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The Red-Eared Slider Turtle: An Animal Model for the Study of Low Doses and Mixtures[1]

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SYNOPSIS. Current risk assessment techniques for almost all chemicals involve establishing a threshold dose, or the dose below which no adverse effects are seen. But threshold doses may not apply in situations where the chemical mimics the actions of an endogenous compound—such as a steroid hormone—that is important to development. In such cases, any exposure may exceed the threshold. Recent studies with the red-eared slider turtle have shown that exogenous estradiol—even when applied to eggshell in doses as low 0.4 ng—will affect sex development during embryogenesis. Considering that only 0.2% of the estradiol applied to the eggshell ends up in the embryo, it becomes apparent that even very low dosages of steroid hormones or their mimics can have profound biological effects. We tested this idea using eight compounds identified in the yolk of alligator eggs from Lake Apopka, Fla. Five of the compounds—the PCB mixture Aroclor 1242, *trans*-Nonachlor, *cis*-Nonachlor, p,p′-DDE, and chlordane—altered sex ratio outcomes when applied to eggshells during development. Aroclor 1242 produced the most powerful effects, shifting the ratio of females almost twofold, while chlordane had the greatest effect when combined with estradiol. Administration of all eight compounds together also increased the ratio of females to males. However, comparing the single-compound exposures at the same dosages indicate that these compounds behave differently in combination than they do singly, emphasizing the need for further studies using chemical mixtures reflecting proportions found in nature. The effect of chlordane and Aroclor 1242 on aromatase activity levels during embryogenesis in the brain and adrenal-kidney-gonad (AKG) complex was also examined. Chlordane, a suspected anti-androgen in this species, did not affect aromatase activity in either the brain or the AKG. However, Aroclor 1242 significantly altered aromatase activity levels in the red-eared slider turtle brain—but not in the AKG—during a crucial developmental period. After this crucial period, Aroclor 1242 caused an increase in aromatase activity in the AKG of embryos just prior to hatch. Additionally, hatchling males treated during embryogenesis with Aroclor 1242 and chlordane exhibited significantly lower testosterone levels than controls in response to follicle-stimulating hormone administration, while chlordane-treated females had significantly lower progesterone, testosterone, and 5α-dihydrotestosterone levels relative to controls. These results are similar to those found in juvenile alligators from Lake Apopka. Males treated with Aroclor 1242 and *trans*-Nonachlor displayed an elevated estradiol response to FSH administration *vs.* control males. Taken together, these results suggest that EDCs exert effects during embryonic development that extend beyond birth. They also suggest that the alterations in sex steroid hormone levels observed in animals from contaminated areas may result from EDC-induced alterations in the neuroendocrine axis controlling gonadal sex steroid hormone production.

INTRODUCTION

Numerous manmade compounds introduced into the environment have been shown to disrupt normal endocrine function during vertebrate development (see Crisp *et al.*, 1998 for review). Among these are pesticides, such as chlordane, DDT, and DDT metabolites, and industrial byproducts such as nonylphenol and polychlorinated biphenyls (PCBs). The effects of these endocrine-disrupting compounds (EDCs) were recognized only after the identification of diethylstilbestrol as another such compound (McLachlan, 1977; McLachlan *et al.*, 1982) and the discovery of animals that suffered

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adverse effects in contaminated areas (Guillette *et al.,* 1994; Fry 1995). These findings prompted an ongoing search for models that will help predict these adverse developmental effects of EDCs before they happen (*e.g.,* Sumpter 1998; Sumpter and Jobling, 1995).

Before the discovery that some compounds can affect an organism's normal endocrine-directed development, most studies involving contaminants centered on their possible carcinogenic effects. Such studies required the identification of the level at which no adverse effects (*i.e.,* cancer) were observed. This dosage level was then divided by a predetermined number (usually 100) to set the acceptable level of exposure for these compounds. Typically these studies are performed with adult animals, whose systems respond quite differently from that of an embryonic vertebrate to subtle changes in endocrine signals. Dosages that do not seem to affect animals exposed as adults can cause profound changes in the exposed embryonic animal, ranging from effects on sexual differentiation to complete alteration of primary sex. Additionally, studies have traditionally examined a single compound at a time, employing a dose-response approach to assessing the effects of potentially harmful compounds (Gaylor *et al.,* 1988). But in the natural environment, animals can be exposed to a mixture of compounds, and new approaches to risk assessment must take this multi-exposure scenario into account.

One problem facing researchers in the field of endocrine disruption is identifying a suitable animal model for *in vivo* studies of EDC effects. The red-eared slider turtle (*Trachemys scripta elegans*) provides one such model. We have demonstrated that the red-eared slider turtle can serve as a marker for endocrine disruption on several levels, from the organismal (sex determination) to the physiological (circulating steroid hormone levels). For a number of reasons, this turtle makes a good model for studying EDCs singly, in mixtures, and in low doses. First, in the red-eared slider turtle, sex is determined by the temperature of the incubating egg, and the exogenous application of steroid hormones or steroidogenic enzyme inhibitors can reproduce temperature's effects (see Crews, 1996 for review). For example, 26°C normally produces male hatchlings, but topically applying estrogen or estrogenic substances in a solvent (ethanol) to the eggshell at this temperature will cause females to result. Alternately, 29.4°C produces hatchlings with a sex ratio heavily skewed towards females. But adding aromatase inhibitor—which effectively halts the production of estrogens—to the eggshell will result in 100 percent males. The lability of sex determination in this turtle offers the opportunity to use gonadal sex as a marker for EDC effects, even the effects of extremely low dosages of EDCs or EDC mixtures. We have also used aromatase activity during development as a marker for EDC effects; aromatase is an enzyme key to female development in this species (Crews, 1996), and its activity exhibits temperature dependence (Willingham *et al.,* 2000). Lastly, the hatchlings exposed to EDCs during embryogenesis provide yet another way to assess endocrine disruption—measurement of steroid hormone levels in their blood.

LOW-DOSE EFFECTS

One concern with traditional risk assessment approaches is the assumption that a threshold exists for a compound's observable effects in an organism, and exposure to levels below that threshold will not cause adverse effects. But when a compound or molecule mimics an endogenous compound or molecule, then the concept of a threshold dose may not apply (Gaylor *et al.,* 1988). For example, estrogen is an endogenous molecule that affects an organism at very low concentrations; *any* additional exposure to a manmade compound that mimics estrogen's effects may result in levels that automatically exceed the threshold for adverse effects in that organism. This scenario applies most intuitively in embryonic organisms whose systems may be far more sensitive to even the most minor fluctuations in the endocrine environment (*e.g.,* vom Saal 1995; vom Saal *et al.,* 1998).

To test the hypothesis that estrogenic compounds may not demonstrate a threshold dose for effects, a retrospective analysis

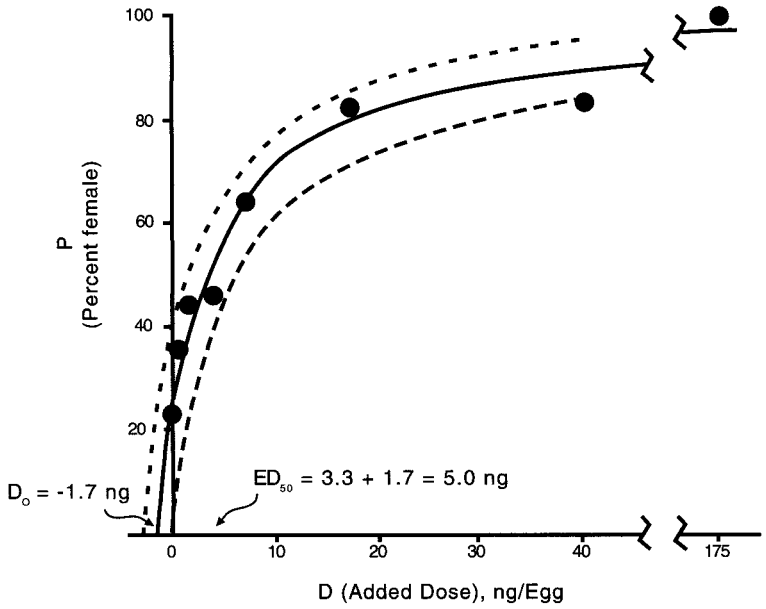

FIG. 1. A large prospective test of the no-threshold model. Eggs (n = 2400) were incubated at 28.6°C (male-biased temperature) and treated with estradiol-17β in dosages of 0, 0.4, 1.6, 4.0, 7.0, 17, 40, and 175ng/egg. The solid line, calculated from the modified Michaelis-Menten [% female = $100(d + 1.7)/5 + (d + 1.7)$], fits with an r^2 of 0.90. The dashed lines indicate 95% confidence intervals. The solid line strikes the dose axis at −1.7 ng/egg, which gives the predicted average endogenous dose. The ED_{50} of 5ng is the sum of the endogenous dose and applied doses (ED_{50} = 3.3 + 1.7 = 5 ng) (Redrawn from Sheehan *et al.*, 1999).

of published data was conducted analyzing the effects of varying doses of estradiol on sex determination in the red-eared slider turtle at three different incubation temperatures (Sheehan *et al.*, 1999). The results of all three retrospective analyses fit the Michaelis-Menten model of a single protein-molecule interaction driving a reversible process.

Using a power-analysis approach that resulted in a large (2,400 eggs) dose-response study, the role of exogenous estradiol and the concept of the threshold hypothesis in this species was tested, as was the idea that the Michaelis-Menten equation can be used to predict an organismal response. In this study, the Michaelis-Menten provided an ED_{50} of 5.0 with a 95% confidence limit of ±2.0 ng (endogenous dose = 1.7 ± 1.3 ng; exogenous dose = 3.3 ± 1.7 ng) and an r^2 = 0.90 for fit of the modified equation. The lowest dose, 0.4 ng/10 g egg, increased the female fraction by 11.4% beyond the temperature control. The most striking feature of these studies is that the curve becomes increasingly linear as the dose approaches zero, reinforcing the concept that a threshold dose may not exist when an exogenous molecule mimics an endogenous one by acting through the same mechanism (Fig. 1).

EFFECTS OF XENOBIOTICS AT AN ORGANISMAL LEVE, SINGLY AND IN COMBINATION

Taking the idea of low-dose effects a step further, we completed a study examining the effects of low, environmentally relevant dosages of EDCs on the sex determination of the red-eared slider turtle. Using compounds in concentrations identified by Heinz *et al.* (1991) in alligator eggs from Lake Apopka, Florida (Table 1), we assessed their ability to significantly increase the ratio of female hatchlings in groups of eggs incubating at a male-biased temperature (28.6°C). Our results demonstrated that five of the compounds were capable of significantly increasing the ratio of female hatchlings beyond that observed in the tem-

TABLE 1. *Compounds identified by Heinz* et al. *(1991) in alligator eggs from Lake Apopka, Florida and applied to red-eared slider turtle eggs to assess their effects on sex ratios (Willingham and Crews, 1999).**

Compound	Amount (ng)/ 10g egg	Concentration (μM)
Dieldrin	1.197	0.63
Toxaphene	0.415	0.22
p,p′-DDD	4.2	2.6
*Cis*Nonachlor	0.356	0.16
Aroclor 1242	0.848	0.53
*Trans*Nonachlor	0.556	0.25
Chlordane	0.451	0.22
p,p′-DDE	28.2	18

* Amounts applied per egg are shown in ng. The μM concentrations of each compound are given.

perature controls (Willingham and Crews, 1999) (Fig. 2). The compounds were applied during embryogenesis in concentrations identified by Heinz *et al.* (1991), and the total amount applied to each egg was usually in the low parts per billion (see Table 1). Considering that studies indicate that less than 0.2% of the compound applied actually reaches the embryo (Crews *et al.*, 1991), these already-low doses may, in fact, be considerably lower.

A previous study examining the effects of PCB mixtures on the red-eared slider found that in combination, some PCB mixtures applied during embryogenesis cause sex reversal from male to female at a normally male-producing temperature (Bergeron *et al.*, 1994). The results of this study pointed to the need to examine other mixtures. Thus, in addition to examining the effects of the Lake Apopka compounds singly, we also looked at the behavior of these compounds in combination. We applied a combination of all eight compounds to eggshells in the concentrations identified by Heinz *et al.* (1991) (Table 1) and found that in combination, they significantly increased

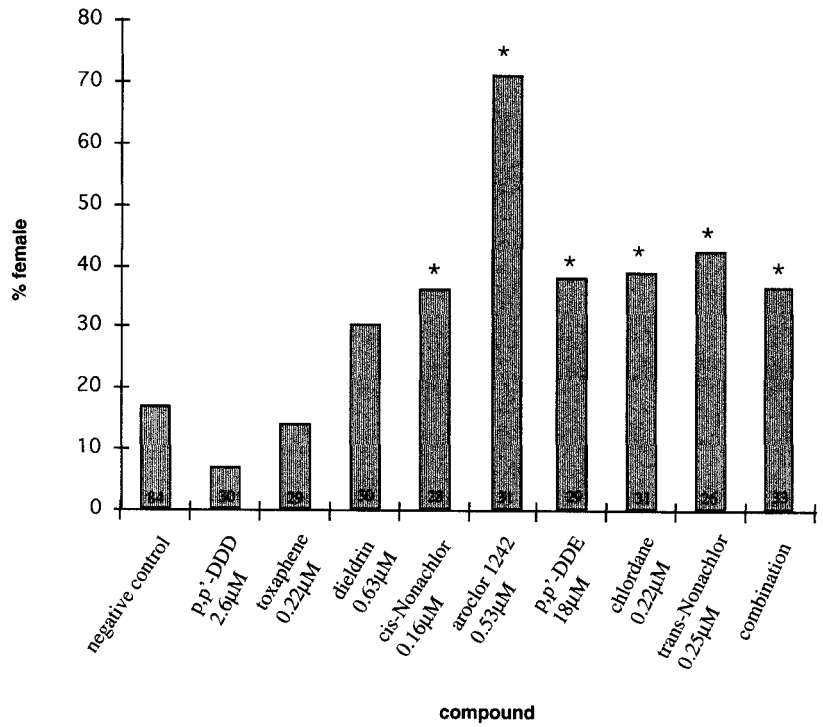

FIG. 2. Effect of endocrine disrupting chemicals on sex determination in the red-eared slider turtle, *Trachemys scripta elegans*, showing percent sex reversal for each treatment. All eggs incubated at 28.6°C, a male-biased sex ratio. Percent reversal for negative (solvent only) control also shown. Numbers in bars indicate sample size. Asterisk indicates significant reversal *versus* negative control for compounds applied singly (Redrawn from Willingham and Crews, 1999).

the ratio of female hatchlings (Willingham and Crews, 1999) (Fig. 2). Additionally, we examined the behavior of each compound in combination with estradiol, and found that one compound—the pesticide chlordane—exerted effects significantly beyond those of estradiol alone (Willingham and Crews, 1999). This result with chlordane suggested the possibility that it may operate as an anti-androgen in this species.

EFFECTS OF XENOBIOTICS ON STERIDOGENIC ENZYME ACTIVITY DURING EMBRYOGENESIS

The results using gonadal sex as a biomarker for endocrine disruption led to questions that focused on how these compounds may exert their effects. We identified another way to assess the effects of EDCs in the red-eared slider turtle. Aromatase, the enzyme responsible for converting aromatizable androgens to estrogens, is a key steroidogenic enzyme in the sex determining pathway of the red-eared slider turtle (Crews, 1996). Inhibiting aromatase will cause males to result at a temperature that normally produces a heavily female-biased sex ratio; applying estrogen—the metabolic product of aromatase activity—to eggs at a male-producing temperature causes females to develop (Crews, 1996).

A few studies point to the idea that some EDCs may act via steroidogenic enzyme pathways to exert their endocrine-disrupting effects. For example, Majdic *et al.* (1996) found that the xenobiotic octylphenol alters steroidogenic-factor 1 (SF-1) expression in rats. Steroidogenic-factor 1 is responsible for aromatase regulation, and it exhibits sex-specific expression in the red-eared slider turtle (Fleming *et al.,* 1999). Compounds that affect SF-1 activity could also affect downstream processes, such as aromatase activity. For this reason, we examined the effects of two compounds—the PCB mixture Aroclor 2142 and the pesticide chlordane—on aromatase activity in the brain and adrenal-kidney-gonad (AKG) complex of the red-eared slider turtle during embryogenesis.

The Aroclor 1242 mixture produced interesting and intriguing results. In a separate study, we found that aromatase activity levels in the brain are higher in embryos from a female-producing temperature than those from a male-producing temperature at a crucial developmental period (Willingham and Crews, 2000). These findings support previous work in other turtle species with temperature-dependent sex determination (Salame-Mendez *et al.,* 1998; Jeyasuria and Place, 1998) and provide evidence for the idea that the temperature-transducing organ in the red-eared slider turtle is the brain. In the studies using Aroclor 1242, we found that the PCB mixture causes a significant increase in aromatase activity in the brain within 24 hr of application to the eggshell and during the temperature-sensitive window (Willingham and Crews, 2000). Additionally, the Aroclor 1242 mixture resulted in significantly higher aromatase activity levels in the AKG just before hatch.

Chlordane is a suspected anti-androgen in this species (Willingham and Crews, 1999), and the PCB mixture exerted the most powerful effects in the studies using the data from Heinz *et al.* (1991) (Fig. 2). We found that chlordane had no effect on aromatase activity levels, indirectly supporting the idea that it is an anti-androgen and exerts its effects by inhibiting the male developmental pathway, rather than interfering with the female-developmental pathway.

EFFECTS OF XENOBIOTICS BEYOND BIRTH

The results of Aroclor 1242's effects in the AKG just before hatch are especially interesting in light of the effects the Aroclor 1242 mixture had on circulating steroid hormones in male hatchlings. Male hatchlings exposed to Aroclor 1242 or chlordane during embryogenesis exhibited significantly lower testosterone levels than controls (Fig. 3), while chlordane-treated females had significantly lower progesterone, testosterone, and 5α-dihydrotestosterone levels relative to controls (Fig. 4). The effects of chlordane on male and female hatchlings can easily be explained if the idea that this compound acts as an anti-androgen is correct.

But the results with Aroclor 1242, when taken together with the increase in aromatase activity in AKG just before hatch, paint a more complete picture. In the red-eared

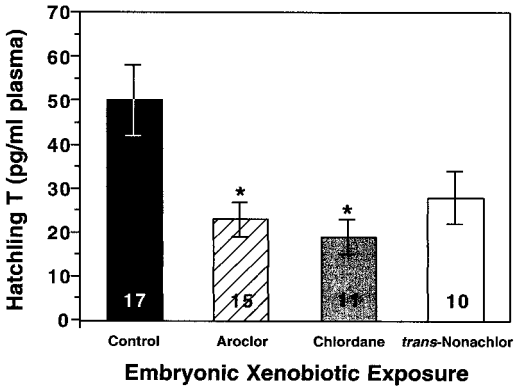

FIG. 3. Plasma testosterone levels of male hatchling red-eared slider turtles (*Trachemys scripta elegans)* as a function of embryonic xenobiotic treatment. Testosterone levels are least-squares means (±1 SE) from the ANOVA described in the text. These means represent the means for each treatment group while controlling for all other independent variables in the experiment. * indicates significance relative to control. Sample sizes for each group are indicated at the base of the histogram bars. Each sample represents plasma pooled from 2–3 individuals (Redrawn from Willingham *et al.,* 2000).

slider turtle, the PCB mixture Aroclor 1242 exerts a powerful organismal effect, altering the sex determining pathway from male to female (Willingham and Crews, 1999) (Fig. 2). During embryogenesis, this PCB mixture also causes an increase in aromatase activity in a crucial organ at a crucial time period (unpublished data, E.J.W. and D.C.). Additionally, Aroclor 1242 exposure results in an increase in aromatase activity in the AKG of this species just before hatch (unpublished data, E.J.W. and D.C.). Just after hatch, testosterone levels in Aroclor 1242-exposed males are low, and estradiol is measurable only in the Aroclor-2142-exposed male hatchlings (Willingham *et al.,* 1999). Because aromatase is the enzyme that converts testosterone to estradiol, lower testosterone levels and higher estradiol levels are an expected outcome of increased aromatase activity. Taken together, these data create a complete picture of Aroclor 1242's effects, working via the aromatase pathway and causing an increase in activity at crucial time periods, and finally exerting its effects even beyond embryogenesis.

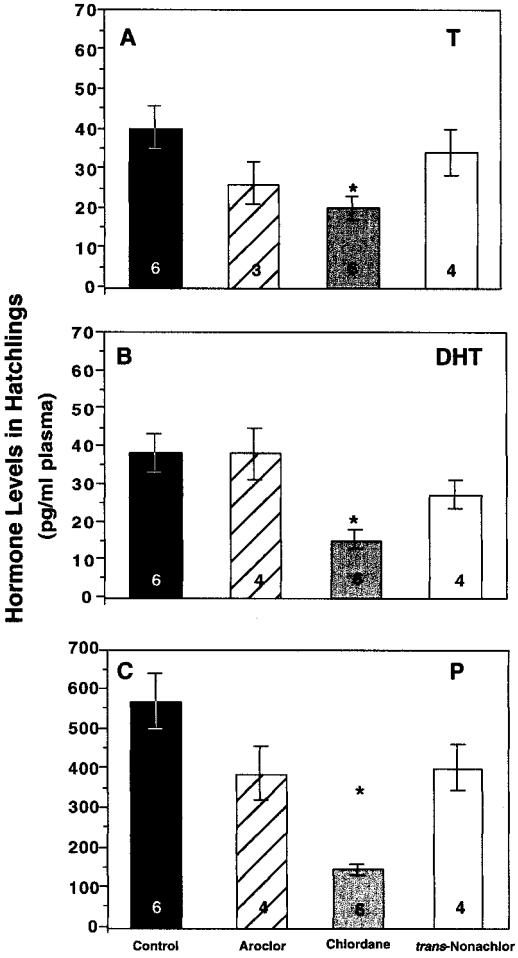

FIG. 4. Plasma testosterone (A), progesterone (B), and 5α-dihydrotestosterone (C) levels of female hatchling red-eared slider turtles (*Trachemys scripta elegans*) as a function of embryonic xenobiotic treatment. Hormone levels are least-squares means as noted in Figure 1. * indicates significance relative to controls. Sample sizes for each group are indicated at the base of the histogram bars. Each sample represents plasma pooled from 2–3 individuals (Redrawn from Willingham *et al.,* 2000).

CONCLUSION

As the studies with Aroclor 1242 and chlordane indicate, the red-eared slider turtle offers a number of different markers for assessing the endocrine-disrupting capabilities of contaminants. The lability in sex determination and ubiquity of this species make it an excellent model for an organismal response to EDCs. In addition, the definitive nature of gonadal sex offers a way

to measure unequivocally effects of mixtures in a whole organism. Beyond an organismal response, identifying the role of aromatase in the sex developmental pathway of this species has also made available a marker for the effects of these compounds on aromatase activity. Lastly, we can measure the effects of EDCs even beyond birth in embryonically exposed animals by examining levels of circulating sex steroid hormones, thereby revealing anomalies even in animals that appear morphologically normal.

Research is beginning to address the interactive nature of EDCs and particularly whether the threshold concept as developed in toxicological research applies to these chemicals. We would predict (Crews *et al.*, 2000) that further research will reveal that for a variety of EDCs a threshold dosage will not be evident since they mimic the actions of endogenous molecules (*e.g.*, estrogen) critical to development. Research is already demonstrating that bioaccumulated EDCs inherited from the mother not only influence the morphological and physiological development of the offspring, but also affect their reproductive behavior as adults. This indicates that such compounds may shape the neural substrates of reproductive physiology and behavior. If this is found to be the case, psychobiological principles suggest that these behavioral alterations will compound and magnify the effects of EDCs over successive generations. Lastly, the issue of species, individual, and even tissue differences in the effects of these compounds continue to be an enigma. While it remains to be demonstrated, it is likely that the sensitivity of a species or an individual to a compound will be found to be related to species-typical circulating concentrations of gonadal steroid hormones.

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