The Role of Estrogen in Turtle Sex Determination and the Effect of PCBs

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In the current model of vertebrate sex determination and sexual differentiation, gonadal sex is fixed at fertilization by specific chromosomes, a process known as genotypic sex determination (GSD). Only after the gonad is formed do hormones begin to exert an influence that modifies specific structures that eventually will differ between the sexes. Many egg-laying reptiles do not exhibit GSD but rather depend on the temperature of the incubating egg to determine the gonadal sex of the offspring, a process termed temperature-dependent sex determination (TSD). Research on TSD indicates that sex determination in these species is fundamentally different in at least one way. Gonadal sex is not irrevocably set by the genetic composition inherited at fertilization but depends ultimately on which genes encoding for steroidogenic enzymes and hormone receptors are activated during the midtrimester of embryonic development by temperature. Incubation temperature modifies the activity as well as the temporal and spatial sequence of enzymes and hormone receptors such that sex-specific hormone milieus, created in the urogenital system of the developing embryo, determine gonad type. Estrogen is the physiologic equivalent of incubation temperature and the proximate cue that initiates female sex determination. There is increasing evidence that some polychlorinated biphenyl (PCB) compounds are capable of disrupting reproductive and endocrine function in fish, birds, and mammals, including humans. Reproductive disorders resulting from exposure to these xenobiotic compounds may include reductions in fertility, hatch rate in fish and birds, and 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. Research on the mechanism through which these compounds may be acting to alter reproductive function indicates estrogenic activity, by which the compounds may be altering sexual differentiation. In TSD turtles, the estrogenic effect of some PCBs reverses gonadal sex in individuals incubating at an otherwise male-producing temperature. Furthermore, certain PCBs are synergistic in their effect at very low concentrations. — Environ Health Perspect 103(Suppl 7):73–77 (1995)

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Introduction

In many reptiles, gonadal sex is determined by the incubation temperature of the egg, a process known as temperature-dependent sex determination (TSD) (*1,2*). Reptiles with TSD exhibit various relationships between temperature and sex ratio (Figure 1). Low temperatures produce females and high temperatures produce males in many lizards and crocodilians, whereas this pattern is reversed in most turtles. A mixture of these patterns is evident in the leopard gecko, the snapping turtle, and in crocodiles, wherein extreme incubation temperatures produce females and intermediate temperatures produce varying ratios of males and females.

Reptiles with TSD do not have heteromorphic sex chromosomes and have little or no genetic predisposition to respond to temperature in particular ways. In TSD each individual has the equal ability to become either a male or a female, and incubation temperature serves as the trigger to initiate the cascade that leads to the development of ovaries or testes. The window of sensitivity to temperature is restricted to the midtrimester of development.

It is remarkable, and convenient, that the temperature differential that results in all-male and all-female offspring is restricted to a few degrees Celsius. Further, the fact that an individual is either a gonadal male or a gonadal female indicates an all-or-none effect of temperature. At intermediate incubation temperatures intersexes are rarely formed; rather, the sex ratio varies. The effect of incubation temperature on sex determination is not due to differential mortality. Further, the sex at hatching is permanent, extending to adulthood.

The Role of Steroid Hormones in Temperature-dependent Sex Determination in Turtles

How is the physical stimulus of temperature transduced into a biological stimulus that acts ultimately on a molecular switch to determine sex? The red-eared slider (*Trachemys scripta*) has been used in this research. In this species an incubation temperature of 26°C produces all males, 32°C produces all females, and 29.2°C, the threshold temperature, produces a 1:1 sex ratio. The incubation temperatures that produce intermediate sex ratios span 1°C (*3*). Experiments indicate that the initial sex-specific changes in the gonads are reversible, but this plasticitiy ends at the sexual commitment of the gonads.

In 1987 at the University of Texas at Austin, we began working on the possibility that incubation temperature determines sex by modulating the nature, quantity, and activity of steroidogenic enzymes in tissues proximal to the genital ridge, thereby acting on steroid precursors to result in localized temperature-specific hormonal milieus (*4*). More recently, this

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Abbreviations used: GSD, genotypic sex determination; TSD, temperature-dependent sex determination; DHT, dihydrotestosterone; E_2, estradiol; T, testosterone; SRY, sex-determining region of Y chromosome.

model has been extended to include temperature regulation of the quantity of different steroid hormone receptors and steroidogenic enzymes and the genes encoding these products, positing that, acting together, enzymes and receptors guide the differentiation of the embryonic gonad to determine sex (*3*). Some of the evidence supporting the hypothesis that TSD female and male sex determination are separate and independently controlled processes that are regulated by steroid hormones is as follows [citations to specific papers are contained in Crews et al. (*3*)]: *a*) hormone sensitivity overlaps with temperature sensitivity; *b*) steroid hormones are concentrated in the mesonephros, adrenals, and the gonads; *c*) there is a synergism between hormones and incubation temperature; *d*) estrogen induces female sex determination, whereas nonaromatizable androgens induce male sex determination; *e*) at the threshold temperature, treatment with dihydrotestosterone (DHT) in combination with estradiol (E_2) causes the formation of ovotestes; *f*) when treated with aromatase inhibitor, eggs incubated at a female-producing temperature produce male hatchlings, whereas eggs incubated at a male-biased temperature produce female hatchlings when treated with 5α-reductase inhibitor; and *g*) incubation temperature modulates the genes regulating estrogen and androgen receptor expression.

Two Models for the Mechanism of Action of Incubation Temperature

It is clear that, in TSD, male and female sex determination are separate processes differentially affected by incubation temperature. This is conceptually different from the organization/default model applied to species having genotypic sex determination (*5*). That is, in TSD organisms females presumably result from the activation of an ovary-determining cascade and simultaneous inhibition of a testis-determining cascade; similarly, males presumably result from the activation of a testis-determining cascade and simultaneous inhibition of an ovary-determining cascade.

Any model for the mechanism of action of incubation temperature must account for the facts presented above to be satisfactory. Two different models (but not necessarily mutually exclusive or totally inclusive) have been put forward to account for how incubation temperature determines sex in TSD reptiles (Figure 2)(*3,6,7*). Central to both models is the fact that testosterone (T) serves as a precursor molecule destined for conversion to DHT (via reductase) or E_2 (via aromatase). Similarly, in both models incubation temperature is hypothesized to activate steroid

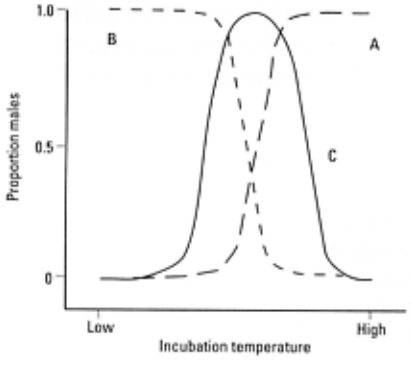

Figure 1. Response of hatchling sex ratio to incubation temperature in various egg-laying reptiles. These graphs represent only the approximate pattern of the response and are not drawn according to any single species. The three patterns recognized presently are (A) only females produced from low incubation temperatures and males at high temperatures, (B) only males produced from low incubation temperatures and females at high temperatures, and (C) only females produced at the temperature extremes, with male production at the intermediate incubation temperatures. Genotypic sex determination also occurs in reptiles with the result that the hatchling sex ratio is fixed at 1:1 despite incubation conditions. Data from Crews (*6*).

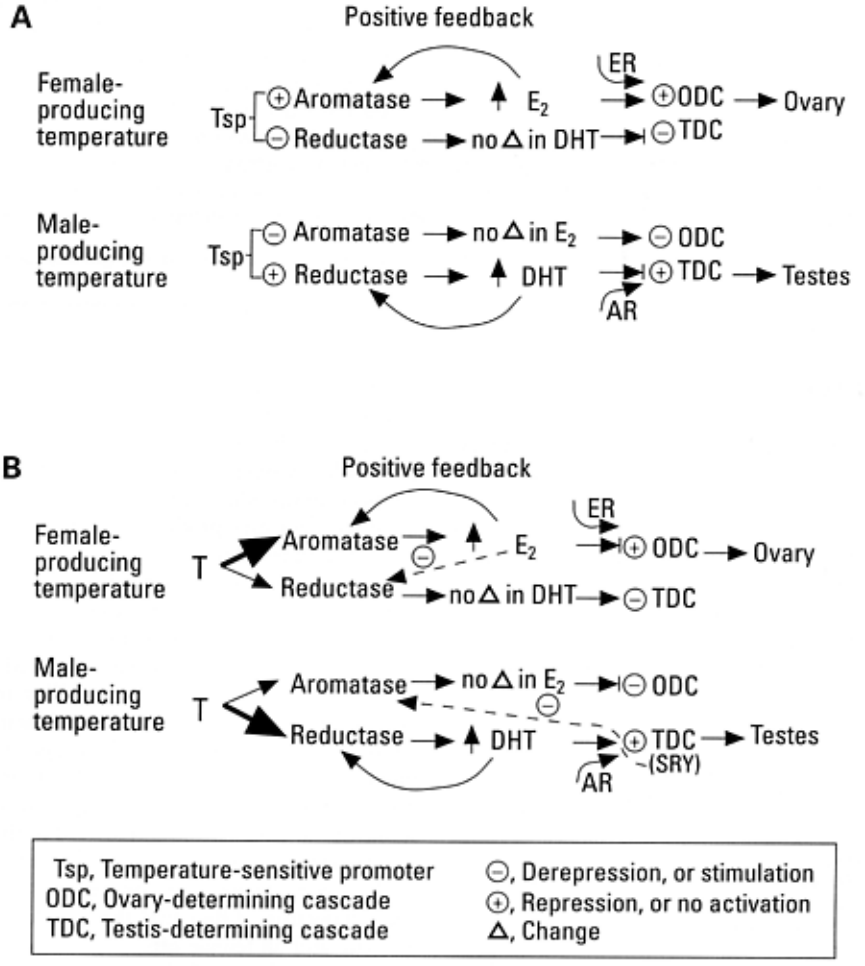

Figure 2. Two models of the mechanism of action of incubation temperature in temperature-dependent sex determination. In both, the temperature experienced during the middle third of incubation initiates the cellular and molecular cascades that result in male or female offspring. In addition to the effects on genes encoding for steroidogenic enzymes, incubation temperature is hypothesized to activate genes encoding for steroid hormone receptors (e.g., male-producing temperature upregulating androgen receptor and female-producing temperature upregulating estrogen receptor). (*A*) Depicts a direct action of incubation temperature on temperature-sensitive promoters on genes encoding for steroidogenic enzymes. (*B*) Depicts an indirect action of incubation temperature. Data from Crews et al. (*3*).

hormone receptor genes [e.g., male-producing incubation temperature upregulates androgen receptor (AR) and female-producing incubation temperature upregulating estrogen receptor (ER)].

The direct model of steroid hormone-mediated sex determination (Figure 2A) hypothesizes that temperature-sensitive promoters are associated with the genes encoding steroidogenic enzymes. At a female-producing temperature, it is possible that the aromatase gene(s) is activated and the reductase gene(s) is inhibited. Similarly, at a male-producing temperature, the gene(s) encoding reductase is activated and the gene(s) encoding aromatase is inhibited. Consequent effects on the concentrations of steroid hormone feed back to increase the amount and activity of steroidogenic enzymes at the level of the urogenital sinus, thereby creating temperature-specific hormonal milieus. For example, a positive feedback exists between E_2 and aromatase at a female-producing temperature in the pond turtle (*8*). It is as yet unproven that a similar feedback relationship may exist between DHT and reductase. In the rat, DHT increases the quantity of reductase mRNA levels (*9*). However, the lack of a dose response of DHT and reductase inhibitor and the absence of a synergism between incubation temperature and DHT-sensitivity suggest that the feedback may be negative in nature. In the hamster, androgens exert a negative control on reductase activity and mRNA levels (*10*). The resulting hormonal milieu would lead to DHT or E_2 binding to specific, high-affinity nuclear receptor proteins (AR and ER, respectively) and, in turn, would bind to the DNA hormone response elements. The consequence would be a stimulation of the transcription of genes associated with the sex-determining cascade of one sex and an inhibition of the expression of genes associated with the sex-determining cascade of the opposite sex.

The indirect model of steroid hormone-mediated sex determination (Figure 2B) does not involve temperature-sensitive promoters but rather feedback control of product formation and hence activation of sex determining cascades. As in the direct model, a positive feedback is hypothesized to exist between aromatase and E_2 whereas reductase is constitutively expressed. At a female-producing temperature, androgen precursor is preferentially metabolized to E_2, thereby causing greater conversion of precursor; E_2 would also inhibit reductase activity. At a male-producing temperature, aromatase does not increase and little or no E_2 is formed. Instead the constitutive expression of reductase causes DHT formation, which in turn acts on AR directly to alter aromatase expression or acts to decrease ER, effectively initiating the testis-determining cascade. There may also exist a *SRY*-like (*SRY* = sex-determining region of the Y chromosome) gene that, when activated, feeds back to further inhibit aromatase expression. Thus, incubation temperature would act only indirectly via the testis-determining gene(s) having a negative feedback on the regulation of aromatase gene expression and leading to suppression of E_2. Support for this hypothesis comes from Haqq et al. (*11*) who demonstrated in the rat that *SRY* may control male development through regulation of aromatase and Müllerian inhibiting substance genes. The mechanism by which this biochemical switching system could operate has been modeled by Jackson (*12*).

The Effect of PCBs on Temperature-dependent Sex Determination

In temperate and tropical zones, reptiles commonly occur at the boundaries of human habitation or in areas of environmental catastrophes and hence could serve as an early warning signal of environmental contamination (*13*). This presents an opportunity to develop a nonmammalian laboratory model system for testing the developmental effects of xenobiotic compounds found in the environment.

There is abundant evidence that man-made products can become environmental toxicants (*14*). For example, some of the effects of toxicants such as polychlorinated biphenyls (PCBs) can have estrogenic effects (*15*) and can induce reproductive anomalies in adult female animals in nature (*14*). The fact that, in the red-eared slider, turtle sex determination can be influenced by minute amounts of exogenous estrogenic ligands provides a sensitive indicator of potential contamination by environmental estrogens. It became obvious that the all-or-nothing nature of the response of red-eared slider turtle embryos to exogenous estrogen could be used as a robust bioassaay for environmental estrogens. Accordingly, we assessed the ability of 11 common PCBs to reverse the effects of a male-producing temperature (*16*). Table 1 demonstrates that only two of the compounds tested, 2′,4′,6′-trichloro-4-biphenylol (F) and 2′,3′,4′,5′-tetrachloro-4-biphenylol (G), have estrogenic activity as indicated by the production of female hatchlings from eggs incubated at a male-producing temperature. In these instances only the high dosage produced females complete with fully developed oviducts. 2′,4′,6′-Trichloro-4-biphenylol showed 100% sex reversal at 100 µg, or just below 9 ppm. In tests using mouse tissue, these same two compounds show an appreciable affinity for ER, due in part to their conformational properties as hydroxybiphenyls (*15,17*). 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

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
E_2 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 at 10 and 100 µg; D and L at 5 and 50 µg; E at 25 and 250 µg; K at 3.35 and 33.5 µg. The E_2 control consisted of 10 µg E_2. Data from Bergeron et al. (*16*).

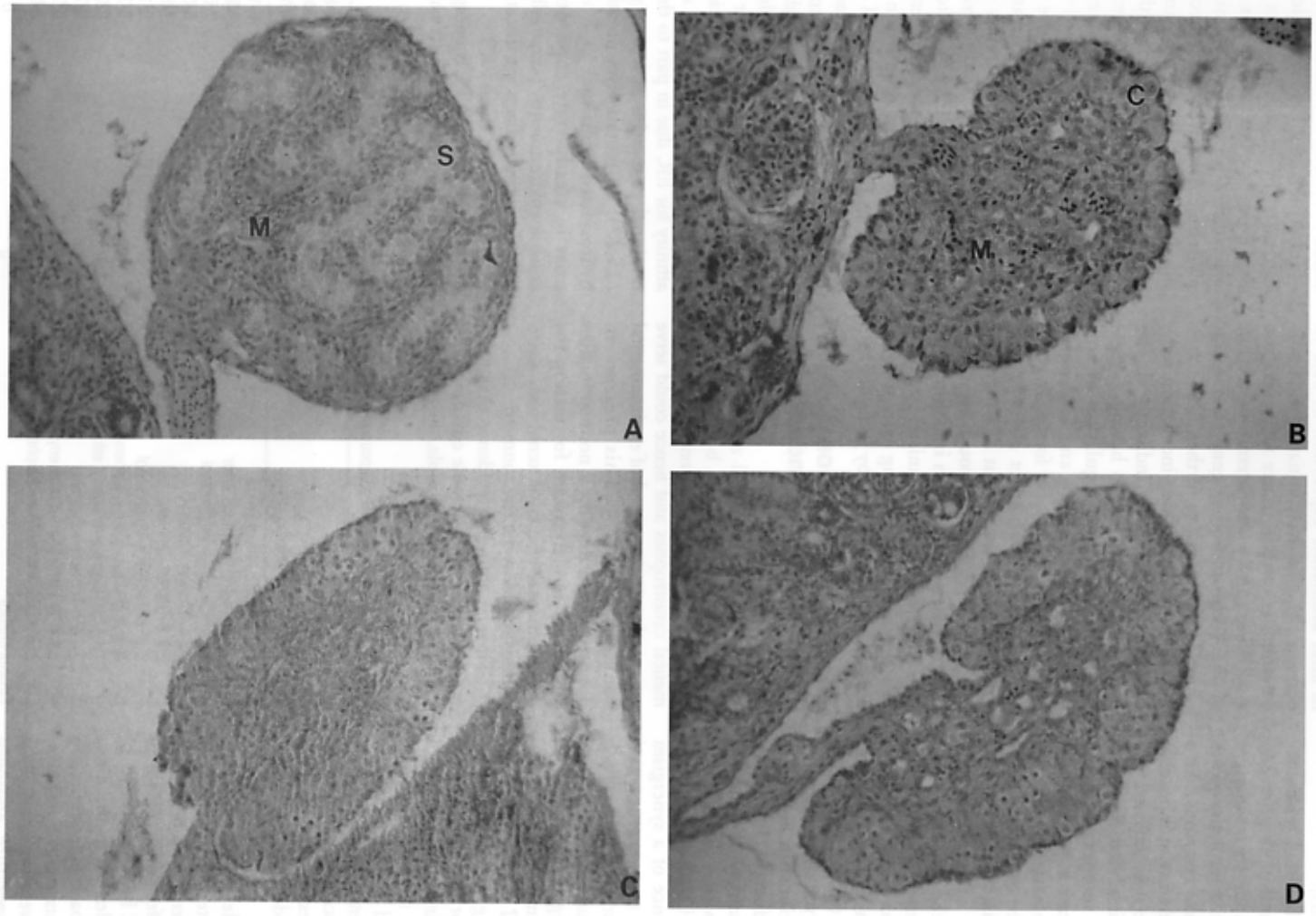

Figure 4. Histological sections of hatchling gonads from male-producing temperature. Note the ovaries possess a distinct cortical region C and a regressed medullary region M whereas the testis has a pronounced medullary region with seminiferous tubules S. (*A*) Temperature-determined testis (temperature control); (*B*) estrogen-determined ovary (hormone control); (*C*) PCB-determined ovary (200 µg 2′,4′,6′-trichloro-4-biphenylol); (*D*) PCB-determined ovary (100 µg compounds 2′,4′,6′-trichloro-4-biphenylol and 2′,3′,4′,5′-tetrachloro-4-biphenylol).

decided in the second series of experiments to look at combinations of the same PCBs (Figures 3 and 4). All eggs were incubated at 27.8° C and received a low (10 µg), medium (100 µg), or high (145–190 µg) dose of compounds. Some eggs received a cocktail of all PCBs except the two that caused sex reversal (F and G). Others were exposed to combined hydroxybiphenyls, again excluding F and G. Last, some eggs were treated with combined nonhydroxylated PCBs. In all three conditions, there was no evidence of sex reversal.

Since we knew compounds F and G showed estrogenic activity at the slightly higher temperature, we decided to try these two compounds at a temperature that produces 100% males (26° C). Both compounds showed significant sex reversal at this temperature. When combined, F and G synergized, which resulted in a significant increase in ovarian development at a dose of 10 µg, or less than 1 ppm; F alone and G alone required at least a 10-fold higher dose to show sex reversal. Exogenous E_2 produces similar results at a dose of 0.5 µg, or 0.04 ppm (*18*).

Such experiments demonstrate that it is possible to use the eggs of reptiles with TSD as biomarkers of environmental contamination. Indeed, recent studies with the alligator have involved assessment of the degree of actual xenobiotic contamination (via physical and biochemical methods) followed by the application of the amount of contamination to incubating eggs under controlled laboratory conditions; results of such studies are similar to those for the red-eared slider (*13*). Further, the mechanism of action of these endocrine mimics appears to involve the disruption of the normal steroid hormone milieau in the developing embryo that normally faciliate sex determination (*19*).

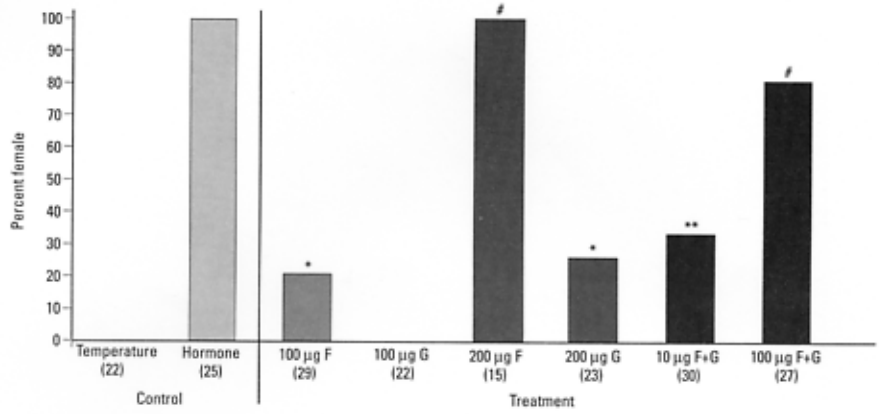

Figure 3. Effect of two estrogenic PCB compounds on sex ratio of the red-eared slider turtle. Eggs were incubated at a temperature that normally produces 100% males (26° C). Temperature control, ethanol alone; hormone control, 10 µg estradiol. PCB compounds included F, 2′,4′,6′-trichloro-4-biphenylol and G, 2′,3′,4′,5′-tetrachloro-4-biphenylol. Sample sizes are presented in parentheses. Significant sex reversal is indicated by $*p \leq 0.03$; $**p = 0.003$; $\#p = 0.0001$. Data from Bergeron et al. (*16*).

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