ($p < 0.05$). Treatment groups are as described in Fig. 1 and text.

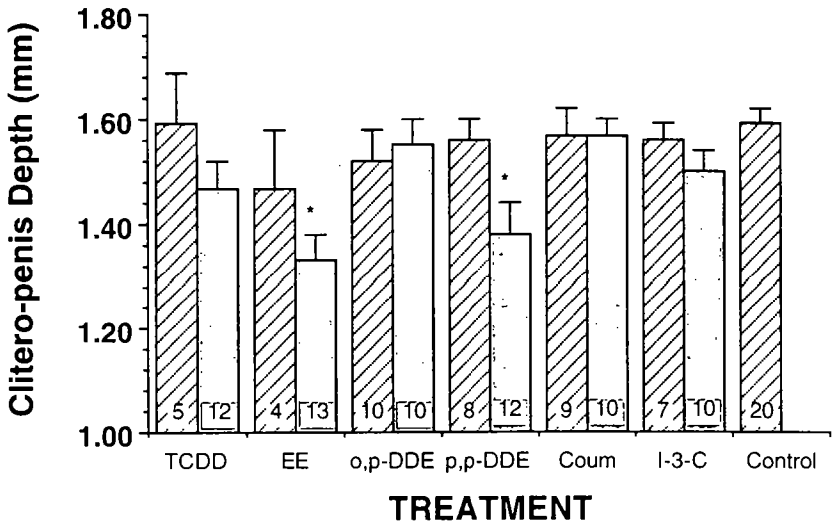


Fig. 3. Effects of *in ovo* xenobiotic exposure on clitero-penis depth (mm) in hatchling *Alligator mississippiensis*. Asterisks indicate groups that are significantly different from controls ($p < 0.0006$). Treatment groups are as described in Fig. 1 and text.

Observed plasma vitellogenin levels did not correlate with xenobiotic treatments in alligators. However, there was a significant correlation in the presence of vitellogenin with the clutch from which eggs were collected. Residue analysis of various egg and hatchling components revealed significant endogenous PCB contamination (i.e., hatchlings with vitellogenin induction were from eggs that possessed up to 1.7 ppm PCB congener 77, a known estrogenic substance).

## 4. Conclusions

Embryonic exposure to several xenobiotic substances results in altered sex ratios in American alligators, a species with temperature-dependent sex determination. Specifically, EE and p,p'-DDE treatments led to significant occurances of female gender determination at male-producing temperatures. A consequence of female gender is phenotypic alteration in clitero-penis size (i.e., animals from EE and p,p'-DDE treatments exhibited shorter, thinner phalli). Morphological alterations in the ovarian stroma of hatchlings treated with estrogenic substances were also evident. Ovaries exhibited an increased number and size of medullary vacuoles in treatment groups recieving EE, Coum and o,p'-DDE. Medullary vacuoles are reported to exhibit aromatase activity during the latter part of embryonic development in this species [Dubowsky and Lang, pers. comm.]. Ovaries from females treated with TCDD exhibited a reduction in medullary vacuole number and size (not shown). The presence of endogenous PCB contamination in alligator eggs used in this study may present a source of confounding effects.

## 5. References

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