Embryonic Treatment with Xenobiotics Disrupts Steroid Hormone Profiles in Hatchling Red-Eared Slider Turtles (*Trachemys scripta elegans*)

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Many compounds in the environment capable of acting as endocrine disruptors have been assayed for their developmental effects on morphogenesis; however, few studies have addressed how such xenobiotics affect physiology. In the current study we examine the effects of three endocrine-disrupting compounds, chlordane, *trans*-nonachlor, and the polychlorinated biphenyl (PCB) mixture Aroclor 1242, on the steroid hormone concentrations of red-eared slider turtle (*Trachemys scripta elegans*) hatchlings treated *in ovo*. Basal steroid concentrations and steroid concentrations in response to follicle-stimulating hormone were examined in both male and female turtles treated with each of the three compounds. Treated male turtles exposed to Aroclor 1242 or chlordane exhibited significantly lower testosterone concentrations than controls, whereas chlordane-treated females had significantly lower progesterone, testosterone, and 5α-dihydrotestosterone concentrations relative to controls. The effects of these endocrine disruptors extend beyond embryonic development, altering sex-steroid physiology in exposed animals. *Key words*: Aroclor 1242, chlordane, PCBs, steroid hormone, xenobiotic. *Environ Health Perspect* 108:329–332 (2000). [Online 22 February 2000]
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Endogenous hormones exert effects throughout embryonic development, after birth, and into adulthood. Compounds in the environment that operate as endocrine disruptors may also have the capacity to affect organisms from conception to death (*1*).

Endocrine-disrupting compounds (EDCs) found in alligator eggs from Lake Apopka, Florida (*2*), have the ability to affect sex determination in the red-eared slider turtle (*Trachemys scripta elegans*), a species with temperature-dependent sex determination (TSD) (*3*). In TSD, the temperature of the incubating egg determines the sex of the animal; low temperatures (< 28.2°C) produce male-biased sex ratios, with temperatures ≤ 27.8°C producing all males; higher temperatures (29.4°C) produce female-biased sex ratios, with temperatures ≥ 31°C producing all females (*4*). Temperature exerts its effects in the middle third of embryonic development, but application of hormones or EDCs during this period can alter the temperature-induced outcome (*3,5,6*). For example, estradiol (E_2) applied to eggs incubated at a normally male-producing temperature causes development of females (*5*). Mixtures of polychlorinated biphenyls (PCBs) also cause female sex determination at male-producing temperatures (*6*), as do chlordane, the PCB mixture Aroclor 1242, and *trans*-nonachlor (*3*). In the current study we examined the effects of each of three compounds—chlordane, the PCB mixture Aroclor 1242, and *trans*-nonachlor—on sex steroid levels in hatchling red-eared slider turtles treated during embryogenesis. Although the hatchlings from groups with sex ratios altered by xenobiotic exposure may appear morphologically normal, we hypothesized that their sex steroid physiology might have been affected as compared to unexposed hatchlings from the same temperature.

Materials and Methods

Embryonic xenobiotic treatment. We purchased turtle eggs from a commercial supplier (Robert Kliebert, Hammond, LA) and candled the eggs in the laboratory to establish viability. Viable eggs were placed randomly (to separate clutches) in groups of 35 into trays containing 1:1 vermiculite:water (wt/vol) and incubated at 28.6°C in computer-controlled incubators. We also monitored the temperature using HOBO temperature loggers (Onset Computer Corporation, Bourne, MA) and by daily readings of in-incubator shelf thermometers. Temperature variation was slight, with a minimum temperature of 28.5 and a maximum of 28.7°C. All turtles examined in this study were incubated simultaneously. We selected the 28.6°C temperature because it is a "threshold temperature" at which females first appear in the sex ratio.

Treatment occurred at Stage 17, during the middle third of development (*5*). Before treatment at Stage 17, all eggs were shifted around among the boxes so that no box contained eggs that all came from the same clutch. Eggs were treated with one of the treatment compounds dissolved in dimethylsulfoxide (DMSO), or with DMSO alone as a vehicle control. We selected the concentrations based on previously published results with these compounds (*3*); specific concentrations and amounts are shown in Table 1. Eggs were spotted once with 5 µL dissolved compound or vehicle and returned to incubators for the duration of development (*5*). After hatch, some turtles in the assay were sexed immediately, whereas others were kept for blood sampling for radioimmunoassay (RIA), after which they also were sexed.

Hatchling FSH challenge and RIA. Blood samples were drawn from 195 turtles (59 temperature control, 51 aroclor-treated, 43 chlordane-treated, and 42 *trans*-nonachlor-treated) at 6 weeks of age. Turtles from each xenobiotic treatment group were randomly assigned to one of three groups for RIA: nontreated control, vehicle-treated control (0.5 mL nanopure water), and follicle-stimulating hormone (FSH)-treated (50 µg ovine FSH dissolved in 0.5 mL nanopure water; 1 U/mg; Sigma, St. Louis, MO). Nontreated turtles were anesthetized on ice and then pithed, whereas vehicle-treated and FSH-challenged turtles were first injected intraperitoneally with either water vehicle (0.5 mL H_2O) or FSH, maintained at room temperature for 3 hr, anesthetized on ice, and then pithed. To obtain an adequate volume of blood from each euthanized turtle (0.15–0.4 mL whole blood), the heart was exposed by removal of a small window from the plastron, the ventricle and atria were cut with microdissection scissors, and blood was collected directly from the hemorrhaging heart with a heparinized 1-cc syringe. Blood was then transferred to 0.5-mL microfuge tubes and centrifuged at 3,000 rpm for 10 min at 4°C. Plasma was drawn off and frozen at -80°C. Upon sacrifice, the gonadal sex of each turtle was diagnosed as described previously (*6*). Briefly, the plastron was removed and the gonads were examined under a dissection microscope. Appearance of gonads (size, shape, vascularization) and assignment of sex was noted, as was presence or absence of oviducts.

We pooled plasma from two to three individuals to make 600 µL samples for RIA

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Table 1. Concentration and amount of each compound administered to each turtle egg based on concentrations found in egg yolks of alligators at Lake Apopka, Florida (*2*).

Xenobiotic	Concentration (μM)	Amount (ng/10 g egg)
Aroclor 1242	0.26	0.424
Chlordane	0.22	0.451
trans-Nonachlor	0.25	0.556

because a pilot study indicated that hormone levels were very low. Consequently, pooled samples included 23 temperature control, 19 Aroclor 1242-treated, 18 chlordane-treated, and 15 *trans*-nonachlor-treated turtles, half of which were FSH-treated. We then assayed the samples for 5α-dihydrotestosterone (DHT), E_2, progesterone, and testosterone using the methods of Tousignant and Crews (*7*). Assay sensitivity was 13 pg 5α-DHT/mL plasma, 13 pg E_2/mL plasma, 106 pg progesterone/mL plasma, and 10 pg testosterone/mL plasma. Intra-assay coefficients of variation were 16% for 5α-DHT, 18% for E_2, 12% for progesterone, and 17% for testosterone. Interassay coefficients of variation were 18% for 5α-DHT, 17% for E_2, 20% for progesterone, and 13% for testosterone.

Statistical analysis. We analyzed sex ratio data using two-by-two contingency tables. All experimental groups for the sex ratio analysis were compared to simultaneously incubated controls using Fisher's exact one-tailed tests. We used one-tailed tests because the results of Willingham and Crews (*3*) demonstrated that the compounds used in this experiment alter temperature-dependent gonadal sex determination, increasing the ratio of females.

Plasma steroid levels were log-transformed to eliminate unequal variances and compared using separate two-way analyses of variance (ANOVAs) for males and females for each steroid. Because the nontreated and vehicle-treated groups for RIA did not differ, they were combined in the analysis and are hereafter described as "RIA control." Thus, independent variables in the two-way ANOVAs were embryonic xenobiotic treatment, FSH challenge, and their interaction. Because the following hierarchical set of comparisons among treatment groups was planned *a priori*, we used the Dunn-Sidák correction for multiple comparisons (*8*). We first determined if the interaction between embryonic xenobiotic treatment and hatchling FSH administration was significant. If this interaction was significant, we determined whether basal steroid levels differed between RIA controls and xenobiotic-treated turtles and whether FSH-induced steroid levels differed between control and xenobiotic-treated turtles, for a total of six comparisons, an experimentwise $\alpha = 0.05$, and a nominal $\alpha = 0.008$ for individual comparisons. If the overall interaction was not significant, we then examined the main effects. If the main effect of xenobiotic treatment influenced steroid levels, we determined which xenobiotic groups differed significantly from the RIA control group, for a total of three comparisons, an experimentwise $\alpha = 0.05$, and a nominal $\alpha = 0.017$ for individual comparisons. All statistics were calculated using Version 3.1 of JMP (SAS Institute, Cary, NC) (*9*) for Apple Macintosh (Apple Computer, Inc., Cupertino, CA).

Results

Sex ratio. Each of the three compounds altered the male:female ratio as compared to that of the temperature controls (108 males:24 females). The male:female sex ratio in this study for each compound was 54:30 for Aroclor 1242 ($p = 0.003$; $\chi^2 = 8.261$); 29:14 for chlordane ($p = 0.041$; $\chi^2 = 3.705$); and 51:23 for *trans*-nonachlor ($p = 0.027$; $\chi^2 = 4.364$).

Males. Circulating levels of testosterone in hatchlings were influenced by xenobiotic treatment during embryonic development [ANOVA, $p = 0.003$; F ratio = 5.31; degrees of freedom (*df*) = 3, 45] (Figure 1). Testosterone concentrations were significantly lower in Aroclor- and chlordane-treated males as compared to RIA control males ($p = 0.003$ and $p = 0.001$, respectively). Although males treated with *trans*-nonachlor tended to have reduced testosterone levels as compared to RIA control males, the difference did not reach the statistical criteria of $\alpha = 0.017$ (i.e., $p = 0.039$). There was no significant effect of FSH administration or a significant interaction between xenobiotic treatment and FSH administration for testosterone levels ($p > 0.05$ for all).

Neither 5α-DHT nor progesterone levels were influenced by embryonic xenobiotic treatment or FSH treatment ($p > 0.05$ for all).

Concentrations of E_2 were not detectable in many (72%) of the samples (i.e., < 13 pg E_2/mL plasma).

Females. Although sample sizes were smaller for females, xenobiotic treatment during embryonic development also significantly influenced plasma testosterone levels in female turtles (ANOVA, F ratio = 4.44; *df* = 3,11; $p = 0.028$) (Figure 2A). Testosterone levels were significantly lower in chlordane-treated females as compared to RIA control females ($p = 0.005$). There was, however, no significant effect of FSH challenge or a significant interaction between xenobiotic treatment and FSH administration on testosterone levels ($p > 0.05$ for all).

Xenobiotic treatment during embryonic development significantly influenced plasma 5α-DHT levels in females (ANOVA, F ratio = 8.72; *df* = 3,12; $p = 0.002$) (Figure 2B). DHT levels were only significantly lower in chlordane-treated females as compared to RIA control females ($p < 0.001$). There was no significant effect of FSH administration or a significant interaction between xenobiotic treatment and FSH challenge on 5α-DHT levels ($p > 0.05$ for all).

Xenobiotic treatment during embryonic development significantly affected plasma progesterone levels in female turtles, which was similar to its effect on testosterone and 5α-DHT (ANOVA, F ratio = 19.94; *df* = 3, 12; $p < 0.001$) (Figure 2C). Progesterone levels were significantly lower only in chlordane-treated females as compared to RIA control females ($p < 0.001$). There was no significant effect of FSH administration or a significant interaction between xenobiotic treatment and FSH challenge on progesterone levels ($p > 0.05$ for all).

Concentrations of E_2 were not detectable in any group of females (i.e., concentrations were < 13 pg E_2/mL plasma).

Discussion

Altered hormone levels in response to embryonic exposure to xenobiotics have been identified in alligators (*10*) and in male rats (*11*); however, the present study provides the first direct link between effects on sex determination and sexual differentiation during embryogenesis and subsequent effects following birth. The three compounds used in this study significantly influenced the normal process of gonadal differentiation, which supports previous results (*3*). Moreover, two of these compounds also exert significant effects after hatch by altering circulating hormone

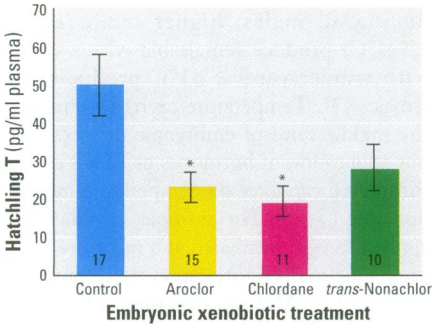

Figure 1. Plasma testosterone (T) concentrations in male hatchling red-eared slider turtles (*Trachemys scripta elegans*) as a function of embryonic xenobiotic treatment. Testosterone concentrations are shown as least-squares means (± 1 SE) from the ANOVA, as described in "Materials and Methods." These means represent the means for each treatment group while controlling for all other independent variables in the experiment. The number in the bar is the sample size. Each sample represents plasma pooled from two to three individuals.
*Significant relative to control ($p > 0.05$).

levels in both male and female turtles. In male hatchlings exposed during embryogenesis, Aroclor and chlordane lowered testosterone levels; in female hatchlings exposed during embryogenesis, chlordane caused a reduction in testosterone, DHT, and progesterone.

Although much of the research in this field has focused on mammals, studies using TSD reptiles are informative because of the many similarities between the patterns of gonadal development and the possibilities for manipulation that TSD reptiles offer (*5*). When gonadal sex is altered by administration of a natural estrogen (e.g., 17β-estradiol) or an aromatase inhibitor during embryogenesis, steroid profiles do not change detectably in the young snapping turtle (*Chelydra serpentina*), another TSD species (*12*). Similarly, no morphologic or histologic differences have been observed between temperature-determined female red-eared slider turtles and females resulting from treatment with natural estrogens (*5*). In juvenile American alligators (*Alligator mississippiensis*) exposed to contaminants in Lake Apopka, the testes appear normal morphologically; however, the circulating concentration of testosterone in males is reduced, as is the activity of aromatase, the enzyme that converts testosterone to E_2 (*10*). These effects have been attributed to contamination of the lake by agricultural runoff and a pesticide spill.

Other species also respond to prenatal exposure to EDCs. Aroclor 1254, another PCB mixture, causes a decline in steroid hormone levels in female Atlantic croakers (*13*). Interestingly, although the testes of alligators experimentally treated *in ovo* with the herbicide atrazine appear normal morphologically, the pattern of aromatase activity is altered compared to that of normal males (*10*). Bass exposed to EDCs in two Florida rivers exhibit decreased testosterone levels and are vitellogenic, suggesting altered steroidogenic enzyme activity (*14*). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin lowers serum androgen levels in adult male Sprague-Dawley rats exposed *in utero*, and it has been proposed that dioxin affects these changes by altering the testosterone synthetic pathway (*11*). Thus, changing the pattern of steroid enzyme activity is one possible mechanism by which EDCs can affect reproductive system function after hatch.

Two key steroidogenic enzymes in red-eared slider turtle sex development are reductase, which biosynthesizes nonaromatizable androgens from testosterone, and aromatase, which metabolizes precursors into estrogens (*15*). A change in steroidogenic enzyme activity causes a shift in the hormonal milieu of an organism; in the red-eared slider turtle, these alterations may result in the change from one gonadal pathway to the other (*5*). Applying E_2 to eggs during incubation at a male-producing temperature results in females, thus mimicking the end point of the pathway triggered at a female-producing temperature (*5*). Xenobiotics may cause a similar shift by altering the sex steroid milieu, either by affecting steroidogenic enzyme production, activity, or degradation rates, or by directly mimicking or inhibiting sex steroid hormones themselves.

Aroclor 1242, the PCB mixture that we used in the current study, lowered concentrations of testosterone in both males and females; in males, Aroclor 1242 appeared to cause an increase in E_2 levels (data not shown). Thus, Aroclor 1242 might act by increasing aromatase activity, resulting in lower testosterone concentrations and an increase in E_2 concentrations. However, Aroclor 1242 does not increase E_2 concentrations in females, which are undetectable in this assay; this may result because the preferred estrogen synthetic pathway in female red-eared slider turtles is to estrone or estriol, rather than to E_2. Both estrone and estriol are more powerful estrogens than E_2 in this species when applied exogenously in sex ratio studies (*5*).

Chlordane caused a significant increase in the female sex ratio in this study, a result that supports previous work (*3*). Chlordane also has been shown to interact with exogenously applied E_2, causing a significant increase in female hatchlings as compared to the effects of E_2 alone (*3*). Additionally, female hatchlings from chlordane-treated groups exhibited lower testosterone, DHT, and progesterone concentrations. These results, taken together, suggest that chlordane is an anti-androgen which complements the effects of E_2; furthermore, chlordane may exert its effects on the sex ratio by reducing androgen concentrations, resulting in an altered steroidal milieu. The subsequent reduction in androgen concentrations could be extreme enough to keep them significantly below those found in normal females.

In some cases, turtles that did not undergo a change to a female pathway still exhibited altered levels of circulating steroid hormones after hatch. Both chlordane and the Aroclor mixture caused a decrease in testosterone concentrations in males. Morphologically, the gonads of these hatchlings appeared normal. These data suggest that chlordane and Aroclor 1242 both acted to alter the internal steroidal milieu, but not to the extent of shifting development to the female sex-determining pathway. Chlordane, once again, could cause lowered testosterone concentrations through its activity as an anti-androgen, whereas Aroclor could have this effect by causing an increase in aromatase activity. One question that arises, however, is why chlordane did not cause a reduction in nonaromatizable androgens in the male hatchlings as it did in the exposed females. Applying increasing concentrations of testosterone to eggs at male-producing temperatures causes concordantly increasing ratios of females to develop; this effect is blocked when testosterone is applied in conjunction with aromatase inhibitor (*15*). Thus, in the presence of excess testosterone, aromatase is the preferential enzyme. But what about lowered concentrations of testosterone, as in the case of the chlordane-exposed males? A possible explanation for the differences in the effects of chlordane is that for the embryos irreversibly differentiated as males, lower testosterone

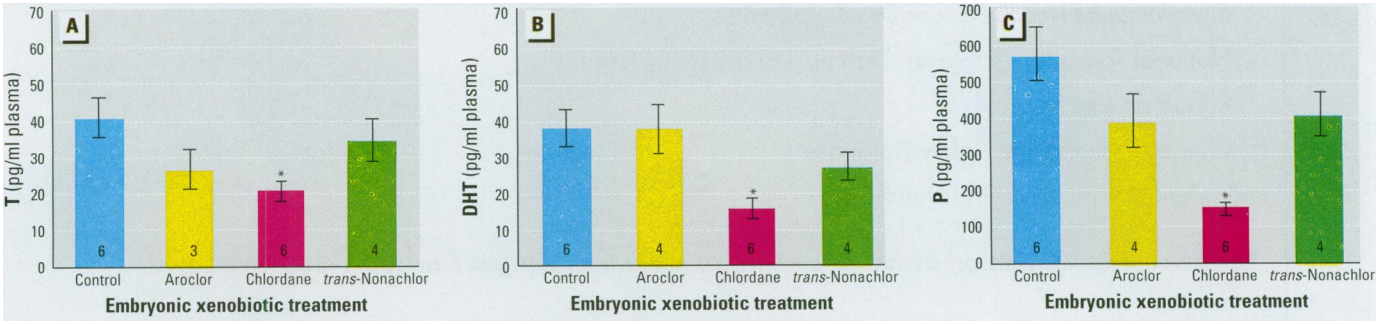

Figure 2. (*A*) Plasma testosterone (T), (*B*) 5α-dihydrotestosterone (DHT), and (*C*) progesterone (P) concentrations in female hatchling red-eared slider turtles (*Trachemys scripta elegans*) as a function of embryonic xenobiotic treatment. Hormone concentrations are shown as least-squares means (± 1 SE) from the ANOVA, as described in "Materials and Methods." The number in the bar is the sample size. Each sample represents plasma pooled from two to three individuals.
*Significant relative to control ($p > 0.05$).

concentrations elicit an increase in reductase activity, so that late-stage embryos and hatchlings have normal concentrations of nonaromatizable androgens. For embryos irreversibly dedicated to the female pathway, a mechanism for maintaining androgen concentrations would not be necessary.

Interestingly, in studies of male and female hatchlings from different incubation temperatures, higher temperatures cause a reduction in the concentration of testosterone (*16*). Additionally, progesterone concentrations decrease with increased incubation temperature. Aroclor and chlordane both caused a reduction in testosterone, and chlordane treatment resulted in decreased progesterone and DHT in females. These results suggest that the Aroclor mixture and chlordane affect the pathway triggered by incubation temperature, and their effects, at least at the level of circulating steroid hormones, mimic those of increasing or decreasing temperature.

trans-Nonachlor did not affect circulating steroid hormone concentrations in either male or female turtles. This result suggests that *trans*-nonachlor may act as a direct estrogen mimic, possibly without aberrant effects on the turtle, much like E_2 acts in snapping turtles exposed during embryogenesis and shifted to a female developmental pathway (*12*). Future studies examining the effects of these compounds on estrogen receptor levels during development will help elucidate this matter.

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