

Alterations in Steroidogenesis in Alligators (*Alligator mississippiensis*) Exposed Naturally and Experimentally to Environmental Contaminants

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Many environmental contaminants alter the reproduction of animals by altering the development and function of the endocrine system. The ability of environmental contaminants to alter the endocrine system of alligators was studied both in a descriptive study in which juvenile alligators from a historically contaminated lake were compared to animals from a control lake and in an experimental study in which hatchling control alligators were exposed *in ovo* to several endocrine-disrupting standards and two modern-use herbicides. Endocrine status was assessed by examining plasma hormone concentrations, gonadal-adrenal mesonephros (GAM) aromatase activity, and gonadal histopathology. In the descriptive study, juvenile alligators from the contaminated lake had significantly lower plasma testosterone concentrations (29.2 pg/ml compared to 51.3 pg/ml), whereas plasma 17 β -estradiol concentrations did not vary when compared to controls. GAM aromatase activity was significantly decreased in the alligators from the contaminated lake (7.6 pmol/g/hr compared to 11.4 pmol/g/hr). In the experimental study, the endocrine-disrupting standards had the expected effects. 17 β -Estradiol and tamoxifen caused sex reversal from male to female, with a corresponding increase in aromatase activity. Vinclozolin had no apparent effect on male or female alligators. Among the herbicides tested, atrazine induced GAM aromatase activity in male hatchling alligators that was neither characteristic of males nor females, although testicular differentiation was not altered. Exposure to 2,4-dichlorophenoxyacetic acid had no effect on the endocrine parameters that were measured. Together, these studies show that exposure to some environmental chemicals (such as atrazine) can alter steroidogenesis in alligators, but the endocrine alterations previously noted for Lake Apopka, Florida, alligators can not be fully explained by this mechanism. **Key words:** alligator, aromatase, atrazine, contaminants, 2,4-D, endocrine disruption, hormones. *Environ Health Perspect* 105:528–533 (1997)

Environmental contaminants alter the reproduction of a number of wildlife species by changing the normal endocrine environment that mediates sexual differentiation and function (1). Many of these endocrine alterations are thought to occur by direct interactions between the contaminants and hormone receptors (2), but the specific mechanisms by which most environmental contaminants cause endocrine disruption are unknown. Although all vertebrates are potentially susceptible to reproductive disruption by endocrine-disrupting contaminants (EDCs), many ectothermic vertebrates are particularly sensitive due to the processes mediating the organization of the reproductive system (3). Unlike birds and mammals, many fish, amphibians, and reptiles exhibit environmental sex determination, by which the gender of the undifferentiated embryo is determined by an environmental variable. In many reptiles, the temperature of egg incubation determines the sex of the offspring (4). Exposure of developing reptile embryos to exogenous chemicals can mimic the effects of temperature on sex determination. For example, when red-eared turtle (*Trachemys scripta*) embryos are incubated at a male-producing temperature and exposed to 17 β -estradiol (E₂) during the window of developmental sex determi-

nation, phenotypically female turtles are produced (5,6). This estrogen-induced sex reversal appears to be dose dependent (7) and suggests that other steroidal agonists, steroidal antagonists, and steroidogenic disruptors could alter normal sexual differentiation. Indeed, Wibbels and Crews (8) found that steroid hormones are not exclusive in their ability to alter normal sex determination, as many estrogen agonists and steroidogenic modifiers mimic and/or reverse the effects of temperature on the differentiation of primary sex organs in red-eared turtles.

The specific mechanisms by which temperature determines gender are unknown, but it is hypothesized that temperature stimulates or suppresses pivotal steroidogenic enzymes (9). These enzymes then propagate a cascade of events leading to the organization of a testis or ovary. This hypothesis is supported by work conducted on the steroidogenic enzyme aromatase. Aromatase converts androgens to estrogens by binding the C₁₉ androgen substrate and catalyzing several reactions, thus leading to a phenolic ring characteristic of estrogens (10). The pivotal role of aromatase in temperature-dependent sex determination is supported by several lines of evidence. First, several studies indicate

that aromatase activity is increased in prospective females during periods coinciding with thermosensitivity (11–13). Second, high doses (50–100 μ g per egg) of testosterone cause feminization of *T. scripta* at a male-producing temperature (8,14). Because testosterone is the precursor to E₂, this phenomenon is thought to be mediated by the enzyme aromatase. Third, administration of an aromatase inhibitor induces male sex determination in both a female unisexual (parthenogenetic) lizard and a turtle with temperature-dependent sex determination (15). Collectively, these studies suggest that aromatase is an enzyme critical to thermosensitive sex determination and is capable of modification by extrinsic factors.

In consideration of these studies, we propose that the endocrine-altering effects of some environmental contaminants may be mediated via changes in the expression or activity of the aromatase enzyme. Two studies, one descriptive and one experimental, were conducted to test this hypothesis. First, juvenile alligators from a control lake and a lake historically contaminated with a number of persistent organochlorines were analyzed for plasma steroid hormones and *in vitro* gonadal-adrenal aromatase activity. Second, embryos from a control lake were exposed to several known hormonal modifiers and two common herbicides, and

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hatchlings were analyzed for egg chorioallantoic fluid (CAF) hormones, plasma steroid hormones, and *in vitro* aromatase activity. Using these studies, we sought to determine whether aromatase function could explain endocrine alterations in alligators exposed to EDCs.

Materials and Methods

Animals and Treatments

For the descriptive study, eggs were collected from six nests on Lake Apopka, Florida (contaminated lake), and six nests on Lake Woodruff National Wildlife Refuge, Florida (control lake), during the first week of July 1995. Lake Apopka is designated as one of Florida's most polluted lakes (16) due to extensive agricultural activities around the lake, a sewage treatment facility associated with the city of Winter Garden, Florida, and a major pesticide spill from the Tower Chemical Company (Clermont, FL). The pesticide spill, which occurred in 1980, consisted primarily of dicofol, but had significant amounts of DDT, DDE, and DDD in the mixture (17). Analysis of alligator eggs taken from Lake Apopka in 1984 and 1985 revealed significant residues of toxaphene, dieldrin, *p,p'*-DDE, *p,p'*-DDD, *trans*-nonachlor, and polychlorinated biphenyls (18). A previous study found evidence of estrogenic contamination among the female alligators of Lake Apopka (19) and, as we wanted to minimize the number of eggs taken from Lake Apopka, we incubated eggs only at a female-producing temperature (30°C) (20). After hatching, alligators were housed at the Sante Fe Teaching Zoo (Santa Fe Community College, Gainesville, FL) in outdoor semiaquatic enclosures. At 9 months of age, the female alligators were transported to the laboratory for tissue collection.

For the experimental study, eggs were collected from seven nests on Lake Woodruff, Florida, during the first week of July, 1995. Eggs were transported to our lab and placed in an incubator at 30°C. One egg from each clutch was opened to stage the embryos. Staging was based on criteria defined by Ferguson (21). Five days after collection (and prior to the temperature-sensitive period when sex determination occurs), eggs were separated into two groups such that half of the eggs from one clutch were incubated at 30°C (female-producing temperature) and half at 33°C (male-producing temperature). Eggs were maintained at approximately 90% humidity using sphagnum moss as incubation material. Within each incubation group, eggs from each clutch were distributed

among six treatment groups of varying dosages (Table 1). One treatment group served as a control and three groups served as endocrine-disrupting standards: E₂; tamoxifen, which acts as an estrogen in embryonic alligators but an antiestrogen in hatchlings (22); and vinclozolin, a potent antiandrogen in rodents (23). The two remaining treatment groups were the modern-use herbicides atrazine and 2,4-dichlorophenoxyacetic acid (2,4-D). Treatments were applied topically to the eggshell in 50 µl of 95% ethanol, a technique frequently used to transport compounds inside reptilian eggshells (7,8). Using this method, Crews et al. (7) found that greater than 89% of the applied compound is incorporated into the embryo. The treatments were applied at stage 21 of embryonic development, the beginning of the critical period of gonadal differentiation (20).

Upon pipping, CAF was collected and frozen at -72°C until steroid hormone analysis. Total protein content in the CAF samples was determined using a commercially available Bradford assay kit (Bio-Rad, Hercules, CA), and CAF steroid hormone concentrations are presented per microgram protein. This was necessary due to differential hydration states of the CAF samples.

Aromatase assay. Hatchlings were individually housed for 10 days prior to tissue collection. Following the collection of blood from the dorsal post-cranial sinus, a lethal injection of sodium pentobarbital (0.4 mg/g) was administered in the sinus. Animals are anesthetized within 30 sec using this method. The right gonadal-adrenal mesonephros (GAM) complex was immediately removed for the aromatase bioassay. Aromatase activity was measured indirectly based on the release of tritium from 1β-³H-androstenedione during aromatization of the substrate into estrogen (24). Briefly, the tissue was placed in 400 µl culture media (RPMI-1640; Sigma Chemical Co., St. Louis, MO) supplemented with 0.8 mM tritiated androstenedione (DuPont NEN

Research Products, Boston, MA; # NET-926). After a 6-hr incubation at 32°C, 300 µl of the media was transferred to a new tube. Chloroform (1.5 ml) was added and the tube was vortexed and then centrifuged for 15 min at 2,000g. A 200-µl aliquot of the aqueous phase was added to a new tube. Five percent charcoal/0.5% dextran (200 µl) was added and the tube was vortexed and then immediately centrifuged for 15 min at 2,000g. Scintillation fluid (5 ml) was added to 300 µl supernatant and the tube was counted on a Beckman scintillation counter (Beckman Instruments, Schaumburg, IL). 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 non-specific 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. Assay sensitivity was defined as twice the mean counts per minute (cpm) of blank tubes, which corresponds to 0.15 pmol/g/hr for the average-weight GAM (0.032 g).

GAMs from three additional control female alligators were used to determine the specificity of the aromatase assay. The left GAM was incubated as above, whereas the right GAM was exposed to media supplemented with the aromatase inhibitor 4-hydroxy androstenedione (100 µM). Alligators exposed to the aromatase inhibitor had significantly lower GAM aromatase activity ($\mu = 0.45$ pmol/g/hr) compared to the individuals incubated normally ($\mu = 3.15$ pmol/g/hr).

Histology. Histology was conducted to determine histological sex in order to document which compounds induced sex reversal. A complete histopathological examination of the GAMs was beyond the scope of this study. The left GAM was preserved in Bouin's fixative, serial sectioned at 7 µm

Table 1. Experimental treatments and dosages that were applied to different groups of eggs

Treatment	Effect	Doses (ppm)
Control ^a	None	None; diluent only
17β-Estradiol	Natural estrogen	0.014, 0.14, 1.4, 14 ppm
Vinclozolin	Androgen antagonist in rodents	0.14, 1.4, 14 ppm
Tamoxifen	Estrogen agonist/antagonist	0.14, 1.4, 14 ppm
2,4-D	Common herbicide, ?	0.14, 1.4, 14 ppm
Atrazine	Common herbicide, ?	0.14, 1.4, 14 ppm

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; ?, effects unknown. A sample size of five eggs was included in each dose-treatment group.

^aEach chemical was solubilized in 95% ethanol prior to topical application on the egg; thus, two control doses were used—one with 95% ethanol and one without. There was no difference between these controls for any of the variables measured.

following paraffin embedding, and stained with a modification of Harris' trichrome staining procedure (25). Gonads were inspected and scored as testis or ovary by two independent researchers. Histological criteria originally reported by Forbes (26) and recently reestablished by Guillette et al. (19) were used to determine sex. In brief, criteria for testis included reduced cortex and medullary sex cord proliferation, whereas criteria for ovaries included hypertrophied cortex, medullary reduction, the presence of lacunae in the medulla, and germ cells in the cortex.

Radioimmunoassays. E_2 and testosterone (T) concentrations were measured in plasma of the 9-month-old descriptive study animals and in plasma and CAF of all hatchlings that provided ample fluids. Radioimmunoassays for E_2 and T were performed as previously described (27) with the following modifications in sample extraction. CAF (750 μ l) was mixed overnight (15 hr) with 2 ml of 95% ethanol. The suspension was centrifuged at 1,200g for 20 min. Duplicate aliquots of supernatant (500 μ l) for each sample were dried under constant air stream. Extraction efficiency averaged 92% for T and 94% for E_2 with this method. For plasma extraction, plasma (125 μ l) was mixed with 4 ml ethyl ether for 1 min. The aqueous layer was frozen in a dry ice-methanol bath (-25°C), and the ether phase decanted into an assay tube. The aqueous pellet was reextracted with ether and the ether added to the assay tube. The ether was dried with constant air stream. Extraction efficiency was consistent and averaged 95% for T and 94% for E_2 . Cross-reactivities of the T antisera (T3-125, Endocrine Sciences, Calabasas Hills, CA) to other ligands are as follows: dihydrotestosterone, 44%; Δ -1-testosterone, 41%; Δ -1-dihydrotestosterone, 18%; 5 α -androstane-3 β ,17 β -diol, 3%; 4-androstene-3 β ,17 β -diol, 2.5%; Δ -4-androstenedione, 2%; 5 β -androstane-3 β ,17 β -diol, 1.5%; estradiol, 0.5%; all other ligands <0.2%. Cross-reactivities of the E_2 antisera (E26-47, Endocrine Sciences) to other ligands are as follows: estrone, 1.3%; estriol, 0.6%; 16-keto-estriol, 0.2%; all other ligands <0.2%. For the plasma radioimmunoassays (RIAs), interassay variance was 15.0% for T and 12.6% for E_2 , and intraassay variance was 3.6% for T and 3.7% for E_2 . For the CAF RIAs, interassay variance was 11.8% for T and 16.1% for E_2 , and intraassay variance was 4.68% for T and 3.5% for E_2 .

Statistics. Hormone concentrations were estimated from raw data with the commercially available Beckman EIA/RIA ImmunoFit software program (Fullerton,

CA). Statistics were performed with the software packages StatView (Abacus Concepts, Inc., Berkeley, CA) and Superanova (Abacus Concepts, Inc.). For the descriptive study, an unpaired t -test was used for between-lake comparisons. In the experimental study, a two-factor analysis of variance (ANOVA) was used to test the effects of treatment and dose on aromatase activities. Where the interaction of treatment and dose was not significant, the factor of dose was removed from the analysis and a one-factor ANOVA was used to test the effects of treatment on hormone concentrations and aromatase activity. Fisher's protected LSD was used as a post-hoc test to discriminate which groups differed significantly.

Results

Descriptive Study—Female Juvenile Alligators

Results of the aromatase enzyme assay are expressed both as fmol/hr and pmol/g/hr. The former is used in other studies of alligator gonadal aromatase activity (13,24) and is presented here for comparative purposes only. A comparison of the female juvenile alligators found that GAM aromatase activity was significantly elevated in Lake Woodruff alligators compared to Lake Apopka alligators (Table 2, Fig. 1B). Mean concentrations of E_2 were not different between lakes ($p = 0.5178$), but concentrations of T were significantly lower in Apopka animals compared to Woodruff animals ($p = 0.05$; Fig. 1A).

Experimental Study—Neonatal Alligators

Four response variables were measured for the alligators treated *in ovo*: sex reversal, CAF hormone concentrations, hatchling plasma hormone concentrations, and GAM aromatase activity. Tables 3 and 4 summarize these results. One-hundred percent sex reversal—male to female—was noted for all dosages of E_2 and tamoxifen. No other treatments caused sex reversal.

Two-way ANOVA revealed that dose had no influence on plasma hormone concentration or gonadal aromatase activity in the eggs incubated at male and female temperatures. Dose did have an influence on CAF E_2 concentrations, but this was due only to the E_2 treatment group. Therefore, dose was removed from the statistical analysis, and a one-way ANOVA was used to determine if differences existed among treatment groups. The treatment group had no significant influence on CAF hormone concentrations, with the exception of tamoxifen treatments on eggs incubated at

a male-producing temperature. These sex-reversed females had significantly more CAF T compared to control males ($p = 0.024$). Neither CAF T concentrations ($p = 0.92$) nor CAF E_2 concentrations ($p = 0.77$) were different between control males and control females.

Plasma E_2 concentrations were not significantly different among treatment groups, but plasma T was different among treatment groups incubated at a male-producing temperature. Tamoxifen- ($p = 0.028$) and E_2 - ($p = 0.027$) treated animals, which were sex reversed, had elevated plasma T concentrations compared to control males. The ratio of E_2 /T was not significantly different for eggs incubated at male or female temperatures. Neither plasma E_2 ($p = 0.72$) nor plasma T ($p = 0.09$) were significantly different between control males and control females.

No differences in aromatase activity were detected among treated eggs at the female-producing temperature, but treatment groups in the male-temperature regime were significantly different. Tamoxifen-treated eggs had significantly more aromatase activity compared to control males ($p = 0.012$). Among the other eggs incubated at a male temperature, both E_2 - and atrazine-treated hatchlings appeared to have elevated aromatase activity. Although not significantly different from control males (E_2 , $p = 0.15$; atrazine, $p = 0.65$), further analysis revealed that these E_2 - and atrazine-treated animals were also not significantly different from control females (E_2 , $p = 0.65$; atrazine, $p = 0.13$).

Discussion

There are numerous mechanisms through which environmental contaminants potentially can cause endocrine alterations (1), and this study has documented that one such mechanism is the alteration of the steroidogenic enzyme aromatase. Results from the descriptive study indicate that GAM aromatase activity is significantly different between control and contaminated juvenile alligators, but this difference does not correspond with the alterations in circulating hormones in these animals. A previous study has shown that juvenile female alligators from Lake Apopka have significantly higher concentrations of plasma E_2 when compared to juvenile females from Lake Woodruff (19). One hypothesis for this increase in plasma E_2 is that aromatase activity is increased in the contaminant-exposed Lake Apopka alligators. However, the current study shows that female alligators from Lake Apopka have a significantly lower mean aromatase activity when compared to females from Lake Woodruff. These results are consistent with

data from *in vitro* cultures of gonads from Lake Apopka and Lake Woodruff juvenile alligators, which show that ovaries from Lake Apopka animals produce significantly less E_2 *in vitro* than do Lake Woodruff animals (28).

Although this study did not detect a difference in plasma E_2 between female alligators from Lakes Apopka and Woodruff, significantly less T was present in the plasma of the female Lake Apopka alligators. Several studies have noted a decreased plasma T concentration in male and female juvenile alligators from Lake Apopka (19, 29). The extent to which such decreases in plasma T alter the physiology of alligators is unknown, but a recent study has shown that mean phallic size in alligators from Lake Apopka is significantly smaller than that of males from Lake Woodruff (29). Because alligator phallic development and size are androgen dependent (30), it is probable that the decrease in plasma T concentrations contributes to an inhibition of penis growth in males. The effects of decreased T on females are unknown.

Results from the experimental study indicate that treatment with exogenous chemicals can alter the endocrine system of developing alligator embryos. Among the endocrine-disrupting standards, E_2 and tamoxifen treatments caused the development of ovaries in embryos incubated at a male-producing temperature. However, neither atrazine nor 2,4-D had such obvious endocrine-altering effects. In an attempt to assess the impact of the treatments on the developing embryo, we measured hormone concentrations in the CAF. The urinary wastes of developing oviparous embryos are stored in a membrane-bound sac called the allantois. As development proceeds, the allantoic membrane fuses with the chorion; the fluid inside is termed CAF. A previous study measured sex steroids in

this fluid and indicated that the measurement of these steroids could provide an assessment of the embryonic hormonal environment (31). Unlike the study by Gross et al. (31), we were unable to detect any difference in steroid hormones between control males and females. The only difference in CAF hormones was for T concentrations between the tamoxifen treatment and controls at a male-producing temperature. Therefore, measurement of steroid hormones in CAF does not appear to be a useful technique for assessing the embryonic hormonal environment of alligators.

Measurement of aromatase activity indicates that the enzymatic activity of

GAM tissue from hatchlings incubated at a male-producing temperature can be altered by exposure to exogenous compounds. None of the treatment groups had an effect on aromatase activity of hatchlings incubated at a female-producing temperature, but several differences were noted among alligators incubated at a male-producing temperature. All dosages of E_2 and tamoxifen caused ovarian differentiation at the male-producing temperature, and aromatase activity in these groups was increased accordingly (although only the tamoxifen-treated animals had aromatase activity that was significantly different from the control males). The aromatase activity of the

Table 2. *t*-Test results from gonadal-adrenal mesonephros aromatase activities of alligators from Lakes Apopka^a and Woodruff,^b Florida

	fmol/hr (± 1 SE) ^c	pmol/g/hr (± 1 SE)
Lake Apopka (<i>n</i> = 12)	204.5 \pm 21.792	7.549 \pm 1.264
Lake Woodruff (<i>n</i> = 14)	364.4 \pm 47.82	11.424 \pm 1.159
<i>df</i>	24	24
<i>t</i> -value	-2.877	-2.262
<i>p</i> -value	0.0083	0.0331

SE, standard error; *df*, degrees of freedom.

^aContaminated lake.

^bControl lake.

^cAromatase activity is expressed as both fmol/hr and pmol/g/hr; statistical analysis was performed on the latter, and the former is presented only to facilitate comparison with other studies (13,24).

Table 3. CAF hormones, plasma hormones, and gonadal-adrenal mesonephros aromatase activity for hatchling alligators treated *in ovo* with various compounds and incubated at a female-producing temperature (30°C).

Treatment	CAF E_2 (ng/ μ g protein)	CAF T (ng/ μ g protein)	Plasma E_2 (pg/ml)	Plasma T (pg/ml)	Plasma E_2/T^a	Aromatase (pmol/g/hr)
Control	126 \pm 38	235 \pm 99	14.1 \pm 5.1	33.3 \pm 6.1	0.54 \pm 0.22	3.3 \pm 0.78
E_2	443 \pm 165	110 \pm 25	13.0 \pm 2.1	25.9 \pm 3.2	0.56 \pm 0.08	3.2 \pm 0.64
Vinclozolin	104 \pm 36	122 \pm 29	25.3 \pm 5.7	27.9 \pm 3.8	0.99 \pm 0.22	3.7 \pm 1.17
Tamoxifen	196 \pm 123	197 \pm 88	27.9 \pm 6.7	34.3 \pm 5.2	0.84 \pm 0.19	5.0 \pm 0.89
2,4-D	305 \pm 137	732 \pm 331	10.7 \pm 1.7	25.1 \pm 3.2	0.48 \pm 0.08	4.9 \pm 1.19
Atrazine	32 \pm 8	65 \pm 15	18.7 \pm 5.0	28.5 \pm 2.6	0.67 \pm 0.17	3.8 \pm 0.75

Abbreviations: CAF, chorioallantoic fluid; E_2 , 17 β -estradiol; T, testosterone; 2,4-D, 2,4-dichlorophenoxyacetic acid. Variation is represented as ± 1 standard error. Histological examination of the gonads revealed that all animals incubated at a female-producing temperature had ovaries. There were no significant differences between treatment group and control females for any of the response variables.

^aThe range of E_2/T ratios are different from those previously presented for alligators by Guillette et al. (19). This is expected because different antibodies (with different sensitivities and specificities) were used in the two studies.

Table 4. CAF hormones, plasma hormones, and gonadal-adrenal mesonephros aromatase activity for hatchling alligators treated *in ovo* with various compounds and incubated at a male-producing temperature (33°C).

Treatment	CAF E_2 (ng/ μ g protein)	CAF T (ng/ μ g protein)	Plasma E_2 (pg/ml)	Plasma T (pg/ml)	Plasma E_2/T	Aromatase (pmol/g/hr)
Control	74 \pm 15	83 \pm 17	11.7 \pm 2.5	19.1 \pm 3.4	0.57 \pm 0.11	0.51 \pm 0.17
E_2^a	328 \pm 159	145 \pm 52	14.0 \pm 4.3	37.5 \pm 4.2 ^b	0.44 \pm 0.09	2.70 \pm 0.66
Vinclozolin	77 \pm 22	165 \pm 46	14.9 \pm 3.3	25.0 \pm 4.8	0.62 \pm 0.07	0.43 \pm 0.20
Tamoxifen ^a	282 \pm 116	427 \pm 119 ^b	18.3 \pm 3.7	37.6 \pm 4.5 ^b	0.55 \pm 0.11	4.34 \pm 1.71 ^b
2,4-D	140 \pm 57	285 \pm 81	8.6 \pm 0.6	28.6 \pm 5.6	0.47 \pm 0.10	0.44 \pm 0.06
Atrazine	69 \pm 18	132 \pm 28	16.7 \pm 4.1	24.3 \pm 3.7	0.65 \pm 0.13	1.21 \pm 0.60

Abbreviations: CAF, chorioallantoic fluid; E_2 , 17 β -estradiol; T, testosterone; 2,4-D, 2,4-dichlorophenoxyacetic acid.

Variation is represented as ± 1 standard error.

^aAll embryos treated with E_2 and tamoxifen were identified as females after histological examination of the gonads.

^bSignificant differences between the treatment group and control males.

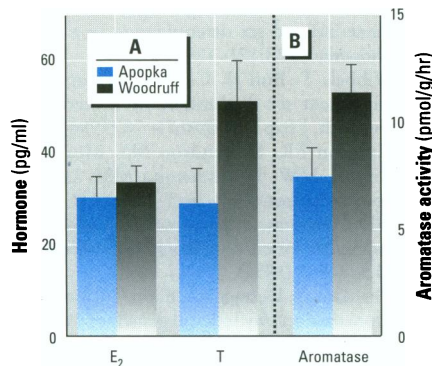


Figure 1. (A) Steroid hormone concentrations and (B) gonadal-adrenal mesonephros aromatase activity for 9-month-old female alligators from Lakes Apopka and Woodruff, Florida. Aromatase activity is significantly higher for animals from Lake Woodruff.

ovaries from these sex-reversed animals was not significantly different from ovaries of control female alligators.

Interestingly, atrazine treatments did not induce sex reversal at a morphological level but did stimulate testicular aromatase activity that was not significantly different from that of ovaries from control animals. Neither vinclozolin nor 2,4-D treatments had any effect on testicular aromatase activity. This suggests that although sex reversal was not induced by atrazine, atrazine altered steroidogenesis in the hatchling alligators such that more estrogen was produced. This increase in circulating estrogen was not detected by radioimmunoassay, although this is expected because there is no difference in control male and female hormone concentrations. A more sensitive and precise assay for E_2 and T could potentially uncover differences in these individual hormones.

Using *in vivo* and *in vitro* techniques, Connor et al. (32) concluded that chloro-triazines do not interact with the estrogen receptor and, thus, any estrogenic or anti-estrogenic effects must occur at mechanistic levels other than the estrogen receptor-ligand interaction. The data presented in this study suggest that atrazine may affect organisms at the level of steroidogenic enzyme activity. The ability of atrazine to alter the activity of steroidogenic enzymes has been noted previously in several studies. For instance, the female offspring of rats treated with atrazine during pregnancy and lactation show increased pituitary 5α -reductase and 3α -hydroxysteroid dehydrogenase activity (33). Conversely, atrazine significantly decreases pituitary 5α -reductase, 3α -hydroxysteroid dehydrogenase, and 3β -hydroxysteroid dehydrogenase activity in adult male rats (34,35). The aromatase enzyme is of the cytochrome P450 enzyme family; therefore, it is likely that many compounds (such as atrazine) increase aromatase activity in a similar manner to that of P450 enzymes involved in detoxification.

Atrazine could cause greater endocrine disruption in alligators hatched in the wild than that revealed by the present controlled laboratory experiment. As explained in the introduction, the temperature of egg incubation determines the sex of many reptiles, including alligators. Previous studies have indicated that steroid hormones and incubation temperature exhibit synergism, such that steroid hormones exert a greater effect at intermediate temperatures that produce both males and females (5,14). For instance, males incubated at a male-producing temperature close to the temperature threshold for female development are more sensitive to the effects of E_2 (5). The

present experimental design used two temperatures that would produce either 100% females (30°C) or 100% males (33°C). However, if eggs were incubated at a temperature closer to that which produces 50% males and 50% females (pivotal temperature), the effect of aromatase and the resultant steroid environment could be magnified. Future studies should explore this hypothesis.

Previous studies have shown that E_2 and tamoxifen treatments promote ovarian differentiation in reptiles incubated at male-producing temperatures (36,37). Although it is not known if such animals are capable of successful reproduction during adulthood, the present study finds no significant difference in aromatase activity between the female control animals and the sex-reversed tamoxifen- and E_2 -treated animals. This would suggest that at least at hatching, exogenously sex-reversed animals are capable of normal steroid production.

The present study has indicated that induction and suppression of aromatase enzyme activity are potential modes of contaminant-induced endocrine disruption in a species with temperature-dependent sex determination. Such disruption may not be limited to species with environmental sex determination, as aromatase function can also be affected in species with genetic sex determination. For example, the newt *Pleurodeles waltl* exhibits a ZZ/ZW system of genetic sex determination in which females are the heterogametic sex and, although sex determination is under gametic control, the sexual differentiation of the gonads can be modified by alterations in temperature (11). When ZW females are incubated at 32°C, aromatase activity in the gonadal-mesonephric complex is decreased to male-like levels. This suggests that although sex determination is genetic, the steroidal environment can be easily manipulated by exposure to various extraneous factors, including compounds that modify steroidogenic enzymes. Indeed, the addition of aromatase inhibitors modifies the sexual differentiation of animals with genetic sex determination. When treated with an aromatase inhibitor, genetically female chickens are masculinized but eggs fertilized by these males are not viable (38). This infertility is species dependent, as genetically female Chinook salmon (*Oncorhynchus tshawytscha*) develop into functional males if exposed to an aromatase inhibitor for as little as 2 hr (39).

This study attempted to determine 1) if the endocrine alterations previously observed for wild alligators could be explained by alterations in aromatase activity and 2) if endocrine disruptions, including alterations

in aromatase activity, could be induced by embryonic exposure to herbicides used extensively in the habitat of these wild alligators. Aromatase activity was significantly lower in female Lake Apopka animals compared to control animals, a result that supports data from *in vitro* culture of Lake Apopka alligator gonads (28). This increased aromatase activity does not, however, explain increases in circulating E_2 that have been described previously for wild Lake Apopka females (19). Embryonic exposure to 2,4-D had no effect on the endocrine parameters measured, but atrazine exposure caused aromatase activity that was characteristic of neither males nor females. Thus, atrazine exposure may induce endocrine alterations in embryonic alligators. These data emphasize the importance of considering the alteration of steroidogenic enzymes when analyzing contaminant-induced endocrine disruption.

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