



The functional and structural observations of the neonatal reproductive system of alligators exposed *in ovo* to atrazine, 2,4-D, or estradiol

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Wild alligators exposed to persistent organochlorine contaminants, municipal waste compounds, and contemporary-use herbicides exhibit reproductive alterations that are thought to be caused by endocrine disruption. This study tests the hypothesis that these alterations, at least in part, result from exposure of alligator embryos to contemporary-use herbicides. Alligator eggs were collected early in development, exposed to estradiol-17 β , atrazine, or 2,4-D (at dosages of 0.14, 1.4, and 14 ppm, plus a dosage of 0.014 ppm for estradiol-17 β only) before the period of gonadal differentiation, and incubated at a temperature that would produce either 100% males or 100% females. Analysis of histology was performed on the gonads and reproductive tracts of hatchlings. In females, epithelial cell height of the Müllerian duct and medullary regression of the ovary were assessed, whereas in males, sex-cord diameter was measured. Eggs incubated at the female-determining temperature produced all female hatchlings, whereas the estradiol-17 β treatments caused the production of females at the male-determining temperature. Neither atrazine nor 2,4-D had this effect. Both Müllerian duct epithelial cell height and medullary regression were increased in estradiol-treated animals, but no differences were noted between herbicide-treated alligators and controls. A previous study found that male alligators exposed to 14 ppm atrazine had elevated gonadal aromatase activity, but there was no difference in sex-cord diameter in this or any other treatment group. Additionally, we observed that hepatic aromatase activity was not altered by *in ovo* exposure to any of the treatments. These results indicate that these herbicides alone are not responsible for the gonadal abnormalities previously reported for juvenile alligators from Lake Apopka and emphasize the importance of analyzing both the function (*i.e.*, steroidogenic enzyme activity) and the structure (*i.e.*, histological analysis) of the reproductive system. Structural assessment alone may be insufficient for detecting subtle endocrine alterations.

Keywords: alligators, atrazine, contaminants, endocrine disruption, herbicides, 2,4-D.

Introduction

Many environmental chemicals cause reproductive abnormalities by eliciting changes in the endocrine control of reproduction. These endocrine-disrupting contaminants (EDCs) can alter endocrine control by changing the rates of hormone production or excretion, altering hormone bioavailability, or directly interacting with hormone receptors as agonists or antagonists (Crain and Guillette, 1997). There is no particular structural class of chemicals that causes endocrine alterations. For instance, some polychlorinated biphenyls (PCBs) are potent EDCs, but many other PCB congeners have no known endocrine-disrupting effect (Bergeron et al., 1994). Although computer modeling can predict many of the compounds that will interact with the human estrogen receptor alpha (Waller et al., 1996), the causative agents of endocrine disruption are often difficult to identify because endocrine alterations can be elicited through mechanisms independent of a receptor.

The American alligator (*Alligator mississippiensis*) population at Lake Apopka, Florida, is an example of a wildlife population that is adversely affected by EDCs. Alligators from Lake Apopka exhibit altered circulating hormone concentrations (Guillette et al., 1994; Guillette et al., 1996a; Guillette et al., 1997; Crain et al., 1998), abnormal gonadal morphology (Guillette et al., 1994), and reduced phallus size (Guillette et al., 1996a) when compared to alligators from reference populations. These alterations suggest that the endocrine control of reproduction has been altered in these alligators. The causative agents of these abnormalities are unknown, but it has been suggested that the abnormalities are related to embryonic exposure to elevated organochlorine contaminants, pesticides, and/or municipal waste products in the lake (Schelske and Brezonik, 1992; Guillette and Crain, 1995; Guillette et al., 1996b). The purpose of this study is to test the hypothesis that the endocrine alterations, at least in part, result from exposure of alligator embryos to the contemporary-use herbicides atrazine or 2,4-D. Endocrine status was assessed by analyses of gonadal histology and hepatic steroidogenic activity.

Materials and methods

Animals and Treatments

Alligator eggs were collected from Lake Woodruff, Florida. Lake Woodruff is a relatively pristine environment, sur-

1. Abbreviations: ANOVA, analysis of variance; DPM, disintegrations per minute; EDC, endocrine-disrupting contaminant; ER, estrogen receptor; GAM, gonad-adrenal-mesonephros; PLSD, protected least squares difference; PCB, polychlorinated biphenyl

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rounded by a National Wildlife Refuge. Eggs from seven nests were equally divided into a number of treatment groups, and this study represents a subset of data from a larger project. Eggs were treated with the test compounds at stage 21 in embryonic development, just prior to the onset of gonadal differentiation (Lang and Andrews, 1994). Eggs were treated with either the estrogenic control estradiol-17 β (Sigma Chemical Co., St. Louis, MO), the herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine; 99% purity), or the herbicide 2,4-D (2,4-dichlorophenoxyacetic acid; 97.6% purity) (both herbicides were obtained from Chem Service, West Chester, PA). Each egg received a single dose of either 12.5 μ g, 125 μ g, or 1250 μ g (in the case of estradiol treatments, there was also a 1.25- μ g treatment group). The average egg weight was 90 g; thus, these treatments represent a minimal embryonic dose of 0.014, 0.14, 1.4, or 14 ppm. Estradiol, atrazine, and 2,4-D were applied topically to the eggshell in 50 μ l of 95% ethanol, a technique frequently used to transport compounds inside reptilian eggshells (Crews et al., 1991; Wibbels and Crews, 1992). One control group was treated with 50 μ l of 95% ethanol, whereas the other control group was not treated. Five eggs from each dose-treatment group were incubated at either a temperature that produces 100% males (33°C) or a temperature that produces 100% females (30°C) (Lang and Andrews, 1994).

Hepatic Aromatase Assay

Hatchlings were individually housed for 10 days prior to tissue collection. Animals were euthanized by administering a lethal injection of sodium pentobarbital (0.4 mg/g) into the postcranial sinus. A section of liver (16–35 mg) was immediately removed for the determination of hepatic aromatase activity. Aromatase activity was measured using previously described procedures (Crain et al., 1997). Briefly, a liver section was placed in 400 μ l of culture media (RPMI-1640; Sigma) supplemented with 0.8 mM tritiated androstenedione (DuPont NEN Research Products; no. NET-926). After a 6-h incubation at 32°C, 300 μ l of the media were transferred to a new tube. Chloroform (1.5 ml) was added and the tube was vortexed and then centrifuged for 15 min at 2000 \times g. A 200- μ l aliquot of the aqueous phase was added to a new tube. Five percent charcoal–0.5% dextran (200 μ l) was added and the tube was vortexed and then immediately centrifuged for 15 min at 2000 \times g. Scintiverse BD (5 ml; Fisher Chemical Co.) was added to 300 μ l supernatant and the tube was counted on a Beckman scintillation counter. Aromatase activity is proportional to the amount of tritium in the scintillation vial and is calculated as a percentage of the total substrate added. After subtracting the nonspecific tritium release, the disintegrations per minute (DPM) of the sample tubes are converted to a percentage of the total DPM added. This percentage is multiplied by the mass of the substrate

added. After adjusting for extraction loss, the value obtained represents the amount of substrate converted to tritiated water, which is proportional to aromatase activity (Ackerman et al., 1981).

Gonadal Aromatase Activity and Histology

Aromatase activity of the right gonad of these animals has been previously published (Crain et al., 1997). The left gonad and its associated mesonephric and adrenal tissue were preserved in Bouin's fixative (Humason, 1972). The preserved tissue was cleared in 75% ethanol, serial sectioned at 7 μ m following paraffin embedding, and stained with a modification of the Harris' trichrome procedure (Humason, 1972).

For the histological analysis, gonads were first categorized as testes or ovaries using criteria initially defined by (Forbes, 1940) and recently elaborated by (Guillette et al., 1994). Criteria for testis included medullary sex cord proliferation and a reduced cortex, whereas criteria for ovaries included hypertrophied cortex, medullary reduction, the presence of lacunae in the medulla, and germ cells in the cortex.

In the ovaries, both Müllerian duct epithelial cell height and the degree of degeneration in the ovarian medulla were measured. A calibrated ocular micrometer was used to measure the columnar epithelial cell height of the Müllerian duct. Three random height measurements of well-defined epithelial cells were taken for each duct. The degree of medullary degeneration was analyzed using a subjective assessment of the percentage of the medullary area filled with lacunae. Five categories were used in the assessment: 0–20%, 21–40%, 41–60%, 61–80% and 81–100% of the medulla having lacunae. Five different measurements were taken at random from each ovary.

In the testes, the diameter of the medullary sex cords was measured using a calibrated ocular micrometer. To make the measurements, five of the largest elliptical sex cords were selected from each testis.

Statistics

A one-way analysis of variance (ANOVA) was used to test the effects of each dose-treatment group on hepatic aromatase activity, sex-cord diameter, and Müllerian duct epithelial cell height. Fisher's PLSD was used as a post hoc test. A Kruskal–Wallis analysis was used to test the degree of ovarian medullary degeneration among the dose-treatment groups.



Results

Hepatic Aromatase Activity

Results of the hepatic aromatase assay are graphically presented in Figure 1A. There was no significant difference between control male and control female animals in hepatic aromatase activity ($p = 0.58$). Likewise, neither atrazine nor 2,4-D treatments had any measurable effect on hepatic aromatase activity in males ($p = 0.52$ for the

interaction of treatment and dose; $p = 0.82$ for the effect of treatment) or females ($p = 0.29$ for the interaction of treatment and dose; $p = 0.43$ for the effect of treatment). For comparison, aromatase activity of the gonad-adrenal-mesonephros (GAM) complex is presented in Figure 1B. These data show significantly elevated aromatase activity in GAMs of male hatchlings treated *in ovo* with atrazine (see Crain et al., 1997) for original presentation of GAM aromatase data).

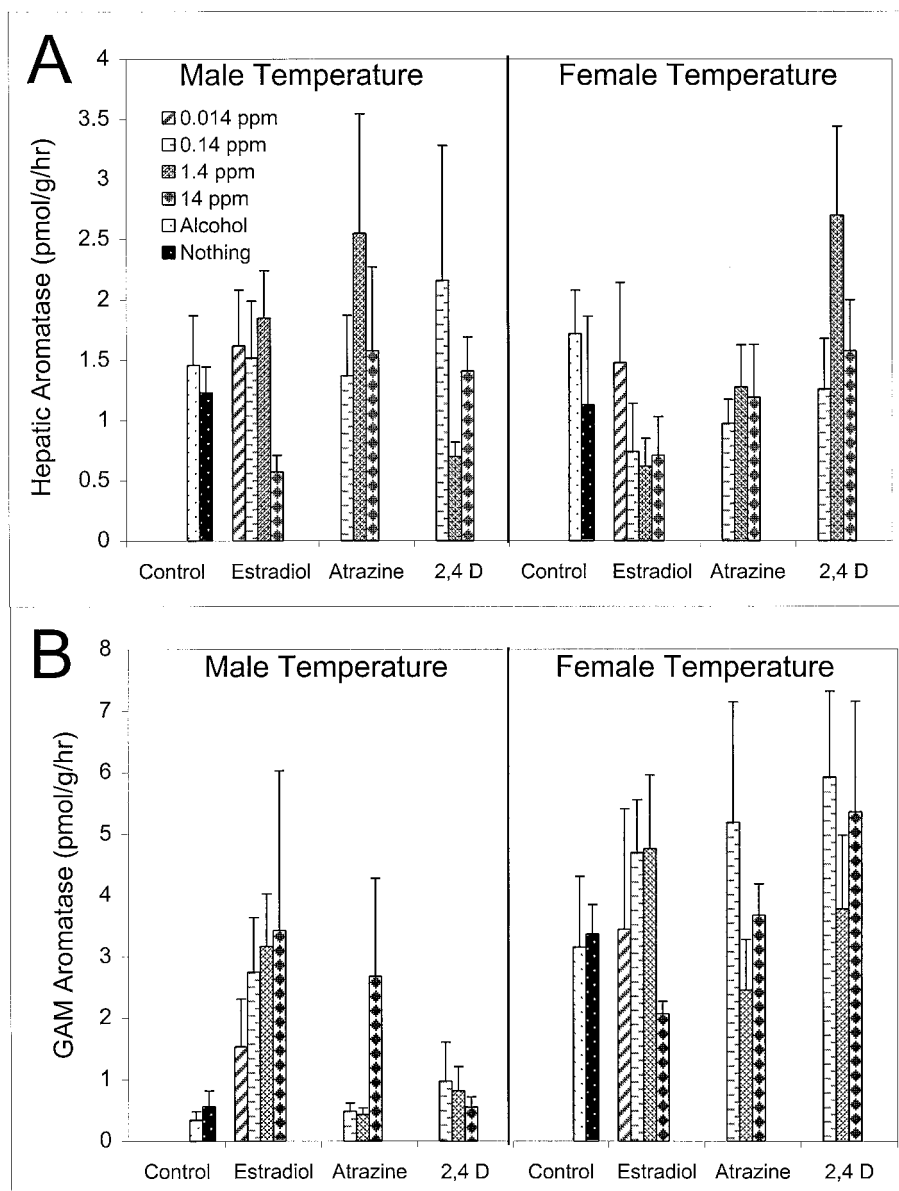


Figure 1. Aromatase activity in the liver (A) and gonad-adrenal-mesonephric (GAM) complex (B) of hatchling alligators that were exposed as embryos to the treatments. After application of the treatments, eggs were incubated at a male-producing temperature or a female-producing temperature. Irrespective of the temperature regime, all alligators exposed to estradiol-17 β were females. The gender of all other alligators conformed to the respective temperature regime. None of the treatments caused a significant change in hepatic aromatase activity. Respective to the gonads, dose had no influence on GAM aromatase activity and, thus, was removed from the statistical analysis. As a group, all atrazine-treated males had GAM aromatase activity that was neither characteristic of males nor females. See Crain et al. (1997) for further discussion of these GAM data.

**Table 1.** Results of the histological analyses

	Percent females	Epithelial cell height (μm)	Medullary degeneration rank ^a	Sex-cord diameter (μm)
<i>Female-producing temperature</i>				
Control: 95% ethanol	100	12.3 \pm 1.77	2.75 \pm 0.25	NA
Control: nothing	100	12.4 \pm 0.52	3.00 \pm 0.41	NA
Estradiol-17 β :				
0.014 ppm	100	12.0 \pm 0.96	3.0 \pm 1.0	NA
0.14 ppm	100	13.0 \pm 1.84	3.67 \pm 0.88	NA
1.4 ppm	100	12.7 \pm 2.67	3.0 \pm 1.0	NA
14 ppm	100	30.7 \pm 1.45 ^b	5.0 \pm 0 ^c	NA
Atrazine:				
0.14 ppm	100	12.3 \pm 1.57	2.75 \pm 0.48	NA
1.4 ppm	100	8.67 \pm 3.81	2.40 \pm 0.40	NA
14 ppm	100	13.3 \pm 3.03	3.60 \pm 0.60	NA
2,4-D:				
0.14 ppm	100	13.4 \pm 1.80	3.17 \pm 0.31	NA
1.4 ppm	100	13.5 \pm 1.74	3.20 \pm 0.58	NA
14 ppm	100	13.5 \pm 0.44	3.25 \pm 0.48	NA
<i>Male-producing temperature</i>				
Control: 95% ethanol	0	NA	NA	80.5 \pm 6.08
Control: nothing	0	NA	NA	80.0 \pm 9.79
Estradiol-17 β :				
0.014 ppm	100	10.7 \pm 1.66	3.0 \pm 0	NA
0.14 ppm	100	11.3 \pm 1.33	2.0 \pm 1.0	NA
1.4 ppm	100	12.7 \pm 0.51	2.8 \pm 0.73	NA
14 ppm	100	29.0 \pm 2.0 ^b	5.0 \pm 0 ^c	NA
Atrazine:				
0.14 ppm	0	NA	NA	75.3 \pm 3.48
1.4 ppm	0	NA	NA	74.5 \pm 5.91
14 ppm	0	NA	NA	70.0 \pm 1.53
2,4-D:				
0.14 ppm	0	NA	NA	75.8 \pm 3.28
1.4 ppm	0	NA	NA	73.3 \pm 10.6
14 ppm	0	NA	NA	73.7 \pm 8.21

^aRanks are defined as: 1 = 0–20%; 2 = 21–40%; 3 = 41–60%; 4 = 61–80%; 5 = 81–100%.

^bSignificantly different from control females and all other treatment groups at $p < 0.001$.

^cSignificantly different from control females and all other treatment groups at $p < 0.05$.

Abbreviations: NA, not applicable.

Gonadal Histology

None of the histological measurements of control and herbicide-treated hatchlings differed significantly (Table 1). Among these groups, all animals incubated at 30°C had ovaries, whereas all alligators incubated at 33°C had testes. This was not the case for the estrogenic control group treated with estradiol-17 β . All eggs treated with estradiol-17 β produced female hatchlings at the male-producing temperature. Epithelial cell height of the Müllerian duct was different among the treatment groups ($p < 0.0001$). This difference is attributed to the significant increase in cell height in the 14 ppm estradiol-17 β treatment groups in both the male ($p < 0.001$) and female ($p < 0.001$) temperature regimes. The regression of the ovarian medulla (measured by the percent of the medulla filled with lacunae) was not different among the groups ($p = 0.27$).

While both herbicides appeared to induce smaller sex cords, this reduction in sex-cord diameter was not signifi-

cantly different ($p = 0.96$) due to a high degree of variation in the sex-cord diameters of all groups.

Discussion

The results of this study suggest that embryonic exposure to 2,4-D or atrazine did not cause significant alterations in gonadal structure or hepatic steroidogenic enzyme activity of hatchling American alligators. A previous study utilizing these same animals found that embryonic exposure to a single high dose of atrazine (14 ppm) caused elevated aromatase activity in the GAM complex of male hatchling alligators (Crain et al., 1997). However, neither atrazine nor 2,4-D caused significant changes in hepatic aromatase activity of male or female hatchling alligators. The absence of any noticeable effect may be due to the early develop-



mental stage at which the animals were exposed. Most endocrine effects that have been previously described for atrazine and 2,4-D are elicited on a functional, normally organized reproductive system (see below). However, the alligators in this study were exposed to atrazine or 2,4-D during the development of the reproductive system. It is possible that 2,4-D or atrazine would alter hepatic aromatase activity in adult animals. For example, exposure to ammonium perfluorooctanoate (C8; a herbicide that, like 2,4-D, stimulates hepatic peroxisome proliferation) causes increased hepatic aromatase activity in adult rats (Biegel et al., 1995), and elevations in circulating estradiol-17 β are proportional to this increase in hepatic aromatase activity (Liu et al., 1996a,b). 2,4-D exposure causes changes in adult spermatogenesis, including reduced spermatozoa motility (asthenospermia), increased spermatozoa death (necrospermia), and increased spermatozoa abnormalities (teratospermia) (Lerda and Rizza, 1991).

Atrazine causes numerous endocrine alterations in adult vertebrates, although the mechanisms underlying these alterations are incompletely understood. In pigs, dietary exposure to atrazine (2 mg (kg body weight)⁻¹ day⁻¹) caused elevated circulating estradiol-17 β prior to the expected onset of estrus (Gojmerac et al., 1996). In these pigs, however, estrus never occurred, and histopathological examination revealed the persistence of the corpora lutea. The exact signals that cause the retention of the corpora lutea are unknown (Norris, 1997), and therefore it is difficult to surmise the mechanism of endocrine disruption in the female pigs. However, antiestrogenic or progestagenic actions would prolong the life of the corpora lutea, and, interestingly, a recent study demonstrated that atrazine has affinity for the alligator progesterone receptor (Vonier et al., 1996). It is plausible that some, if not most, of the endocrine-disrupting effects of atrazine are mediated *via* atrazine functioning as a steroid hormone antagonist. Atrazine acts as an antiandrogen in the rat prostate, interfering with the formation of the dihydrotestosterone-androgen receptor complex (Kniewald et al., 1979, 1987, 1995; Simic et al., 1991). Atrazine may also act as a weak antiestrogen in the rat uterus, as atrazine is capable of weak inhibition of estrogen-stimulated responses (Tennant et al., 1994a). This antiestrogenic effect may be mediated by events other than or in addition to estrogen receptor (ER) binding, as atrazine exhibits little (Tennant et al., 1994b; Tran et al., 1996) or no (Connor et al., 1996) binding to the ER. Indeed, it has been suggested that most of the antiestrogenic activity of atrazine is independent of a direct interaction with the ER (Tennant et al., 1994b; Connor et al., 1996).

Results of this study suggest that neither atrazine nor 2,4-D has an estrogenic effect on the developing alligator reproductive system. First, neither herbicide caused the produc-

tion of females at the male-producing temperature, whereas all doses of estradiol-17 β (even as low as 0.014 ppm) caused feminization of prospective males. Second, the more subtle estrogenic effects noted for the animals exposed to a high dose of estradiol-17 β were not noted in the alligators exposed to atrazine or 2,4-D. Estradiol (14 ppm) caused a significant increase in the degeneration of the ovarian medulla and a significant increase in the height of Müllerian duct epithelial cells. However, the high dose of estradiol necessary to elicit these responses indicates that these histological endpoints are not as sensitive as gender reversal.

Much of the confusion surrounding the way(s) that atrazine and 2,4-D cause endocrine disruption is likely a factor of the compounds having varying effects in different tissues and at different developmental and reproductive stages. For example, embryonic alligators exposed to high dosages (14 ppm) of atrazine exhibit elevated aromatase activity in the gonad/adrenal complex (Crain et al., 1997). In essence this would result in estrogenicity, as aromatase is the steroidogenic enzyme responsible for the formation of estradiol-17 β . The effects of atrazine on juvenile or adult alligator testes are unknown, but studies in the adult male rat prostate suggest that atrazine acts as an antiandrogen. Future studies to investigate endocrine-disrupting effects in wild populations should take into consideration the developmental and reproductive stages as factors that could affect the mode of action of a suspected endocrine disruptor.

Previous studies have noted endocrine abnormalities in the alligators living in Lake Apopka, Florida. One of the most pronounced abnormalities noted for Lake Apopka animals is abnormal gonadal histology. Juvenile Apopka females have polyovular follicles and polynuclear oocytes, whereas juvenile Apopka males have testes with poorly organized seminiferous tubules with aberrant bar-shaped nuclei (Guillette et al., 1994). Ovaries and testes in the present study had none of these characteristics. In addition, Müllerian duct epithelial cell height and percent of the medulla filled with lacunae appeared unaffected by exposure to atrazine and 2,4-D during this stage of development. Therefore, it does not appear that exposure to these herbicides is responsible for the gonadal abnormalities previously reported for juvenile alligators from Lake Apopka. Future studies should examine the effects of other EDCs that are present in Lake Apopka.

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