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Temperature-Dependent Sex Determination in Reptiles: Proximate Mechanisms, Ultimate Outcomes, and Practical Applications

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ABSTRACT In many egg-laying reptiles, the incubation temperature of the egg determines the sex of the offspring, a process known as temperature-dependent sex determination (TSD). In TSD sex determination is an "all or none" process and intersexes are rarely formed. How is the external signal of temperature transduced into a genetic signal that determines gonadal sex and channels sexual development? Studies with the red-eared slider turtle have focused on the physiological, biochemical, and molecular cascades initiated by the temperature signal. Both male and female development are active processes—rather than the organized/default system characteristic of vertebrates with genotypic sex determination—that require simultaneous activation and suppression of testis- and ovary-determining cascades for normal sex determination. It appears that temperature accomplishes this end by acting on genes encoding for steroidogenic enzymes and steroid hormone receptors and modifying the endocrine microenvironment in the embryo. The temperature experienced in development also has long-term functional outcomes in addition to sex determination. Research with the leopard gecko indicates that incubation temperature as well as steroid hormones serve as organizers in shaping the adult phenotype, with temperature modulating sex hormone action in sexual differentiation. Finally, practical applications of this research have emerged for the conservation and restoration of endangered egg-laying reptiles as well as the embryonic development of reptiles as biomarkers to monitor the estrogenic effects of common environmental contaminants.

Key words: Sex determination, sexual differentiation, reptiles, temperature-dependent sex determination, behavior, steroidogenic enzymes, aromatase, reductase, estrogen, androgen, steroid hormone receptors

INTRODUCTION

Much progress has been made in our understanding of sex determination and differentiation in a variety of organisms. Indeed, until about 25 years ago, it was assumed that all amniote vertebrates (mammals, birds, and reptiles) shared similar genotypic sex-determining (GSD) mechanisms. Then in 1966 Madeline Charnier discovered that in the egg-laying lizard *Agama agama* the incubation temperature of the egg determines the sex of the hatchling. This process of temperature-dependent sex determination (TSD) has now been demonstrated in many turtles, some lizards, and all crocodilians [Bull, 1980; Ewert and Nelson, 1991; Janzen and Paukstis, 1991].

In mammals, birds, and many other gonochoristic vertebrates (separate sexes in separate individuals), gonadal sex is determined at fertilization by specific chromosomes and results in a 1:1 sex ratio. In mammals, a gene on the Y chromosome (designated *Sry*) channels gonadal development to result in testes and the individual to develop a male-typical phenotype; the absence of this gene results in the formation of ovaries and a female-typical phenotype (Fig. 1, top). Thus, the *Sry* gene serves to switch the developmental cascade leading to maleness. However, *Sry* is part of a large family of genetic transcripts, and comparative studies in a variety of species have not found other *Sry*-like genes to be sex-linked in nonmammals [Tiersch *et al.*, 1991, 1992].

Unlike GSD, where it is not possible to deviate the primary sex ratio from unity [Beamer and Whitten, 1991], species with TSD do not inherit sex-specific chromosomes from their parents, and gonadal sex is

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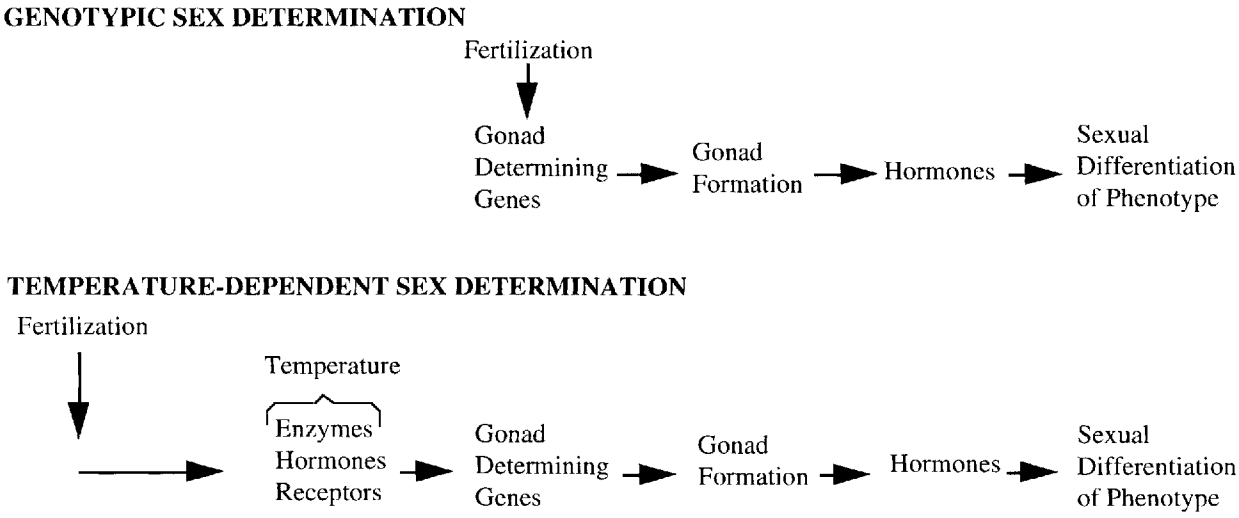


Fig. 1. In the current model of vertebrate sex determination and sexual differentiation (top), gonadal sex is fixed at fertilization by specific chromosomes, a process known as genotypic sex determination. Only after the gonad is formed do hormones begin to exert an influence, modifying specific structures that eventually will differ between the sexes. Research on reptiles with temperature-dependent sex determination indicates that sex determination in these species is fundamentally different in at least one way (bottom). Gonadal sex is not irrevocably set by the genetic composition inherited at fertilization, but rather depends ultimately on which genes encoding for steroidogenic enzymes and hormone receptor are activated during 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.

not determined at fertilization. In TSD each individual has equal potential to become male or female and there is little, or no, genetic predisposition for specific responses to incubation temperature. Rather, incubation temperature serves as a switch that initiates the cascade that leads to the development of testes and suppresses the cascade that leads to the development of ovaries (and vice versa) (Fig. 1, bottom).

Reptiles with TSD may offer model systems to better understand the events that comprise sex determination and sexual differentiation in amniote vertebrates. Mammals and birds share their most recent common ancestry with reptiles. It has been suggested that environmental sex determination may have been the precursor to GSD [Ohno, 1967; Janzen and Paukstis, 1991]. Before such promise can be realized, basic information both on how sex is determined as well as how sexuality develops in TSD species is required. Our purpose here is to summarize a set of studies conducted in this laboratory on two species, the red-eared slider and the leopard gecko. An obvious starting point is to determine how TSD is achieved. We consider the physiological, cellular, and molecular mechanisms of TSD. A second issue concerns the functional outcomes of TSD on characteristics other than gonad phenotype. We demonstrate how the temperature experienced during embryonic development modifies the morphology, physiology, and behavior of the adult. Finally, we provide new information on some practical applications of this research such as benefiting the conservation of *threatened* and *endangered* species and monitoring environmental contamination.

PROXIMATE MECHANISMS OF TEMPERATURE-DEPENDENT SEX DETERMINATION

How is the physical stimulus of temperature transduced into a physiological signal that ultimately acts on a molecular switch to determine sex? We have used the red-eared slider (*Trachemys scripta*) to address this question. In this species, an incubation temperature of 26°C produces all males, 31°C produces all females, and 29.2°C is the *threshold temperature* that produces approximately a 1:1 sex ratio (Fig. 2). The following four points have been established in this and other species:

1. Sex determination is sensitive to both the duration and magnitude of incubation temperature [Wibbels *et al.*, 1991a].
2. Sex determination remains labile to temperature changes through the early stages of gonadal differentiation and initial sex-specific changes in the gonads are reversible [Wibbels *et al.*, 1991a,b]. The temperature-sensitive period corresponds to the middle of embryogenesis (= sex determination period), beginning prior to the differentiation of the gonads into recognizable ovaries or testes but ending well before hatching [Wibbels *et al.*, 1991a].
3. Temperature exerts an "all-or-none" effect on the

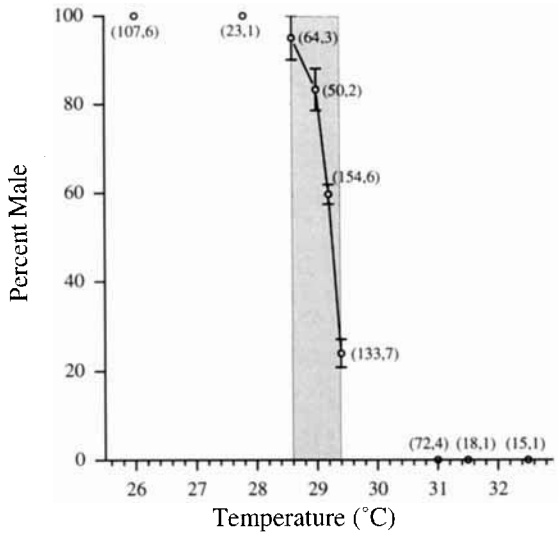


Fig. 2. Relationship between incubation temperature and sex ratio in the red-eared slider turtle. Depicted is the mean sex ratio obtained over a 4-year period. Total number of individuals followed by the number of replicate experiments are shown in parentheses; standard error indicated by vertical bar. Stipple indicates transitional temperature.

ovarian or testicular nature of the gonad; intersex individuals are rarely produced (<.001 incidence) [Crews *et al.*, 1991].

4. During the window of temperature sensitivity, the effect of temperature appears to be cumulative; the longer an embryo is maintained at a particular temperature, the more extreme the new temperature must be to override the effects of the original temperature [Wibbels *et al.*, 1991b].

Our studies explore one general model suggested originally by Claude Pieau: incubation temperature determines sex by altering the hormone environment of the sexually indifferent embryo, channeling development into a male or a female direction. Specifically, incubation temperature acts on the expression of genes encoding for steroidogenic enzymes and steroid hormone receptors, which together guide the differentiation of the embryonic gonad into testes or ovaries (Fig. 1, bottom). What is the evidence supporting this hypothesis? In oviparous vertebrates, the yolk is a rich repository of hormones and their precursors [Bern, 1990]. These precursor molecules are modified by steroidogenic enzymes in the biosynthesis of androgens and estrogens. If incubation temperature modulates the nature, quantity, and activity of steroidogenic enzymes in the embryo, these enzymes could act on steroid precursors to produce temperature-specific hormonal milieus in the embryo. Hydroxysteroid dehydrogenase (HSDH) enzymes are involved in testosterone and estrogen biosynthesis (3β-HSDH and 17β-HSDH) and 3α-HSDH is involved in the synthesis

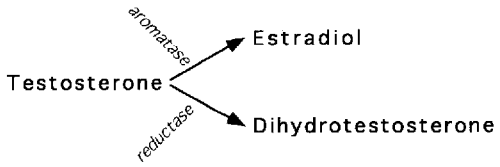


Fig. 3. Biosynthesis of testosterone to dihydrotestosterone (via reductase) and estradiol (via aromatase).

of other androgens. We assessed the activity of 3α-, 3β-, and 17β-HSDH enzymes before, during, and after the temperature-sensitive period in the red-eared slider turtle. HSDH reaction product was observed in the liver, adrenal gland, and kidney before and during the temperature-sensitive window at either incubation temperature and in the genital ridge during the last trimester of development [Thomas *et al.*, 1992]. Similar findings have been reported for the green sea turtle (*Chelonia mydas*) [Merchant-Larios *et al.*, 1989] and the European pond turtle (*Emys orbicularis*) [Pieau, 1973].

Testosterone (T) is the precursor of both dihydrotestosterone (DHT) and estradiol (E2), being converted by reductase to DHT and by aromatase to E2 (Fig. 3). The ontogenetic pattern in aromatase activity parallels that of HSDH enzymes. In the European pond turtle, aromatase activity is greatest in embryos incubating at a female-producing incubation temperature compared to embryos incubating at a male-producing temperature; indeed, there appears to be a positive feedback between E2 and aromatase at a female-producing temperature [see review by Pieau *et al.*, 1994]. Furthermore, initial studies show aromatase levels to be higher before, compared to after, the temperature-sensitive period in the kidney–adrenal–gonadal complex of the red-eared slider [J. Bergeron, A. Wozniak, J. Hutchison, and D. Crews, unpublished data]. No information is available on the ontogenetic pattern of reductase activity.

If incubation temperature modifies the steroid microenvironment in the embryonic turtle, which in turn determines gonadal sex, then the nature and pattern of steroid hormone secretion may differ with incubation temperature. Dorizzi *et al.* [1991] found higher estrogen content in gonads of European pond turtle embryos that were incubating at a female-producing incubation temperature compared to embryos incubating at a male-producing temperature during the temperature-sensitive window. In the red-eared slider turtle, the steroid content in the urogenital tissues (kidney–adrenal–gonad) of embryos incubated at male- and female-producing temperatures differs [White and Thomas, 1992a]. This finding suggests that the onset of steroidogenesis occurs only after the onset of the temperature-sensitive period and not before. Consistent with this interpretation is the finding that in *in vitro* experiments, ovine follicle-stimulating hormone (FSH) fails

to stimulate steroidogenesis in isolated gonads until after the temperature-sensitive period but does elicit significant responses in the kidney and adrenal before or during the temperature-sensitive period [White and Thomas, 1992b].

FUNCTIONAL OUTCOMES OF TEMPERATURE-DEPENDENT SEX DETERMINATION

Since sex determination in TSD depends fundamentally on incubation temperature, is the process of sexual differentiation similarly affected? Before this question can be answered, it is necessary first to review briefly our understanding of sexual differentiation in vertebrates. It has been established that in mammals, androgenic steroid hormones along with other protein hormones (e.g., müllerian inhibiting substance) produced by the embryonic testes, cause the development of male-typical morphological, physiological, and behavioral traits; if testes are not formed, a female phenotype results. Thus, in various mammals, perinatal castration of genetic males results in adults having female-typical morphology, physiology, and behavior, whereas perinatal androgen treatment of genetic females produces adults with a masculinized phenotype.

The modern perspective of sexual differentiation was developed from work on species exhibiting genotypic sex determination (GSD). Initially the embryo is bipotential, having the properties of both sexes. During development of the male the wolffian ducts develop into the epididymides and the vasa deferentia and the müllerian ducts degenerate; in the case of female development, the müllerian ducts become the oviducts and uterus and the wolffian ducts degenerate. An analogy often is made between the development of the urogenital duct system and the brain circuitry underlying sexual behavior. That is, there exists in each individual dual neural substrates underlying sexual behavior, one subserving male-typical behaviors such as mounting and intromission behavior and one subserving female-typical behaviors such as sexual receptivity. Like the duct systems, the development of these structures is differentially affected during development by gonadal hormones. Thus, in mammals, the genetic male is said to be masculinized and defeminized and the genetic female as being feminized and demasculinized (Fig. 4, top). This has been termed the orthogonal model of sexual differentiation.

Given the "all-or-none" nature of TSD, it is evident that in such species maleness does not develop independently of femaleness (and vice versa) and that we must envision another model of sexual differentiation. Originally, sexual differentiation, or masculinity and femininity, were viewed as representing ends of a single continuum (Fig. 4, bottom); in such a unidimensional model individual differences would arise from the degree of masculinization and corresponding defeminization, or feminization and corresponding demasculinization. For such a model to have heuristic value in understanding sex differences in TSD species, two important issues must be addressed. First, are individuals from temperatures that produce both males and females sexually equivalent to those individuals from incubation temperatures that produce all females or all males? That is, are all females (or males) similar regardless of incubation temperature? Second, is the temperature that produces one sex more potent than the temperature that produces the opposite sex? In other words, could a certain temperature serve as an organizer in TSD species in a fashion similar to the role of steroid hormones in GSD species?

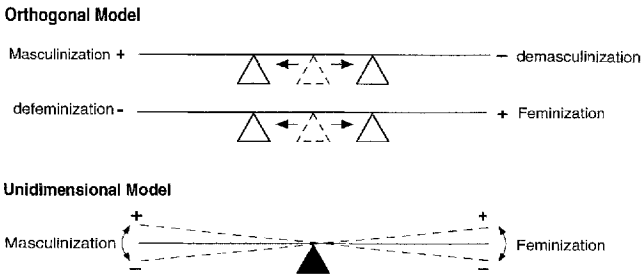


Fig. 4. Two models of sexual differentiation in vertebrates. (Top) Orthogonal model that posits that the dimensions of masculinity and femininity are separate and independent. This model applies to species having genotypic sex determination. (Bottom) Unidimensional model which posits that masculinity and femininity are at opposite ends of the same continuum and hence functionally linked. This model may apply to species with temperature-dependent sex determination.

Because it takes 5–7 years for the red-eared slider turtle to reach reproductive maturity, we have employed the leopard gecko (*Eublepharis macularius*) as an animal model for the study of the functional outcomes of TSD. This lizard species is of moderate size (40–90 g), reaches sexual maturity at approximately 45 weeks of age, and is fully grown at 65 weeks of age. Incubation of leopard gecko eggs at 26°C produces only female hatchlings, 30°C produces a female-biased sex ratio (80:20), and 32.5°C produces a male-biased (20:80) sex ratio. An incubation temperature of 35°C (at or near the lethal maximum) again produces only females [Viets *et al.*, 1993]. By studying animals that differ only in (1) incubation temperature experienced, as well as (2) their prenatal, (3) and/or postnatal hormonal manipulation, it is possible to determine the extent to which differences between and within the sexes are due to incubation temperature and to what extent they are due to the individual's gonadal sex. For example, females determined by incubation temperature can be compared to females determined by estrogen at male-producing temperatures.

Adult leopard geckos exhibit marked sexual dimorphisms in morphology, physiology, and behavior. For example, specialized secretory pores anterior to the clo-

aca are open in males; in females from low incubation temperatures they are closed, whereas in females from higher, male-biased incubation temperatures, they are open. The onset of sexual maturity and plasma hormone levels in leopard geckos varies in a similar fashion as a function of the incubation temperature of the egg. Females from a male-biased incubation temperature take longer to reach sexual maturity [Tousignant and Crews, 1994a] and have significantly lower circulating concentrations of E2 and significantly higher T [Gutzke and Crews, 1988] levels. Males from a female-biased incubation temperature have significantly higher levels of E2 [Tousignant and Crews, 1994a] as adults than do males from a male-biased incubation temperature.

This spectrum of effects evident in females from male-biased incubation temperatures is reminiscent of the well-known masculinizing effects following administration of exogenous androgens on neonatal female mammals and the more recently discovered intrauterine position effect in polytocous mammals. In mammals, the embryonic environment influences behavioral expression in adults. Sociosexual behavior of adults varies according to intrauterine positioning of male and female fetuses and is a consequence of exposure to the hormones from fetal neighbors. For example, in rats, mice, and gerbils, females situated next to males in utero (2M) have a masculinized adult phenotype, compared to females situated next to females (0M) [vom Saal, 1981]. Furthermore, 2M females exhibit a higher intensity of aggression toward female stimulus animals and a decrease in attractivity to male stimulus animals, compared to 0M females. In the gerbil, intrauterine position affects the age of maturity of females; 0M females mature early whereas 2M females mature late [Clark *et al.*, 1988]. Early-maturing (0M) females have lower androgen levels and higher estradiol levels than those of late-maturing (2M) females [Clark *et al.*, 1991]. Could an incubation temperature that produces predominantly males act in an analogous fashion by altering the neuroendocrinology of the females born at these temperatures?

MATERIALS AND METHODS

Freshly laid turtle eggs are obtained commercially (Robert Kliebert, Hammond, LA). After transport to our laboratory, eggs are held at room temperature until viability is established by candling. They are then placed in containers with moistened vermiculite (vermiculite:water, 1:1) and the containers placed in reach-in incubators (Precision) programmed to provide a constant temperature. Embryonic development is monitored by candling eggs and by dissecting 2–4 eggs approximately twice a week to verify specific development stages, based on criteria described by Yntema [1968]. In studies involving experimental treatment, eggs are incubated until stage 17, the approximate midpoint in the temperature-sensitive period in this species [Wibbels *et al.*, 1991a]. Eggs are then randomized into control and experimental treatments. In all experiments, eggs in control groups at each incubation temperature receive a single treatment consisting of 5 μl of 95% ethanol; inhibitor and/or ligand is also dissolved in 5 μl of 95% ethanol. After receiving treatments, all eggs are returned to their respective incubators until they hatch. Otherwise methods are as described in the text and figure legends or in the references therein.

Turtles are euthanized within two weeks after hatching. Gonadal sex and developmental status of the Müllerian ducts are assessed by examination under a dissection microscope. The gonads of hatchling red-eared slider turtles are relatively well differentiated and, with rare exception, appear distinctly testicular or ovarian when viewed under a dissection microscope [Crews *et al.*, 1991; Wibbels *et al.*, 1991a]. Ovaries are long and flat whereas testes are shorter, more round, and have visible seminiferous tubules [Crews *et al.*, 1991]. The developmental status of both the cranial and the caudal halves of the müllerian ducts is examined and scored as either absent, regressed but visible, normal (as in a typical female hatchling), or hypertrophied. A phallus is also noted if present. In the initial studies all gonads were processed for histological examination [Crews *et al.*, 1991; Wibbels *et al.*, 1991a]. In all instances, the histological assessment of sex has coincided with macroscopic assignment of sex. In subsequent studies, the gonads of three to five individuals from each experimental group are processed for histological examination of the gonad to confirm sex assignment.

RESULTS AND DISCUSSION

Proximate Mechanisms of TSD

Is there evidence that steroid hormones are the physiological equivalent of temperature in the sex determination cascade? We have examined the possible role of steroid hormones in both female and male sex determination, although most of our work to date has centered on estrogen and female sex determination.

Estrogen-sensitivity is correlated with temperature-sensitivity. Administration of exogenous estrogen will override the effect of a male-producing incubation temperature so that all of the hatchlings will be female [Crews *et al.*, 1989, 1991]. The period of sensitivity to E2 coincides with the mid-trimester window of temperature-sensitivity; only in this period are incipient males induced to become female [Wibbels *et al.*, 1991a]. Also, the feminizing ability of E2 varies with incubation temperature. Incubation temperature and E2 synergize, with E2 exerting a significantly more potent effect as the threshold temperature is approached (Fig. 5). Finally, the morphological changes

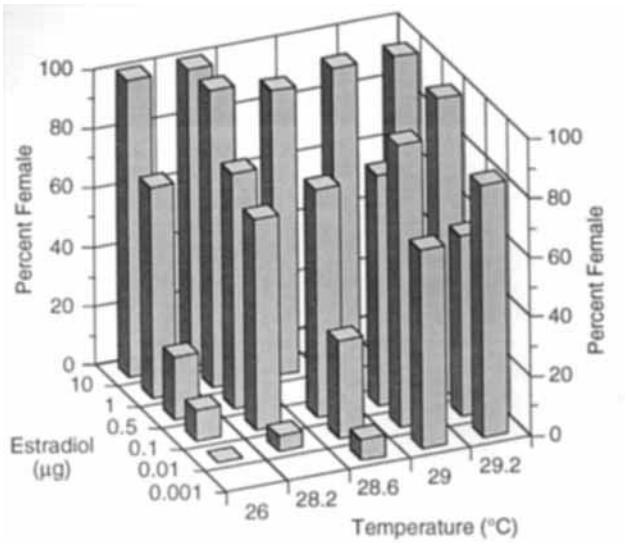


Fig. 5. Interaction between incubation temperature and exogenous estradiol in determining female development in the red-eared slider turtle. Plotted is the female-inducing ability of exogenous estrogen (see Wibbels *et al.*, 1991, for mathematical formula). In all studies, eggs were spotted with hormone in alcohol or with alcohol only. Sample sizes average 26 individuals, ranging from 22 to 40; total number of individuals depicted = 706. Not all cells filled because certain groups were not tested. Cells that appear to counter general trend are within experimental error.

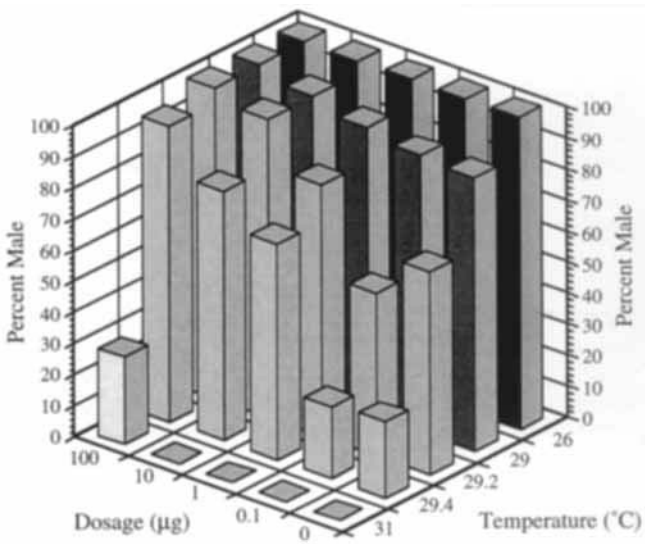


Fig. 6. Administration of the aromatase inhibitor Ciba Geigy 16949A induces male development. In all studies eggs were spotted with the chemical in alcohol or with alcohol only. Sample sizes average 25 individuals, ranging from 21 to 28; total number of individuals depicted = 623.

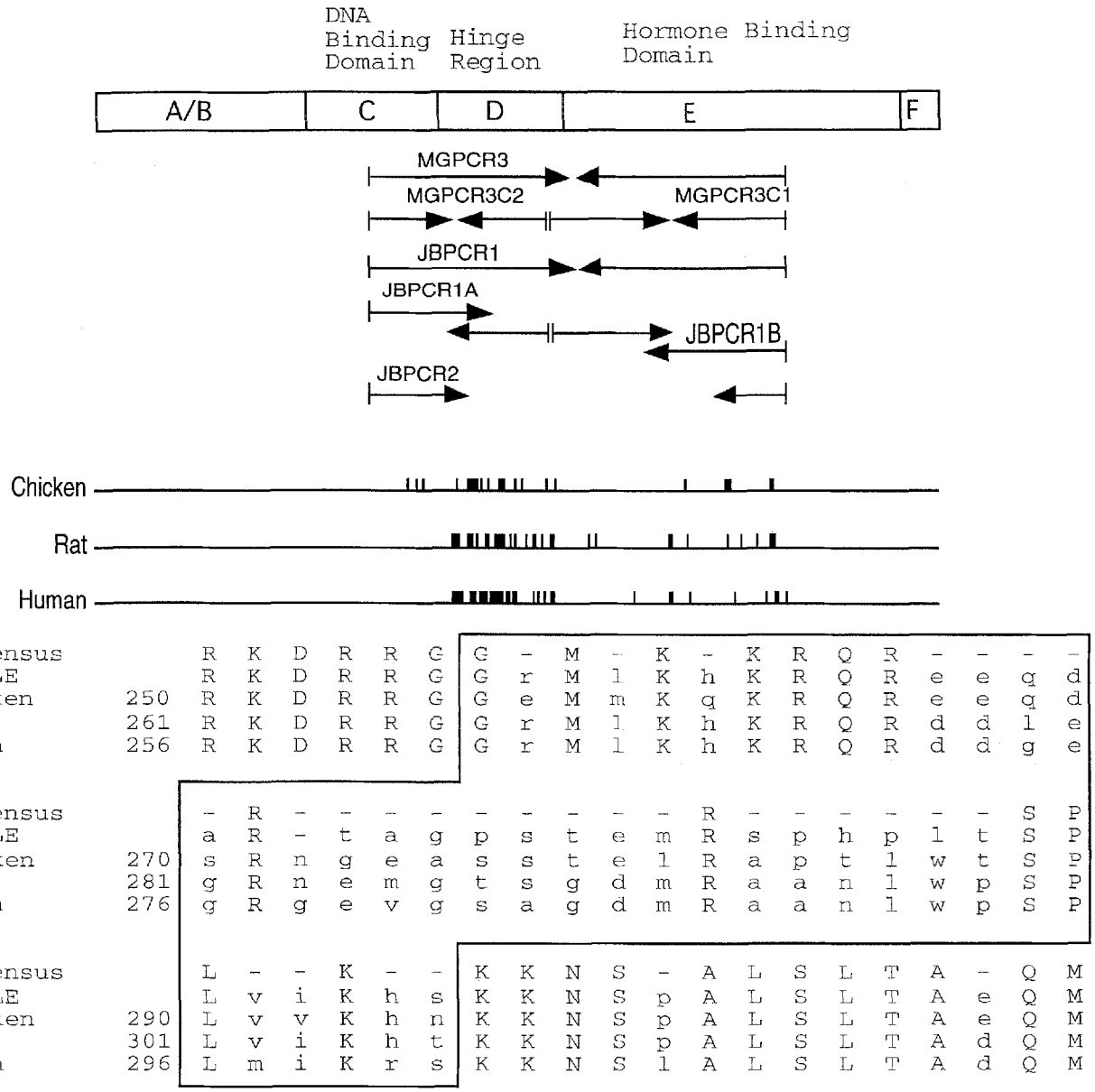
induced by exogenous estrogen administration are identical to those induced by incubation temperature [Wibbels *et al.*, 1993].

Estrogen response is specific to estrogen-like compounds. If E can act as a physiological parallel to female-producing temperature in TSD, does it do so by a hormone receptor mechanism and is it specific to estrogen? We have evaluated the effect of a variety of steroid hormones and their agonists and antagonists at both male- and female-producing temperatures. Embryos incubated at a male-producing temperature are feminized by the estrogen agonists diethylstilbestrol (DES) and R2858, but not by the androgen agonist R1881 [Wibbels and Crews, 1992]. Interestingly, treatment of eggs incubating at a male-producing temperature with T also feminizes approximately one-half the individuals, presumably through the aromatization of T to E2; at a female-producing temperature, comparable dosages of T or E have no discernible effect. Exogenous progesterone, corticosterone, and DHT do not feminize embryos. These results suggest that steroid-induced feminization is mediated via an estrogen-specific receptor.

Aromatase inhibitors will block female development. The biosynthesis of E2 depends on the aromatization of T to E (Fig. 3). Administration of either of two aromatase inhibitors (Ciba Geigy CGS 16949A or CGS 20267) to eggs incubating at female-producing temperatures will cause male development (Fig. 6). This response is temperature sensitive, with lower dosages required as the threshold incubation temperature is approached. Similarly, administration of T and Ciba Geigy 16949A to eggs incubating at 31°C results in 100% of the hatchlings being male.

Estrogen is concentrated in specific tissues and cells. Estrogen target areas during the temperature-sensitive period have been identified using autoradiography [Gahr *et al.*, 1992]. Hyperfilm autoradiography shows uptake primarily in the liver, the kidney–adrenal–gonad area, and the bony structures before, during, and after the period of sex determination in embryos incubated at female- or male-producing temperatures. Emulsion autoradiography reveals that during and after the temperature-sensitive window, labeled cells were found primarily in the liver, kidney, adrenal, and oviduct. Labeled cells in the gonad are distributed evenly throughout the medulla and the cortex.

Distribution and quantity of estrogen-responsive target cells are known. It is assumed, but remains to be proved, that the estrogen-concentrating cells identified by autoradiography contain estrogen receptor (ER) and not simply estrogen-binding protein. Our first effort at documenting this was an instructive failure. In ten turtle species tested, spanning six families, there was no immunoreactivity to mammalian monoclonal antibodies (e.g., H222Spy and H226Spy), although these same antibodies did react with embryonic and adult tissue from various lizards and the alligator [M. Gahr and D. Crews, unpublished data]. Since we were unable to find an available antibody



Consensus		R	K	D	R	R	G	G	-	M	-	K	-	K	R	Q	R	-	-	-	-
TURTLE		R	K	D	R	R	G	G	r	M	l	K	h	K	R	Q	R	e	e	q	d
Chicken	250	R	K	D	R	R	G	G	e	M	m	K	q	K	R	Q	R	e	e	q	d
Rat	261	R	K	D	R	R	G	G	r	M	l	K	h	K	R	Q	R	d	d	l	e
Human	256	R	K	D	R	R	G	G	r	M	l	K	h	K	R	Q	R	d	d	g	e
Consensus		-	R	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-	-	S	P
TURTLE		a	R	-	t	a	g	p	s	t	e	m	R	s	p	h	p	l	t	S	P
Chicken	270	s	R	n	g	e	a	s	s	t	e	l	R	a	p	t	l	w	t	S	P
Rat	281	g	R	n	e	m	g	t	s	g	d	m	R	a	a	n	l	w	p	S	P
Human	276	g	R	g	e	v	g	s	a	g	d	m	R	a	a	n	l	w	p	S	P
Consensus		L	-	-	K	-	-	K	K	N	S	-	A	L	S	L	T	A	-	Q	M
TURTLE		L	v	i	K	h	s	K	K	N	S	p	A	L	S	L	T	A	e	Q	M
Chicken	290	L	v	v	K	h	n	K	K	N	S	p	A	L	S	L	T	A	e	Q	M
Rat	301	L	v	i	K	h	t	K	K	N	S	p	A	L	S	L	T	A	d	Q	M
Human	296	L	m	i	K	r	s	K	K	N	S	l	A	L	S	L	T	A	d	Q	M

Fig. 7. Schematic of turtle estrogen receptor (tER) gene. For these studies, mRNA was isolated from oviduct target tissue of an adult red-eared slider. With PCR primers designed to the most conserved regions of the DNA- and hormone-binding domains of ER gene, DNA fragments were amplified from the cDNA prepared from this mRNA. These products (three independent clones each of which spans 0.9 kb) were subcloned and, based on the nucleic acid composition, the amino acid sequence determined. Top portion shows functional domains of tER with subclones indicating regions sequenced to date below. The hatch marks in the middle portion are a graphic representation of regions where tER differs from chicken, rat, or human ER. The lower portion presents the deduced amino acid sequence for tER compared to the corresponding regions of published sequences for ER of other species. Boxed segment of the sequence signifies the hinge region. Dashes in the consensus sequence indicate nonconserved amino acids.

that recognized turtle estrogen receptor (tER), an alternative strategy was undertaken involving the cloning and sequencing of tER (Fig. 7). Comparison of the sequence of the tER is interesting in two regards. First, it affirms the high degree (>90%) of similarity with the published sequences of the DNA- and steroid-binding domains of the human, rat, and chicken ER. Second, it reveals great differences within the hinge region between the two domains; only 45% similarity exists.

Using nucleotide probes, we have localized and quantified tER–mRNA during the critical periods of sex determination. In situ hybridization located cells containing tER in the liver, adrenal, kidney, oviduct, and the

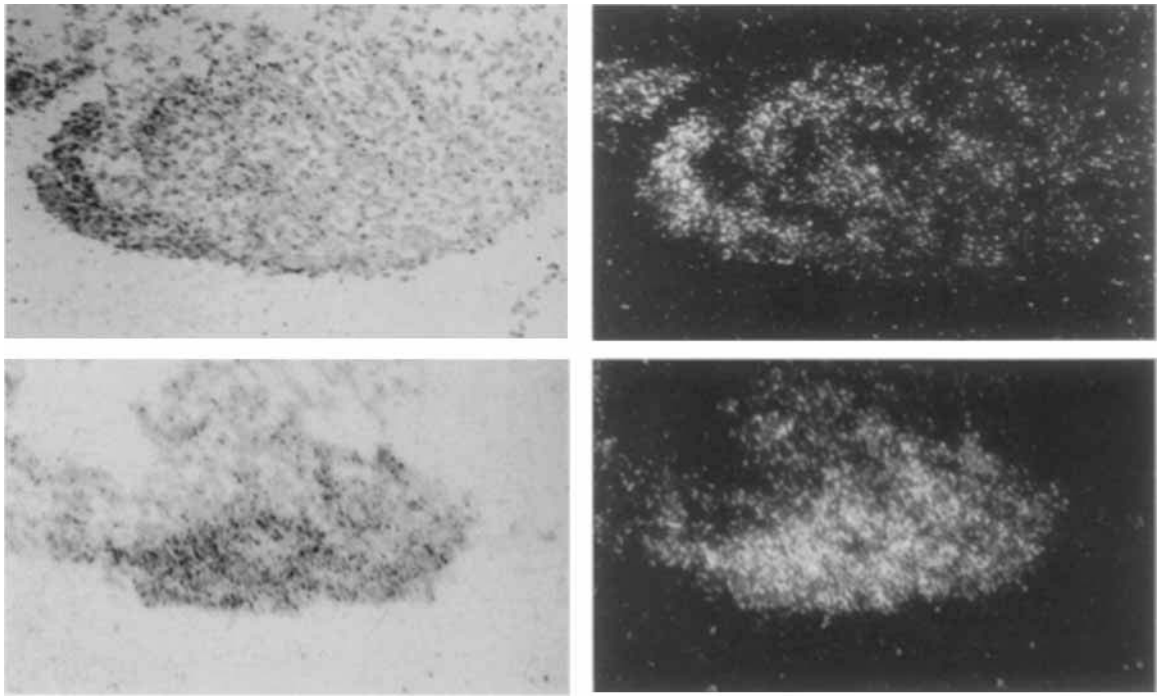


Fig. 8. In situ hybridization of ER-mRNA in the developing gonad in embryos incubating at a male- and a female-producing temperature. Light-field micrography (upper left) of testis and corresponding dark-field image indicating gene transcript (upper right). Light-field micrography (lower left) of ovary and corresponding dark-field image indicating gene transcript (lower right). ^{35}S-labeled antisense ER riboprobe was generated using T7 polymerase transcription of cDNA clone MGPCR3C2, as shown in Figure 7. Tissues are 20 micron cryosections, fixed immediately after sectioning. Hybridization was performed in 50% formamide, 10% dextran sulfate, 0.3M NaCl, 10 mM Tris–Cl (pH 8.0), 1 mM EDTA, and 1 × Denhardt's solution at 45°C. Slides were washed at 50°C in sodium citrate solutions of increasing stringency, prior to a 2-week exposure using emulsion.

gonad (Fig. 8). We hypothesize that the mechanism of action of temperature in TSD includes the regulation of the genes coding for steroid hormone receptors. Preliminary studies using the ribonuclease protection assay (RPA) to quantify tER–mRNA reveal that tER–mRNA varies in abundance with both developmental stage and incubation temperature [J. Bergeron, T. Wibbels, and D. Crews, unpublished data].

Androgens play a role in male sex determination. Although exogenous E2 overcomes the effects of a male-producing temperature and induces female development, neither exogenous T nor DHT can induce male development at an incubation temperature that produces all females. However, if eggs incubating at a threshold temperature receive exogenous DHT, most or all of the offspring will be male [Wibbels *et al.*, 1992] (Fig. 9). Significantly, simultaneous administration of DHT and E to eggs incubating at a threshold temperature will cause hatchlings to have ovotestes (Fig. 10); such individuals are never observed normally in the red-eared slider. We presently are using the same strategy employed in the research with estrogen to determine: under what conditions DHT is effective, when DHT exerts its action, the hormone specificity of male determination, and the relationship between incubation temperature and DHT.

Reductase inhibitors will block male development. The fact that exogenous DHT will induce male development when administered to eggs incubating at a threshold temperature suggests that male development is mediated by an androgen receptor. Testosterone is reduced to DHT by the enzyme reductase (Fig. 3), and administration of the reductase inhibitor 4MA to incubating eggs prevents male development at both threshold or male-biased incubation temperatures (Fig. 11). Comparable results are obtained with MK906. Administration of T and reductase inhibitor to eggs incubating at a male-producing temperature results in the production of female hatchlings.

Feedback model for hormone action in sex determination in TSD. Taken together, our results suggest that female and male sex determination are separate processes that are differentially affected by incubation temperature, rather than by the organization/default system characteristic of genotypic sex determination. Thus, females may result from the activation of an ovary determining cascade and inhibition of a testis determining cascade; conversely,

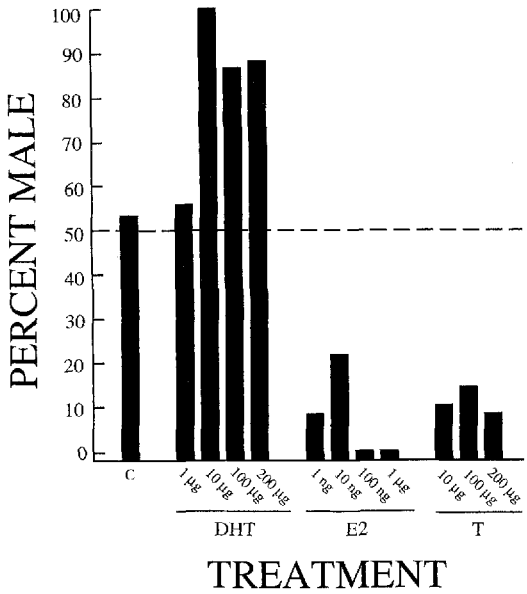


Fig. 9. The role of estrogen in stimulating female development and androgen in stimulating male development in eggs incubated at the threshold temperature (29.2°C) in the red-eared slider. C, ethanol Control; DHT, dihydrotestosterone; E2, estradiol; T, testosterone. Sample sizes range from 20–30 per group. Dashed line represents 50:50 sex ratio.

Fig. 10. The ovotestis that results from simultaneous administration of estrogen and androgen on eggs incubated at an intermediate temperature. Note the thick membrane separating the cortical and medullary components.

males may result from the activation of a testis determining cascade and inhibition of an ovary determining cascade (Fig. 12). Central to the steroid hormone-mediation hypothesis (Crews *et al.*, 1989, 1991; Dorizzi *et al.*, 1991; Pieau *et al.*, 1994) is the role of T as a precursor molecule destined for conversion to DHT (via reductase) or E2 (via aromatase). One hypothetical model (Figure 12, top panel) postulates that at a female-producing temperature the gene(s) encoding for aromatase are activated and the gene(s) encoding for reductase remain at constitutive levels (or perhaps suppressed via negative feedback control of E2), resulting in increased E2 production. For example, Claude Pieau and colleagues (reviewed in

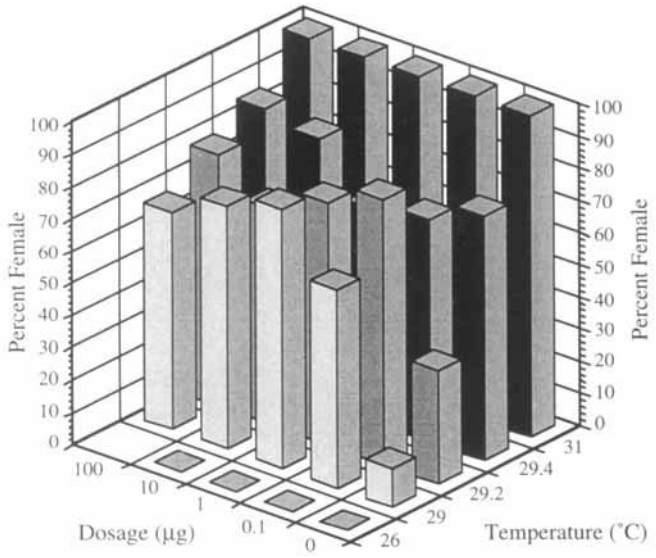


Fig. 11. Administration of the reductase inhibitor 4-MA induces female development. In all studies, eggs were spotted with the chemical in alcohol or with alcohol only. Sample sizes average 25 individuals, ranging from 21–30; total number of individuals depicted = 632; 100-μg dosage at 26°C not filled because group not tested.

Pieau *et al.,* 1994) have documented a positive feedback relationship between E2 secretion and aromatase production at a female-producing temperature in the European pond turtle. On the other hand, at a male-producing temperature the gene(s) encoding for reductase may be enhanced and the gene(s) encoding for aromatase inhibited, resulting in increased DHT production. As yet unproven, a positive feedback relationship similar to that between aromatase and E2 may exist between DHT and reductase. Alternatively, the finding of a lack of a dose-response in the effect of the reductase inhibitor and the absence of a dynamic between DHT sensitivity and incubation temperature as occurs between E2 sensitivity and incubation temperature indicates that the gene(s) for reductase are constitutively expressed at the various incubation temperatures. Thus, incubation temperature may act only indirectly via the testis determining gene(s) having a negative feedback on the regulation of aromatase gene expression, leading to suppression of E2 (Figure 12, bottom panel). For example, Haqq *et al.* (1993) demonstrated recently in the rat that the putative male-determining factor (SRY) may control male development through regulation of aromatase and MIS genes.

We propose further that incubation temperature activates the gene(s) encoding for steroid hormone receptors (e.g., male-producing temperature upregulating AR and female-producing temperature upregulating ER). Whatever the actual enzymatic regulation, the resulting hormonal milieu would lead to the binding of DHT and E2 to specific, high-affinity, intranuclear receptor proteins (AR and ER, respectively), which in turn would activate the putative receptors such that the hormone-hormone receptor complex would bind to hormone response elements on the DNA. The consequence of such events 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.

Such a model would account for the following facts: (1) The effect of temperature or exogenous steroids is all-or-none; that is, individuals are either male or female. (2) Incubation at a threshold temperature results in a 50:50 sex ratio, rather than intersexes being formed. (3) Intersexes can be formed experimentally by the simultaneous administration of DHT and E2 to eggs incubating at a threshold incubation temperature or by high dosages of T at female-biased incubation temperatures. (4) Exogenous E2 will overcome the effects of male-producing temperatures, and there is a correlation between estrogen-sensitivity and temperature-sensitivity. (5) Exogenous DHT cannot overcome the effects of a female-producing temperature; although exogenous DHT will induce male development in eggs incubating at a threshold incubation temperature, there is no apparent dynamic between incubation temperature and sensitivity to DHT. (6) Steroid-induced gonadal feminization is specific to ER-responsive estrogens whereas gonadal masculinization appears to be specific to AR-responsive androgens; and (7) Administration of an aromatase inhibitor will block female development and induce male development, whereas administration of a reductase inhibitor will block male development and induce female development, in eggs incubating at intermediate incubation temperatures.

Functional Outcomes of TSD

Incubation temperature has a permanent and cumulative effect on the morphology, physiology, and behavior of the individual.

Incubation temperature and gonadal hormones have separate roles in sexual differentiation. It must be appreciated that in species with TSD, incubation temperature and gonadal sex co-vary. Thus, any difference between individuals could be due to the incubation temperature of the egg, the gonadal sex of the individual, or both factors combined. If the contribution of each is to be assessed, they must be dissociated. Two approaches can be used to assess the relative contribution of each in the process of sexual differentiation: (1) comparing individuals of the same sex from different incubation temperatures, and (2) hormonally manipulating embryos to reverse gonad phenotype. Thus far the data indicate that while gonadal sex is important in shaping sex differences, it is

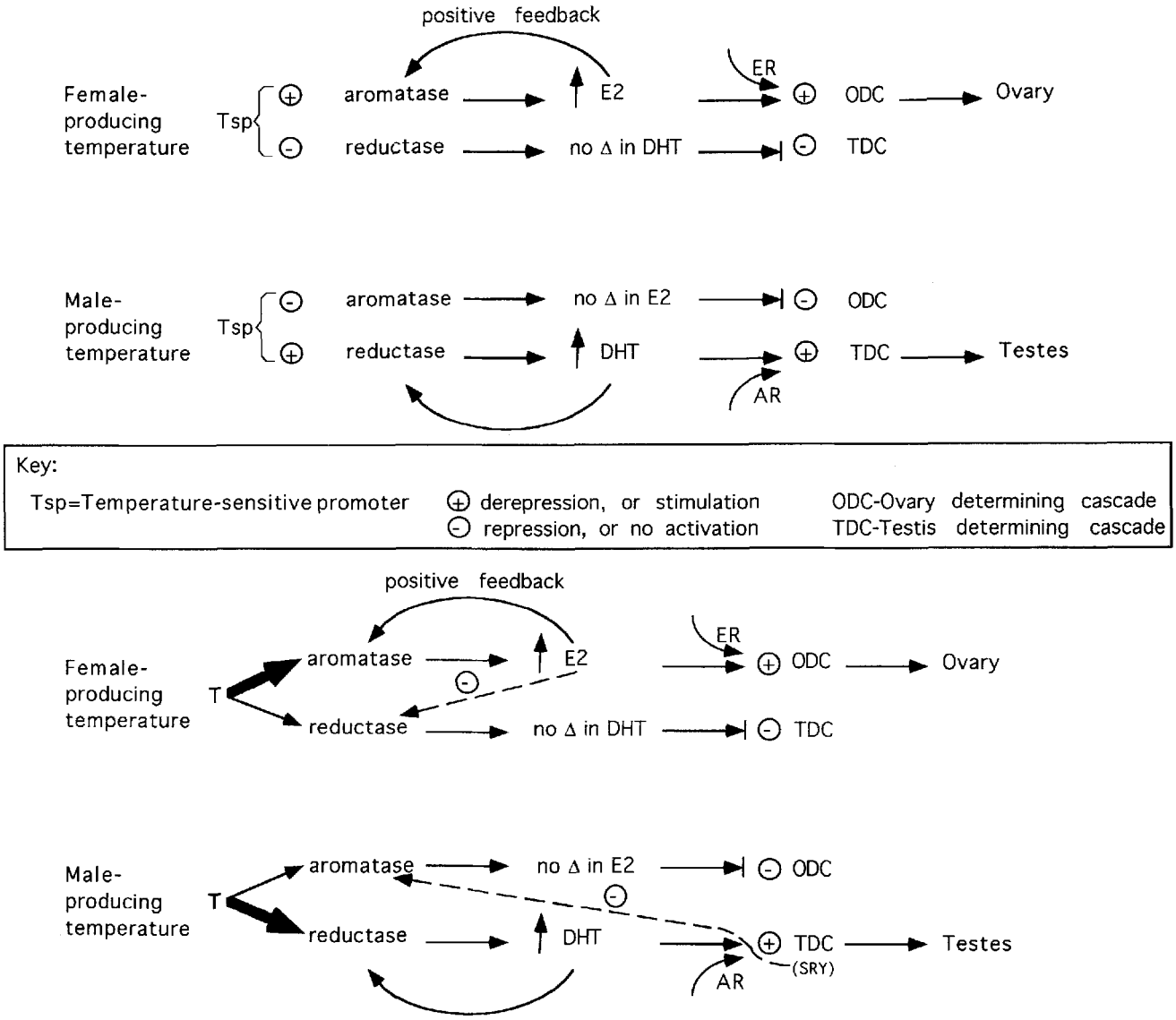


Fig. 12. Two hypothetical scenarios of temperature-dependent sex determination in the red-eared slider (*Trachemys scripta*). The temperature experienced during the middle third of incubation initiates the cellular and molecular cascades that result in male or female offspring. Temperature may act via a temperature-sensitive promoter directly on genes encoding for steroidogenic enzymes, resulting in changes in the steroid hormone milieu (Top panel). Alternatively, temperature may act indirectly via steroid hormone feedback control of steroidogenic enzyme activity and by testis determining factors (an SRY-like gene product) acting on aromatase production (Bottom panel). In both models, 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) in turn contributing to the activation of ovary and testis determining cascades.

secondary in importance to incubation temperature. This is seen particularly in the growth rate and behavior of adults.

In the leopard gecko, the male is the larger sex. However, females from a male-biased incubation temperature grow faster and larger than do females from a female-biased incubation temperature, indeed growing as rapidly and as large as males from lower, female-biased incubation temperatures [Tousignant and Crews, 1994a] (Fig. 13A). If the ovaries are removed from females from low incubation temperatures on the day of hatching, they grow as males from a higher incubation temperature (Fig. 13B). Ovariectomy in adulthood does not cause a similar weight gain, suggesting a developmental effect only. Finally, when the effect of gonad type and incubation temperature is experimentally dissociated using estrogen treatment of eggs at a male-biased incubation temperature, these

estrogen-determined females grow as would control, temperature-determined males (Fig. 13C).

Incubation temperature also has a profound effect on the frequency of species-typical aggressive behavior. In the initial stages of aggression the animal raises and arches its body, standing on its toes and waving the tail slowly. This is followed by a lateral orientation of the animal's body toward the stimulus animal and a slow approach. If the stimulus animal does not flee, then it will be attacked. Submissive behavior consists initially of the animal flattening its body so that the ventrum is pressed to the substrate; at this initial stage the tail tip is twitched. If the aggressive animal approaches, the submissive animal will thrash its tail rapidly and then flee.

Aggression appears to be a male-typical trait in the leopard gecko. Females usually show little or no aggression in response to males, whereas males will posture and often attack another male as he approaches; males rarely attack females. Females from female-biased incubation temperatures are less likely to be aggressive toward male stimulus animals compared to females from a male-biased incubation temperature [Flores *et al.*, 1994] (Fig. 14). Hormone-determined females from a male-biased temperature are as aggressive as temperature-determined females from a male-biased temperature. This finding suggests that the aggressive behavior in a female leopard gecko is less affected by ovarian hormones than by incubation temperature.

In a sexual encounter, the male will slowly approach the female, touching the substrate or licking the air with his tongue. There is an attractivity pheromone contained in the skin of females that elicits the characteristic tail vibration of males where the tail is vibrated rapidly, creating a buzzing sound [Mason and Gutzke, 1990]; females have never been observed to exhibit this tail vibration behavior, even if treated with exogenous androgen. Thus, attractivity is a female-typical trait measured by a sexually active male's courtship behavior toward a stimulus animal. Females from a male-biased incubation temperature are less attractive than are females from female-biased incubation temperatures [Flores *et al.*, 1994] (Fig. 14). Hormone-determined females from a male-biased incubation temperature are both attractive and aggressive.

Practical Applications

The preservation and restoration of *endangered* or *threatened* species is becoming a more pressing problem every day. This is particularly the case with oviparous reptiles, many of which are classified as threatened or endangered. This situation is due in large part to the exploitation of reptiles as an important protein resource (meat and eggs) in Third World countries. In addition, the leather and shell byproducts of many reptiles represent substantial commercial opportunities.

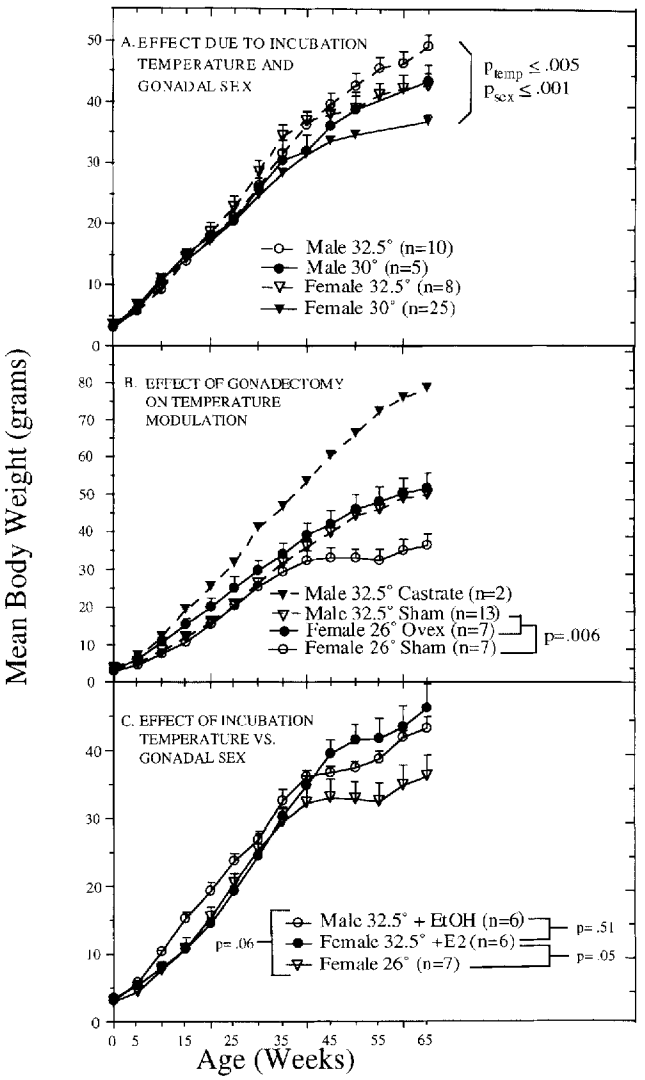


Fig. 13. The relative influence of incubation temperature and gonadal sex on body growth in the leopard gecko. Illustrated is the average body weight (±SE) of individuals from different incubation temperatures or hormonal manipulations. In the leopard gecko, 26°C produces only females, 30°C produces a female-biased (80:20) sex ratio, and 32.5°C produces a male-biased (20:80) sex ratio. **A:** Males and females incubated at either 30.0° or 32.5°C. **B:** Animals incubated at either 26° or 32.5°C and then receiving a sham operation or surgical castration on the day of hatching. **C:** Eggs incubated at 26° or 32.5°C and receiving estrogen or ethanol at the mid-trimester of development. Each individual was weighed weekly from hatching until adulthood; only the data for 5-week intervals are presented here. After hatching, all animals were raised in isolation while exposed to a 14:10-hr/30°:18°C daily photothermal regimen and fed a standard diet. In B the castrated male sample size initially was believed to be nine, but laparoscopy and RIA for androgens in the circulation revealed that in 7/9 instances the castrations were incomplete. Interestingly, only two female individuals were found to have a partial ovariectomy as adults and in one of these females, the records indicate that one gonad was lost in the body cavity after detachment; this ovary has attached to the liver and yolks follicles but does not ovulate them. This finding is consistent with the literature indicating that an intact neural connection to the gonad is necessary for ovulation.

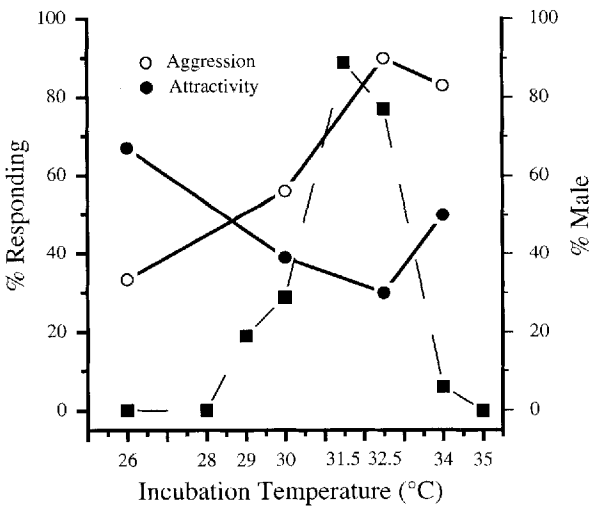


Fig. 14. Effect of incubation temperature on aggression, a male-typical trait, and attractivity, a female-typical trait, in female leopard geckos. Higher incubation temperatures result in a greater number of females exhibiting aggression and decreasing levels of attractivity. Measure of aggression (○) was the female's response to a male stimulus. Attractivity (●) was measured by the male's interest in courting the female. Dashed line represents the sex ratio resulting from different incubation temperatures.

Unfortunately, black market trade of wild-caught specimens has further aggravated this problem.

Because incubation temperature determines the sex ratio at birth, understanding TSD has profound consequences for breeding programs. More than a decade ago Mrosovsky and Yntema [1980] and Morreale *et al.* [1982] suggested that then present practices of excavating, moving, and artificially incubating turtle eggs could result in the production of mostly, if not all, male individuals. This fate has been realized [Shaver *et al.*, 1988; Taubes, 1992; Behler, 1993].

Incubating eggs at female-producing temperatures would seem to be the most effective method for generating female offspring. This would be true if (1) the dynamics of the temperature–sex ratio interaction have been established empirically for the species in question, and (2) there is ready access to controlled environment chambers. Often times we are dealing with so few eggs that experimental validation is impossible. This can result in a frustrating "hit and miss" strategy. Further, for many countries and even entrepreneurs in the United States the cost of precision controlled environment chambers necessary to determine the pattern of TSD of a particular species is prohibitive. Even if incubators are affordable, nesting sites are often in remote and inaccessible areas, making controlled temperature studies even more costly. Furthermore, in these areas, power failures are common, with the result that valuable eggs die.

The technique of spotting eggs with estrogen to induce female development potentially circumvents these negative features.[1] It provides an easy and inexpensive solution to a pressing problem. Estrogen-spotting of eggs has no mortality associated with the application of hormone and is absolutely effective in producing only female offspring [Crews *et al.*, 1991]; the efficacy of the estrogen-spotting method for ensuring the production of females now has been demonstrated in a variety of threatened or endangered species: Mugger's crocodile, Olive Ridley sea turtle, the freshwater Cagle's map turtle, and the New Caledonian gecko. Further, long-term studies with the leopard gecko indicate that the fecundity of such estrogen-determined females is indistinguishable from that of temperature-determined females [D. Crews, unpublished data]. We have also established a long-term breeding pond to monitor the egg-laying of estrogen-determined red-eared slider turtles once reproductive age is reached.

The potential impact of the estrogen-spotting technique can be seen in the following example. Let us assume that 10 breeding females exist and that each female will produce 30 eggs each year. Let us assume also that the young become sexually mature in their third year. Finally, we will assume that a 1:1 sex ratio occurs in unmanipulated normal animals in the population. In all examples no mortality is considered and each female produced has equal fecundity. [These assumptions are clearly unrealistic, but any decreases in production will be equivalent in both the normal and the manipulated animals.] With estrogen-spotting to ensure 100% females, the number of females will increase exponentially. Thus, estrogen-treatment will result in 10,200 females being produced over a 4-year period, compared to the 2,700 females produced normally. At the end of seven years this difference becomes 633,100 versus 56,150!

The timing and amount of estrogen applied to the reptile egg are critical. At the appropriate time and in the proper dosage, estrogen can override the effects of temperature and result in healthy female hatchlings that grow normally and lay eggs as adults. However, if applied too early in development or in too great an amount, the result is increased embryonic mortality and, in those animals that survive to hatch, stunted growth with reduced reproduction or, in extreme instances, intersexuality with consequent failure to breed as adults [Tousigant and Crews, 1994b; Gross and Guillette, 1994]. Thus, the phenomenon of TSD also makes clear another practical application, namely the use of turtle embryos as a bioassay for environmental quality. In temperate and tropical zones, reptiles

[1]The estrogen-spotting procedure is patented ("A Method for Preferential Production of Female Turtles, Lizards, and Crocodiles," U.S. Patent 5,201,280) with all rights assigned to Reptile Conservation International, Inc., a tax exempt, nonprofit organization devoted to the conservation of threatened and endangered reptiles; RCI makes this technology available free of charge to established conservation groups.

TABLE 1. Effects of Some PCB Compounds on Sex Determination*

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
EtOH Control	0	0
E2 Control	100	100

*Eleven compounds were applied to eggs incubated at 27.8°C in two doses per compound. 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 E2 Control consisted of 10 μg E2.

commonly occur at the boundaries of human habitation and/or in areas of environmental catastrophes and hence could serve as an early warning signal of environmental contamination [Gross and Guillette, 1994]. This is possible because the commercial value of some turtle and crocodilian species is so great that eggs are available in large numbers. 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 manmade products can become environmental toxins [Colborn and Clement, 1992]. For example, some of the effects of toxins, such as polychlorinated biphenyls (PCBs), can have estrogenic effects [Korach *et al.*, 1988] and induce reproductive anomalies in adult female animals in nature [Colborn and Clement, 1992]. The fact that in the red-eared slider turtle species sex determination can be influenced by minute amounts of exogenous estrogenic ligands provides a sensitive indicator of potential contamination by environmental estrogens. Using the all-or-nothing nature of the response of red-eared slider turtle embryos to exogenous estrogen, we recently assayed 11 common PCB's [Bergeron *et al.*, 1994]. 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), were found to 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. The former compound 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 [Korach *et al.*, 1988; McKinney *et al.*, 1990]. 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 by direct contact or through the food chain [McKinney *et al.*, 1990].

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 μg), or high (145–190 μg) dosage of compounds. Some eggs received a cocktail of all PCBs, except for the two that caused sex reversal (F and G). Others were exposed to combined hydroxybiphenyls, again excluding F and G. Lastly, some eggs were treated with combined non-hydroxylated 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, resulting in a significant increase in ovarian development at a dose of 10 μg or <1 ppm, whereas F alone and G alone required at least a 10-fold higher dose to show sex reversal. Exogenous E2 produces similar results at a dose of 0.5 μg, or 0.04 ppm [Wibbels *et al.*, 1991].

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/or 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 [Gross and Guillette, 1994].

CONCLUSIONS

Sex determination is the product of coordinated gene expression. The ability to manipulate sex in TSD permits a degree of unparalled control, enabling study of the normal pattern of gene expression not possible with other amniote vertebrate species having genotypic sex determination. The work on TSD is important for four reasons. First, it draws attention to the fact that temperature and steroid hormones can play a pivotal role in sexual development in an amniote vertebrate. In TSD, temperature initiates a cascade of events, probably involving steroid hormones, and culminating in sex determination. It has long been thought that in GSD, steroid hormones (from the mother or embryos) are not involved in gonad formation. The work with TSD reptiles indicates that this conclusion may be premature. Similarly, temperature has not been adequately investigated as a factor in steroid hormone action despite numerous studies documenting how both hormone responsiveness as well as hormone action are completely dependent on temperature; it is remarkable that virtually all studies of the interaction of steroid hormones and their receptors have been conducted at 4°C.

Second, TSD may represent the evolutionary precursor to GSD. Thus, temperature effects in sex determination and sexual differentiation may be masked in homeotherms. Alternatively, temperature sensitivity could still be present but, because it has not been investigated in mammals, remains unknown.

The third reason this work is important is that it speaks directly to the issue of female sex determination. Most effort today focuses on elucidating the mechanisms underlying male development; female development has been relegated to a passive or default state. The reptiles with TSD show that female development must be an active process just as is male development. That is, this work emphasizes that being male also means *not* being a female; similarly, being a female means *not* being a male.

Finally, it is possible to find benefit from such work in conservation efforts to restore endangered or threatened reptile populations. Furthermore, the demonstration that environmental temperature and steroid hormones and corresponding ligands play pivotal roles in sex determination in TSD provide a model system with which to study the action of xenobiotics such as pesticides and other environmental estrogens in the control of gene expression during development.

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