PCBs as Environmental Estrogens: Turtle Sex Determination as a Biomarker of Environmental Contamination

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Polychlorinated biphenyls (PCBs) are widespread, low-level environmental pollutants associated with adverse health effects such as immune suppression and teratogenicity. There is increasing evidence that some PCB compounds are capable of disrupting reproductive and endocrine function in fish, birds, and mammals, including humans, particularly during development. Research on the mechanism through which these compounds act to alter reproductive function indicates estrogenic activity, whereby the compounds may be altering sexual differentiation. Here we demonstrate the estrogenic effect of some PCBs by reversing gonadal sex in a reptile species that exhibits temperature-dependent sex determination. *Key words*: biomarker, environmental estrogens, gonadal sex, PCBs, sexual differentiation, temperature-dependent sex determination. *Environ Health Perspect* 102:780–781 (1994)

Many polychlorinated biphenyls (PCBs) are industrialized chemicals such as those used in adhesives, fire retardants, and waxes (*1*). As a function of physical and chemical properties such as lipid solubility and low rate of degradation, PCBs persist in the environment; thus, individuals in industrialized nations are exposed to high levels of these compounds (*2,3*). Because chemicals of this nature tend to bioaccumulate, for instance, within food chains, they eventually reach measurable levels in human tissues or in nutrient systems such as placental cord blood, breast milk, and egg yolks. This may result in an individual being exposed to high levels during development, a time when toxic effects may be more detrimental than those seen in adults (*3,4*).

Reproductive disorders resulting from exposure to xenobiotic estrogens may include reductions in fertility, lower hatch rates in fish and birds, and decreased viability of offspring, as well as alterations in hormone levels or adult sexual behaviors, all of which have further implications, particularly in wildlife population dynamics (*3–5*). In addition, there is increasing suspicion that effects of estrogenic compounds are correlated to disorders of the male reproductive system, including increased occurrence of prostatic and testicular cancers (*6,7*). There is a need for a sensitive bioassay with which the developmental effects of environmental estrogens can be determined. Reptiles with temperature-dependent sex determination (TSD), in which the incubation temperature of the egg determines the sex of the individual, may provide such a bioassay. Evidence of PCBs acting as estrogens in a TSD species may contribute to a clearer understanding of how estrogens may lead to reproductive dysfunction.

The red-eared slider turtle, *Trachemys scripta*, is a species that possesses a TSD pattern in which warm incubation temperatures, (e.g., 31°C) produce all female hatchlings, whereas cooler incubation temperatures (e.g., 26°C) produce all male hatchlings; intermediate temperatures (between 29.0 and 30.0°C) result in varying ratios of males and females (*8,9*). In reptiles with TSD, the actions of estrogen mimic those of temperature with regard to gonadal differentiation (*10,11*). In the red-eared slider, as in many other TSD species, exogenous estrogens applied to the eggshell during the period of sexual differentiation can counteract the effects of male-producing temperatures and induce ovarian development (*12–15*).

Materials and Methods

Eggs for these experiments were purchased from Robert Kliebert (Hammond, Louisiana) and incubated on a layer of vermiculite: water (1:1) in temperature-controlled chambers (27.8 or 26°C). We monitored embryonic development by candling eggs during early stages, then by dissecting a small sample of eggs approximately twice a week to determine specific developmental stages. At the beginning of the period of gonadal differentiation (stage 17: approximately 4 weeks from the date eggs are laid), which coincides with the developmental stage at which the embryos are sensitive to the effects of exogenous estradiol (*10,11*), eggs were randomly assigned to treatment groups and spotted with either PCB compounds (Ultra Scientific, Hope, Rhode Island) in 95% ethanol, estradiol-17ß (Sigma, St. Louis, Missouri) in ethanol as a positive (hormone) control, or with ethanol alone as a negative (temperature) control. Incubation was continued at the experimental temperatures until hatch (approximately 7 more weeks at this temperature), at which time we dissected the hatchlings to determine resulting sex ratios. We determined gonadal sex and status of genital ducts by visualization under a dissection microscope and verified sex histologically, as described previously for this species (*8,9*). In all cases that the gonad was female, the oviducts or Müllerian ducts were present. In some cases in which testes developed, the Müllerian ducts were also present, indicating estrogenic activity.

Results and Discussion

In the first experiment, 11 different PCB compounds believed to be estrogenic (*16*) were applied individually to eggs incubated at 27.8°C. As shown in Table 1, each compound was administered at 2 doses, 15 eggs per dose. The eggs average 11.4 g (±0.21) each. Comparison of the resulting sex ratios showed that two compounds, 2',4',6'-trichloro-4-biphenylol (compound F) and 2',3',4',5'-tetrachloro-4-biphenylol (compound G), significantly reversed sex at a male-producing temperature (Fisher's Exact Test, $p < 0.001$). The former compound showed 100% sex reversal at 100 µg, or just below 9 ppm. In tests using mouse tissue, these same two compounds showed an appreciable affinity for estrogen receptor, due in part to their conformational properties as hydroxybiphenyls (*16,17*). As metabolites of other PCBs, hydroxylated PCBs such as F and G may exist in steady-state concentrations in aquatic environments, potentially exposing wildlife to their effects via direct contact or through the food chain (*17*).

Because purified PCB compounds are rarely found in the environment, we decided in the second series of experiments to look at combinations of the same PCBs. All eggs were incubated at 27.8°C and received a low (10 µg), medium (100 mg), or high (145–190 µg) dose of compounds in ethanol. Some eggs received a cocktail of all PCBs except the two that caused sex reversal (F and G). Others were exposed to combined hydroxybiphenyls, excluding F and G. Lastly, some eggs were spotted with combined nonhydroxylated PCBs. In all three conditions, there was no evidence of sex reversal.

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Table 1. Effects of some PCB compounds on sex determination[a]

Compound	% Hatchlings with female gonads (low dose/high dose)	% Hatchlings with oviducts (low dose/high dose)
A 2',5',-dichloro-3-biphenylol	0/0	0/14
B 2,2',4-trichlorodiphenyl ether	7/0	21/0
C 2,2',5'-trichloro-4-biphenylol	0/0	7/0
D 2,3,4'-trichlorobiphenyl	7/0	0/0
E 2,3',5-trichlorobiphenyl	0/8	7/8
F 2',4',6'-trichloro-4-biphenylol	0/100	0/100
G 2',3',4',5'-tetrachloro-4-biphenylol	4/50	8/71
H 2,4,4',5-tetrachlorodiphenyl ether	0/0	0/0
J 2,4,4',6-tetrachlorobiphenyl	7/0	0/7
K 2,4,4'',6-tetrachloro-*p*-terphenyl	0/0	0/7
L 2',3',4',5,5'-pentachloro-2-biphenylol	0/0	0/0
Ethanol control	0	0
Estradiol-17ß control	100	100

[a]Eleven compounds were applied to eggs incubated at 27.8°C in two doses per compound. The doses were: A, B, C, F, G, H, J = 10 µg, 100 µg; D, L = 5 µg, 50 µg; E = 25 µg, 250 µg; K = 3.35 µg, 33.5 µg. The estradiol control consisted of 10 µg estradiol-17ß.

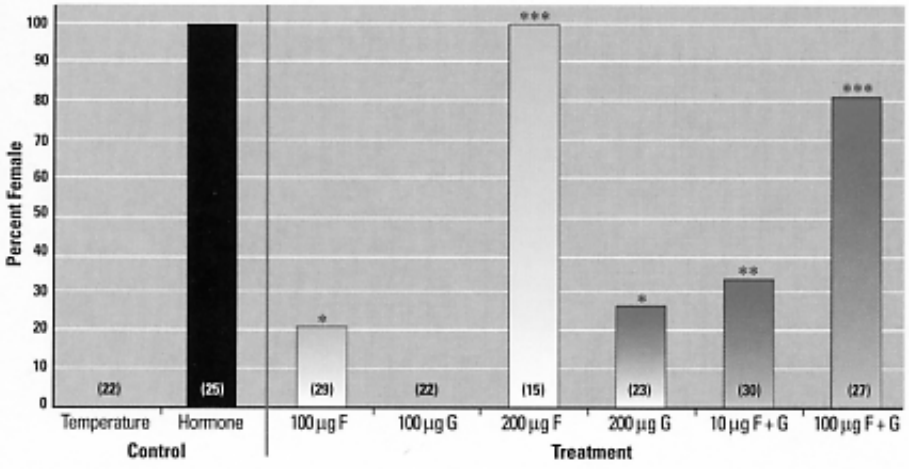

Figure 1. Percentage of female hatchlings at a temperature that normally produces 100% males (26°C) after treatment with two estrogenic PCB compounds in ethanol. Controls: temperature, ethanol alone; hormone, 10 µg estradiol-17ß. PCB compounds: F, 2',4',6'-trichloro-4-biphenylol; G, 2',3',4',5'-tetrachloro-4-biphenylol. Sample sizes in parentheses. Significant sex reversal is indicated by $*p \leq 0.03$; $**p = 0.003$; $***p = 0.0001$.

Because we knew compounds F and G showed estrogenic activity at the slightly higher temperature, we decided to test these two compounds at a temperature that produces 100% males (26°C). Both compounds showed significant sex reversal at this temperature (Fig. 1). When combined, F and G synergized, resulting in a significant increase in ovarian development at a dose of 10 µg, or less than 1 ppm (Fisher's Exact Test, $p < 0.01$), whereas F alone and G alone required at least a 10-fold higher dose to show sex reversal. Exogenous estradiol-17ß produces similar results at a dose of 0.5 µg, or .04 ppm (*9*). As with the earlier experiments involving compounds F and G, whenever the gonad was feminized, the oviducts were retained, and in some cases where testes developed, there were also Müllerian ducts present.

The nature of TSD in this and other reptile species provides a useful system in which to assay the extent of estrogenic activity found in xenobiotic compounds. This report contributes laboratory evidence of the effect of PCBs on sex determination, emphasizing the usefulness of a TSD species as a biomarker to assess environmental contamination and serve as a warning of conditions threatening wildlife populations. The PCB levels reported here as effective in disrupting normal gonadal differentiation in the turtles are comparable to average levels of PCBs found in human breast milk in industrialized nations (*3*). In addition, this study supports the contention that environmental estrogens, through their action on reproductive development, have the potential to alter wildlife populations as well as contribute to reproductive dysfunction in humans (*3–7*). Future studies investigating the mechanisms through which these estrogenic compounds act to affect sex differentiation will continue to shed light on a human contribution to the environment.

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Estrogenicity of Environmental PCBs

The paper by Bergeron, Crews, and McLachlan, "PCBs as Environmental Estrogens: Turtle Sex Determination as a Biomarker of Environmental Contamination" (*EHP* 102:780–781) presents data on the estrogenic activity of 11 chlorinated biphenyls or diphenyl ethers, or hydroxylated derivatives thereof, selected so as to represent a variety of structural types. Some of the PCB types examined (e.g., compounds A, C, D, E, and J) arise from PCB congeners actually detectable in the commercial Aroclors (*1*) and hence also in environmental samples, whereas other PCB types (e.g., those with heavily uneven chlorination of the two rings, as in compounds F,G, and L) arise from PCB congeners that are not detectable in either the Aroclors themselves (*1*) or even environmentally transformed PCB compositions (*2*). It is noteworthy that the only compounds found to have statistically significant estrogenic activity (compounds F and G) both represented 4-hydroxylation products of PCB congeners belonging to the nonenvironmental group, whereas the five compounds representative of PCB structures actually present in the environment were all negative. In short, the results present zero evidence that environmental PCBs present risk of estrogenic activity.

This is hardly what the authors claim, however. In their discussion they state (p. 781):

> This report contributes laboratory evidence of the effect of PCBs on sex determination . . . and serve as a warning of conditions threatening wildlife populations. The PCB levels reported here as effective in disrupting normal gonadal differentiation in the turtles are comparable to average levels of PCBs found in human breast milk in industrialized nations.

This most misleading statement represents a false alarm that can only impede the search for the environmental contaminants that actually do present estrogenic risk.

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Response to Hamilton

There are several points we would like to address in our response. First of all, the compounds studied were chosen because these particular congeners were believed to be estrogenic based on their conformational structure. The primary reason for conducting this type of study was to show the effectiveness of a temperature-dependent sex determined (TSD) species as a tool in assessing estrogenic activity *in vivo*. This point is furthered by an earlier report in *EHP* by Guillette et al. (*1*). While the PCB compounds we used may not be primary components of commerically available PCB mixtures, there are parallels between these compounds and other PCB congeners in the pattern of *ortho*-chlorine substitutions, as Korach et al. (*2*) indicate. It is important to study how these structures affect a developing organism in order to understand how PCBs can act as estrogens. Though these particular congeners may not be currently used in the readily available mixtures, McKinney et al. (*3*) make reference to goals of using PCBs that are easily detoxified via hydroxylation. If such considerations lead to composition of commercially available PCB mixtures away from congeners that exhibit a dioxinlike toxicity, these considerations should include assessment of the estrogenic activity of these compounds. The TSD system can be used to test such mixtures *in vivo*. Furthermore, the appearance of hydroxylated PCB congeners in "nature" is an emerging issue: for example, Bergman et al. (*4*) report that the hydroxylated forms of heavily chlorinated (penta- or heptachlorinated) biphenyls were among the most retentive forms of PCBs found in blood from humans or seals. Clearly, this class of molecules may have environmental significance which is only now being appreciated.

The second point that we would like to emphasize regards the potential for second-generation exposure to PCBs as environmental estrogens. While the congeners that we found to clearly exhibit estrogenic activity may not be produced in the commercial PCB mixtures, or even found in soil and water samples, they may exist within animal tissue during metabolism of the environmental compounds. Maternal metabolic by-products may affect the reproductive system at a critical stage in development of the offspring, producing the detrimental estrogenic effect on the second generation. This is particularly a concern when enhanced estrogenic effects are produced by the synergy of different combinations of low-level PCBs, an issue brought to light by the study in question.

Finally, we share Dr. Hamilton's concern that false alarms may impede identification of contaminants that actually present risk of estrogenic activity. However, Dr. Hamilton's cited quotation of our discussion, and his interpretation of it, require clarification so as not to be misleading. Dr. Hamilton's abbreviated quote would lead readers to believe that it is our report which we say serves as a warning of threatening environmental conditions. In fact, the passage he omitted clearly identifies "the usefulness of a TSD species as a biomarker" to serve in the capacity which Dr. Hamilton apparently ascribes to our report.

Our findings support the call from the scientific community for the need to further study the mechanisms of estrogenic activity of environmental contaminants. These findings, together with the growing body of evidence that a number of environmental compounds mimic estrogens and do have an effect on developing reproductive systems, provide ample indication for further investigation of these mechanisms and their outcomes. Our report suggests a model by which to continue these efforts, and we appreciate the continued interest in our work and the opportunity to address questions regarding it.

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