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Sex Reversal Effects of Environmentally Relevant Xenobiotic Concentrations on the Red-Eared Slider Turtle, a Species with Temperature-Dependent Sex Determination

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Xenobiotics suspected of being estrogenic—the PCB aroclor 1242 and the pesticides toxaphene, dieldrin, *p,p′*-DDD, *cis*-Nonachlor, *trans*-Nonachlor, *p,p′*-DDE, and chlordane—were examined for their ability to override a male-producing incubation temperature and result in female hatchlings in the red-eared slider, a turtle with temperature-dependent sex determination. Compounds were assayed in the environmentally relevant concentrations detected in alligator eggs from Lake Apopka, Florida, singly, in concert with one another, and with estradiol. Compounds assayed alone and resulting in significant sex reversal were *trans*-Nonachlor, *cis*-Nonachlor, aroclor 1242, *p,p′*-DDE, and chlordane. When administered with estradiol, only one of the compounds, chlordane, caused sex reversal at significant levels. When applied together, however, the eight compounds assayed resulted in significant sex reversal.

Key Words: endocrine disruption; environmental estrogens; pesticides; PCBs; turtles; contaminants.

Compounds in the environment that mimic hormones have become a major focus of research in the past decade (see Crisp *et al.*, 1998, for review). Those compounds that imitate estrogen-dependent phenomena are labeled environmental estrogens (EEs) and include pesticides such as chlordane, *p,p′*-DDD, and *p,p′*-DDE and industrial byproducts such as nonylphenol and PCBs (Bergeron *et al.*, 1994; for review see Crisp *et al.*, 1998; Danzo, 1997; Ersochenko, 1981; Fry, 1995; Jobling and Sumpter, 1993; Vonier *et al.*, 1996). Embryos exposed *in ovo* via maternal contamination and animals exposed constantly to an aquatic environment contaminated with such compounds may be at risk. The Florida red-belly turtle (*Chrysemys nelsoni*) was found to have suffered deleterious effects, including the development of ovotestes, from DDT contamination of Lake Apopka, Florida (Guillette *et al.*, 1994). In fact, of all turtles sampled from that area, no normal males were observed.

The red-eared slider, *Trachemys scripta elegans*, like many other turtle species and all crocodilians, lacks sex chromosomes (Janzen and Paukstis, 1991); instead its sex is determined by the incubation temperature of the egg. For *Trachemys scripta*, this means that eggs incubated at 26°C will produce male hatchlings, whereas eggs incubated at 31°C produce female hatchlings. Intermediate temperatures produce mixed-sex ratios, with 29.2°C resulting in a 50:50 ratio. Temperature can be manipulated experimentally to produce the desired sex (Wibbels *et al.*, 1991). Applying estrogens to the eggs of this species during incubation overrides male-producing temperature effects and produces females, even at incubation temperatures that normally result in males (Crews *et al.*, 1989). This

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characteristic makes *T. scripta* excellent for assaying the effects of suspected EEs; it has already been established that certain PCBs alone and in pairs reverse sex in this species from male to female (Bergeron *et al.*, 1994). But other suspect EEs remain untested, including some of those found contaminating the Lake Apopka area.

The contaminants in Lake Apopka are not limited to *p,p′*-DDE/DDT. Heinz *et al.* (1991) found levels of several other compounds in alligator eggs from around the lake, including dieldrin, toxaphene, *cis*- and *trans*-Nonachlor, aroclor 1242, chlordane, and *p,p′*-DDD. Additionally, Vonier *et al.* (1996) found that some of these chemicals were capable of binding alligator estrogen receptor (ER), and therefore might possibly operate via that pathway for estrogenic effects. In the current study, environmentally relevant concentrations of compounds from the Lake Apopka area (Heinz *et al.*, 1991) were tested for their hormone-like activity in the red-eared slider turtle. Additionally, these compounds were assayed with a dose of estradiol to determine their interactions with estrogen. Only one compound reversed sex significantly compared to positive control when combined with estradiol. When applied all together, the compounds reversed sex significantly beyond control, as did five of the compounds when applied singly.

METHODS

Turtle eggs were purchased from a commercial supplier (Robert Kliebert, Hammond, LA) and transported to the lab for candling, used to establish viability. Viable eggs were placed in groups of 35 into trays containing 1:1 vermiculite:water. Eggs were incubated at 28.6°C in computer-controlled incubators, where temperature was also monitored by HOBO temperature loggers (Onset Computer Corporation) and by daily recording of in-incubator shelf thermometer readings. Eggs were randomized prior to treatment to avoid clutch effects. For refinement of the assay and to ascertain the level of developmental sensitivity of the species to these compounds, eggs were incubated at the higher end of the male-producing temperature, 28.6°C, which typically produces a male-biased sex ratio. The University of Texas at Austin has an approved Assurance from the Office of Protection from Research Risks of the National Institutes of Health (Assurance No. A1496). All protocols used in these studies have been approved and reviewed by the Institutional Animal Care and use Committee of the University of Texas at Austin.

Treatment occurred at Stage 17, during the middle third of development—a window of time identified as the temperature-sensitive period—during which sex determination can be manipulated. Turtles hatch at stage 26. Eggs were treated in various concentrations of treatment compound dissolved in solvent, in this case, DMSO for organochlorines and ethanol for the estradiol treatment. Eggs were spotted in 5-μl dosages and returned to incubators for the duration of development (Crews and Bergeron, 1994). Control groups received a 5-μl dose of solvent alone (negative control) for single treatments. For treatments combining suspect EE and estradiol, a dose of 0.01 μg estradiol alone served as positive control. EE concentrations were based on those given by Heinz *et al.* (1991) (Table 1). Eggs were treated with (i) environmentally relevant concentrations, (ii) a combination of all chemicals at those concentrations, and (iii) each compound at the given concentration in concert with estradiol (0.01 μg/egg).

We analyzed sex ratio data using two-by-two contingency tables. All experimental groups (i.e., EEs with or without estradiol) were compared to the appropriate control groups (i.e., with or without estradiol) using Fisher's exact one-tailed tests. One-tailed tests were done because we expected a priori that the compounds

TABLE 1
Concentrations of Heinz *et al.* (1991) and Purity of Each Compound Used in the Sex Reversal Assay

Compound	Concentration (μM)	% Purity
Toxaphene	0.22	100
Dieldrin	0.63	100
trans-Nonachlor	0.25	99.5
Aroclor 1242	0.53	100
cis-Nonachlor	0.16	98.1
p,p′-DDD	2.6	98.6
p,p′-DDE	18	100
∂-Chlordane	0.22	98.9

Note. Compounds for the sex reversal assay were obtained from AccuStandard Inc. (New Haven, CT).

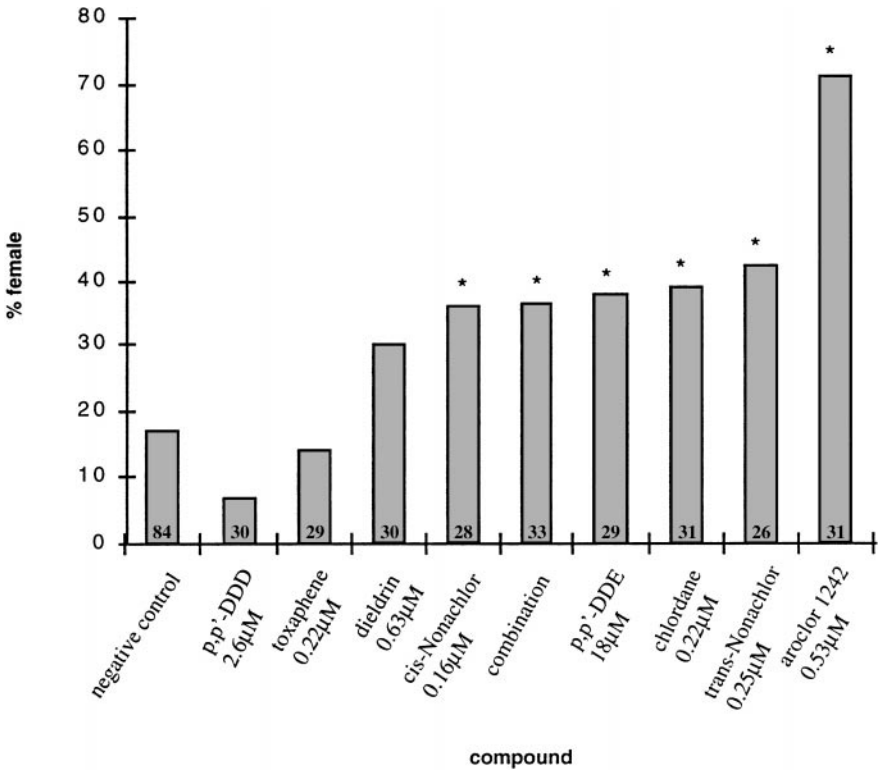

FIG. 1. Effect of xenobiotics on sex determination in the red-eared slider turtle, *Trachemys scripta elegans*, showing percentage of reversal from each treatment. All eggs incubated at 28.6°C. Percentage of reversal for negative (solvent only) control also shown. Numbers in bars indicate sample size. *Significant reversal versus negative control for compounds applied singly.

used in this experiment would be estrogenic (natural estrogens produce females at male-producing temperatures). Version 2 of JMP for Apple Macintosh was used for all statistical procedures (SAS Institute, 1989).

RESULTS

Of those compounds assayed singly, *trans*-Nonachlor, *cis*-Nonachlor, aroclor 1242, *p,p′*-DDE, and chlordane caused significant reversal beyond control (Fig. 1, Table 2), whereas chlordane caused significant reversal when applied in concert with estradiol (Fig. 2, Table 2). The most estrogenic compound acting alone was the PCB aroclor 1242 ($P < 0.001$), resulting in a higher rate of sex reversal than the estradiol control (71% vs 64%). However, aroclor 1242 did not result in a greater rate of reversal when combined with estradiol. Chlordane was the only compound that resulted in an increased rate of sex reversal when combined with estradiol (89% reversed vs 64% estradiol control; $P < 0.05$).

Three compounds had no effect singly in this assay. Toxaphene-treated eggs produced as many females as temperature alone (negative control), and its effects were no different from control in combination with estradiol ($P = 1.0$). Dieldrin and *p,p′*-DDD also had no significant effect when administered alone.

The combination of all compounds without estradiol resulted in significant reversal ($P < 0.05$), but showed no additive effects. Results of all the compounds together in combination with estradiol were not significantly above the estradiol control (80% vs 64%).

Positive and negative control results were consistent with results from previous assays with this species at this temperature (Crews *et al.*, 1996; D. Crews, unpublished data). This species normally resolves clearly as male or female; occasionally, an ovotestes arises or the presence of oviducts (usually regressed) is identified in males. No group had any animals presenting ovotes-

TABLE 2
Showing Level of Significance of Each Treatment versus Negative (Vehicle Only) and Positive (Estradiol Treatment) Controls

	Versus negative control (vehicle only)	Versus positive control (0.01 μg E2)
Vehicle only	—	<0.001
E2 only	<0.001	—
Aroclor 1242	<0.001	NS
Aroclor 1242 + E2	<0.001	NS
cis-Nonachlor	<0.05	NS
cis-Nonachlor + E2	<0.001	NS
Chlordane	<0.05	<0.05
Chlordane + E2	<0.001	<0.05
p,p′-DDD	NS	<0.001
p,p′-DDD + E2	<0.001	NS
p,p′-DDE	<0.05	<0.05
p,p′-DDE + E2	<0.001	NS
Dieldrin	NS	<0.01
Dieldrin + E2	<0.001	NS
Toxaphene	NS	<0.001
Toxaphene + E2	NS	NS
trans-Nonachlor	<0.01	NS
trans-Nonchlor + E2	<0.001	NS
All in combination	<0.05	<0.05
All in combination + E2	<0.001	NS

tes. Three groups had a single male with regressed oviducts: the negative control, the chlordane-treated group, and the group treated with a combination of all the compounds.

DISCUSSION

Studies of the impact of the compounds tested have revealed widespread deleterious effects. *p,p′*-DDE has been hypothesized to be responsible for the reduction in phallus size in male alligators found in Lake Apopka, Florida (Guillette *et al.*, 1994). Recent studies suggest that *p,p′*-DDE or a combination of compounds may be responsible for abnormal phallus and other reproductive abnormalities observed in the alligators (Guillette *et al.*, 1996). DDT and *p,p′*-DDE have greatly affected the reproductive success of several bird species, especially those that consume fish from contaminated lakes. For example, the Forster's tern, which eats fish from Green Bay in Lake Michigan, has experienced severely reduced reproductive success, a reduction attributed to the organochlorine bioaccumulation in the fish (Kubiak *et al.*, 1989). The compounds also cause eggshells of some species to thin, increasing mortality (Eroschenko *et al.*, 1981). Gulls appear to be especially susceptible to eggshell thinning, population declines, and skewed sex ratios (Fry, 1995).

As mentioned before, aquatic reptiles are not immune to EE effects. Lake Apopka, Florida, the site of a pesticide spill and extensive agricultural pesticide pollution, has been a staging ground for examining the effects of environmental compounds on aquatic reptiles. Both the American alligator and the red-belly slider turtle exhibited signs of feminization deleterious to the populations (Guillette *et al.*, 1994, 1996). The suspected cause of feminization was the pesticide DDT and its metabolites. Heinz *et al.* (1991), found DDT's metabolites as well as other pesticides and the PCB aroclor contaminating the lake and also the eggs of the American alligator. Guillette has since demonstrated that *trans*-Nonachlor, dicofol, and *p,p′*-DDD are estrogenic in the American alligator, causing sex reversal from male to female, but that *p,p′*-DDE does not have this effect (personal communication). However, other evidence indicates that *p,p′*-DDE can be estrogenic in this alligator species (Matter *et al.*, 1998). Interestingly, the estrogenic activity of these compounds may be species or genus specific: Clark *et al.* (1998) found that in the Tiger salamander (*Ambystoma tigrinum*), *p,p′*-DDE acts as an estrogen, but DDT behaves like an antiestrogen. Podreka *et al.* (1998), after administering *p,p′*-DDE to the eggs of the green sea turtle *Chelonia mydas*, found no changes in sex ratios, hatchling size, or hatchling weight.

Prior to the present research, an unequivocal *in vivo* assay has not been performed to demonstrate a direct connection between the compounds detected by Heinz and the organismal effect of sex reversal. Vonier *et al.* (1996) showed that these compounds in the concentrations identified by Hienz *et al.* (1991) reduced estradiol binding to alligator ER by 57% in a competitive binding assay. All but three of the compounds applied in the present assay were estrogenic when administered alone, but differed greatly in their results when applied in combination with estradiol. We conclude that acting separately, aroclor 1242, chlordane, *cis*- and *trans*-Nonachlor, and *p,p′*-DDE all have estrogenic effects and are capable of reversing sex significantly beyond controls in this system. Yet none of the EE/

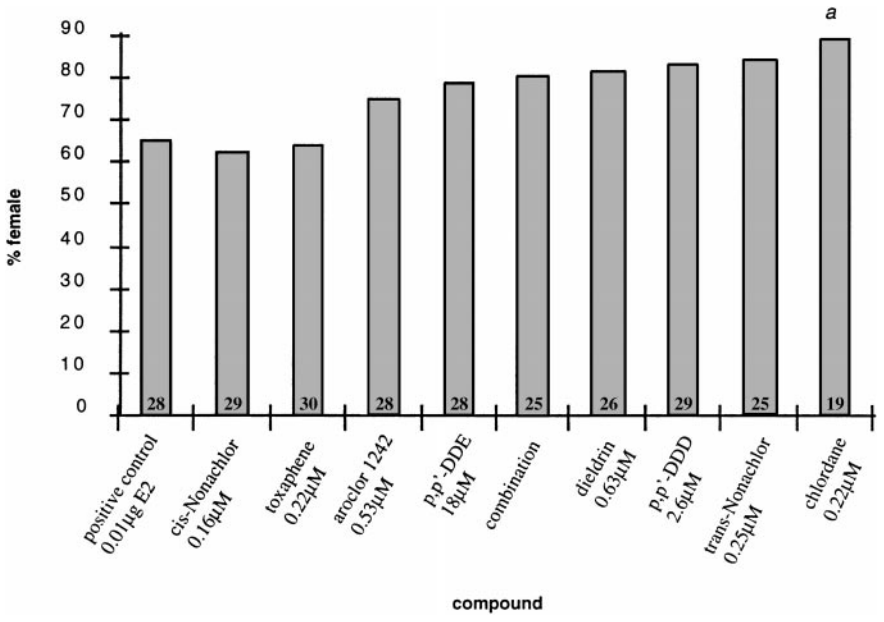

FIG. 2. Effect of certain xenobiotics in combination with estradiol on sex determination in the red-eared slider turtle, *Trachemys scripta elegans*, showing percentage of reversal from each treatment. All eggs incubated at 28.6°C. Compounds were assayed in combination with 0.01 µg estradiol. Percentage reversal for E2 control also shown. Numbers in bars indicate sample size. [a]Significant reversal versus positive control for compounds applied in combination with estradiol.

estradiol combinations resulted in 100% reversal, which would be expected if the effects were additive.

Only one compound, chlordane, showed significant reversal in combination with estradiol (Table 2). *trans*-Nonachlor, *cis*-Nonachlor, and *p,p′*-DDE all gave similar results when administered alone, but showed no significant effects when acting in concert with estradiol. The compounds applied together in the absence of estradiol resulted in a reversal significantly beyond control, but were in no way additive (Table 2). All the compounds together with estradiol did not cause significant reversal. Many explanations are possible for this lack of additivity. The interaction of the compounds is a complicated one, as demonstrated by the alligator ER studies (Vonier *et al.,* 1996). These studies showed that chlordane, toxaphene, and dieldrin had no ability to compete with ER; however, when the three compounds were added to a mix of *p,p′*-DDE, *p,p′*-DDD, *trans*-Nonachlor, *cis*-Nonachlor, and aroclor, they increased the ability of that mix to compete with ER by 14%.

The reason for these complications may lie in the interaction of one or two compounds, or all of them simultaneously. For example, Kelce *et al.* (1995) found that *p,p′*-DDE is a potent inhibitor of the mammalian androgen receptor (AR). Thus, *p,p′*-DDE may have its feminizing effects by inhibiting masculinization. Chlordane showed no binding affinity for alligator ER (Vonier *et al.,* 1996), and to our knowledge, it has not been assayed for its binding capacity with AR. Yet it demonstrated the strongest effect in combination with estradiol, leading to the possibility that it is an AR antagonist.

Another possibility is that some apparent estrogen mimics may operate independently of ER binding and work via a signaling pathway involving cell membrane receptors. Some of the compounds that have not demonstrated ER binding may in fact be operating by this alternate pathway. ER can be activated independently of direct ligand binding (Bunone *et al.,* 1996; Curtis *et al.,* 1996). Research has demonstrated that ER activation can take place via the binding of epidermal growth factor (EGF) to its cell membrane receptor (Bunone *et al.,* 1996; Curtis *et al.,* 1996). This binding triggers a signaling cascade involving the MAP kinase pathway and phosphorylation of ER. It is possible that rather than being mimics of ER ligands, some of these

compounds instead are EGF mimics and affect ER via a different route.

Many questions remain to be answered about possible species differences in sensitivity and the long-term effects of environmental estrogens. Studies examining the long-term effects of such compounds have yet to be performed, even though research on the effects diethylstilbestrol in mammals indicates that *in utero* exposure to this powerful synthetic estrogen hampers adult fertility in exposed offspring (McLachlan, 1977; McLachlan *et al.*, 1982; and see Golden *et al.*, 1998, for review).

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