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REVIEW

Temperature-Dependent Sex Determination: The Interplay of Steroid Hormones and Temperature

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ABSTRACT—Sex determination is the product of coordinated gene expression. Mutational analyses have yielded great progress in our understanding of mammalian sex determination, and insight into the evolution of this sex chromosome system would be valuable. Mammals arose from turtle-like reptiles, and in many turtles the incubation temperature of the egg determines the sex of the offspring, a process known as temperature-dependent sex determination. There is mounting evidence that sex steroid hormones are the physiological equivalent of incubation temperature and serve as the proximate trigger for male and female sex determination. Temperature appears to accomplish this end by acting on genes coding for steroidogenic enzymes and sex steroid hormone receptors. The ability to manipulate sex determination in turtles both by temperature and by sex steroid hormones extends our understanding of the evolution as well as the physiology and molecular biology of sex determination.

INTRODUCTION

Conventional wisdom is that sex determination in the "higher" or amniote vertebrates (mammals, birds, and reptiles) is initiated by the sex chromosomes inherited from the parent. For example, in mammals, a specific gene on the short arm of the Y chromosome (designated as SRY or the sex determining region of the Y chromosome) triggers a genetic cascade that results in the bipotential gonad developing into testes. The hormones secreted by the testes in turn act throughout the body to sculpt the male phenotype. In the absence of SRY, the gonads develop as ovaries and a female phenotype occurs. Both sexes however have the genetic machinery to develop as males or females, since in transgenic studies have shown that placing SRY in an X or female background develop as males.

It has long been thought that in amniote vertebrates steroid hormones have no role in determining the type of gonad that will develop. However, there are several lines of evidence that call this assumption into question. First, administration of estrogen to newborn male opossums will cause the gonad to change into an ovary (Burns, 1961; see also Shaw *et al.*, 1988). Second, transplantation of gonadal primordia from genetic females under the kidney capsule of adult males results in the formation of testicular somatic cells including Sertoli and Leydig cells (c.f., Taketo *et al.*, 1984). Finally, there is good evidence that the hormone levels of both parents at the time of fertilization are related to the sex of the offspring (James, 1986, 1987, 1989, 1992). In mice and gerbils a female's intrauterine position, and hence her hormonal environment as an embryo, affects the sex ratio of litters she produces as an adult (Clark *et al.*, 1993; Vandenbergh and Huggett, 1994).

Another piece of conventional wisdom is that the secondary sex ratio in amniotes is fixed; changes in the environment, whether internal or external, have no effect on the 1:1 (male-to-female) sex ratio. Indeed, thousands of years of selective breeding has consistently failed to alter that fundamental unity. However, there is strong evidence in both rodents and humans that the sex ratio can vary significantly from 1:1 depending upon the time mating occurs in relation to ovulation. For example, in humans, at least two studies show that if intercourse occurs two days before or after ovulation as estimated by the fall in basal body temperature, significantly more female than male offspring will result (Guerrero, 1974; Harlap, 1979). In the gerbil, the sex ratio in the right and left uterine horns differ significantly, with male fetuses developing in the right uterine horn significantly more frequently than would be expected by chance, and vice versa for the left uterine horn (Clark and Galef, 1990). As is common in mammals, the vascularity of the ovaries is asymmetrical, with the right ovary having a greater blood supply than the left ovary. It is well known that vascularity changes the temperature within a tissue. Is it possible that the sex ratio difference in the uterine horns is a result of these differences in ovarian vascularity?

Thus, there are exceptions to the rule that sex determination in mammals is independent of both sex steroid hormones and changes in the environment. To determine if these exceptions are trivial, or indicate something fundamental about the sex determination process, we must turn to the evolutionary ancestors of mammals. Mammals arose from a turtle-like tetrapod 350 million years ago. Within turtles, gonadal sex can be

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determined by two mechanisms, sex chromosomes or incubation temperature. Temperature-dependent sex determination occurs only in oviparous reptiles that lack sex chromosomes. Although SRY-like sequences have been found in reptiles, there is no suggestion of a sex-determining role (Coriat *et al.*, 1993, 1994; Spotila *et al.*, 1994; Tiersch *et al.*, 1991, 1992). Temperature-dependent sex determination is believed to be a primitive sex determining system and perhaps the precursor of sex determination by sex chromosomes (Bull, 1983). Thus, it is possible that here-to-fore unsuspected temperature-dependent dynamics are present in species with sex chromosomes but, because of homeothermy, they are masked.

THE RED-EARED SLIDER TURTLE: AN ANIMAL MODEL FOR THE STUDY OF TEMPERATURE-DEPENDENT SEX DETERMINATION

My laboratory has been working for the past six years with the red-eared slider turtle, (*Trachemys scripta*) as an animal model for the study of temperature-dependent sex determination. This species was chosen in part because its eggs are available commercially in large numbers. In addition, the pattern of temperature-dependent sex determination is so extreme that only males or only females are produced over wide ranges of incubation temperatures (20–28.6°C produce all males, 29.4–35°C produce all females), with a narrow (~1°C), intermediate range producing both sexes (Crews *et al.*, 1994). It important to note that the individuals produced at these intermediate incubation temperatures are not hermaphrodites, but are distinct males or females. Further, incubation temperature exerts its effect only during the mid-trimester of development (Wibbels *et al.*, 1991a). Thus, small changes in incubation temperature acting during a narrow time window completely switch the path of sex determination.

ESTROGENS AND AROMATIZABLE ANDROGENS ARE THE PHYSIOLOGICAL EQUIVALENT OF A FEMALE-PRODUCING INCUBATION TEMPERATURE

Sex steroid hormones appear to be the physiological equivalent of incubation temperature and the proximate trigger for sex determination in the red-eared slider. Administration of exogenous estrogen or their aromatizable androgen precursors to eggs incubating at a male-producing temperature exerts an "all-or-none" effect; hermaphrodites are not produced (Crews *et al.*, 1991; Wibbels *et al.*, 1991a). Although it could be that exogenous estrogen alters sex determination by alternate and overriding metabolic pathways rather than by manipulating the temperature-dependent pathways, this interpretation is unlikely given the following facts.

(i) Estrogen dose-response. Varying amounts of estradiol-17b cause varying sex ratios in hatchlings incubated at a male-producing temperature (Crews *et al.*, 1991). The *threshold* dosage is approximately 1.0 μg estradiol-17β per 10 g egg incubating at 26°C, and 0.01 μg at 29.2°C.

(ii) Critical period. The temperature-sensitive window is in the middle third of development, extending from Stage 15, when the foot is a rudimentary paddle, to Stage 20, when the claws are formed and the outline of scales evident on the skin. The ability of estradiol-17β to counteract the effects of a male-producing temperature also has a critical period that corresponds to the temperature-sensitive window (Gutzke and Chymiy, 1988; Wibbels *et al.*, 1991b).

(iii) Specificity of estrogen response. Steroid-induced feminization is mediated via an estrogen-specific receptor. Embryos are feminized by the estrogen agonists estradiol benzoate, diethylstilbestrol, and R2858 (Crews *et al.*, 1989; Wibbels and Crews, 1992). The androgen agonist R1881, progesterone, corticosterone, and dihydrotestosterone (a non-aromatizable androgen), do not feminize embryos. Interestingly, estrone, estradiol-17β, and estriol are not equipotent at an all-male producing temperature, but are physiologically equivalent at higher temperatures (Fig. 1). Unlike estradiol-17β, estrone and estriol binding with the estrogen receptor changes with temperature (Sasson and Notides, 1983a, b). Finally, treatment of eggs incubated at a male-producing temperature with testosterone or androstenedione also feminizes approximately one-half of the individuals, presumably through the aromatization to estradiol-17β (Crews and Bergeron, 1994; Crews *et al.*, 1995; Wibbels and Crews, 1992, 1995).

(iv) Tissues concentrating estrogen. Autoradiography revealed that estrogen is concentrated primarily in the liver, the adrenal-kidney-gonad (AKG) area, and bony structures of all Stages and both male- and female-producing incubation temperatures (Gahr *et al.*, 1992).

(v) Aromatase inhibitors block female development. Application of aromatase inhibitor to eggs incubating at a female-producing temperature results in male hatchlings (Fig. 2) (Wibbels and Crews, 1994; Crews and Bergeron, 1994; see also Dorizzi *et al.*, 1994; Richard-Mercier *et al.*, 1995; Rhen and Lang, 1994). Administration of both testosterone and aromatase inhibitor at a male-producing incubation temperature blocks the T-induced feminization and all offspring are male. Similarly, administration of testosterone and aromatase inhibitor at a female-producing incubation temperature results in all male offspring.

(vi) Equivalence of estrogen and temperature. Synergism occurs when the effect of two factors together have a greater effect than the sum of their separate effects. Synergism between incubation temperature and steroid hormone treatment is consistent with the hypothesis that these factors work in the same biochemical pathway for sex determination. Exogenous estradiol-17β exerts a significantly more potent effect as the *threshold* temperature (that incubation temperature that produces a 1:1 sex ratio) is approached (Crews and Bergeron, 1994; Wibbels *et al.*, 1991b). Further, the morphological changes occurring in response to exogenous estrogen are indistinguishable from the changes induced by incubation temperature (Wibbels *et al.*, 1993).

(vii) Treatment of embryos with estradiol-17β does not

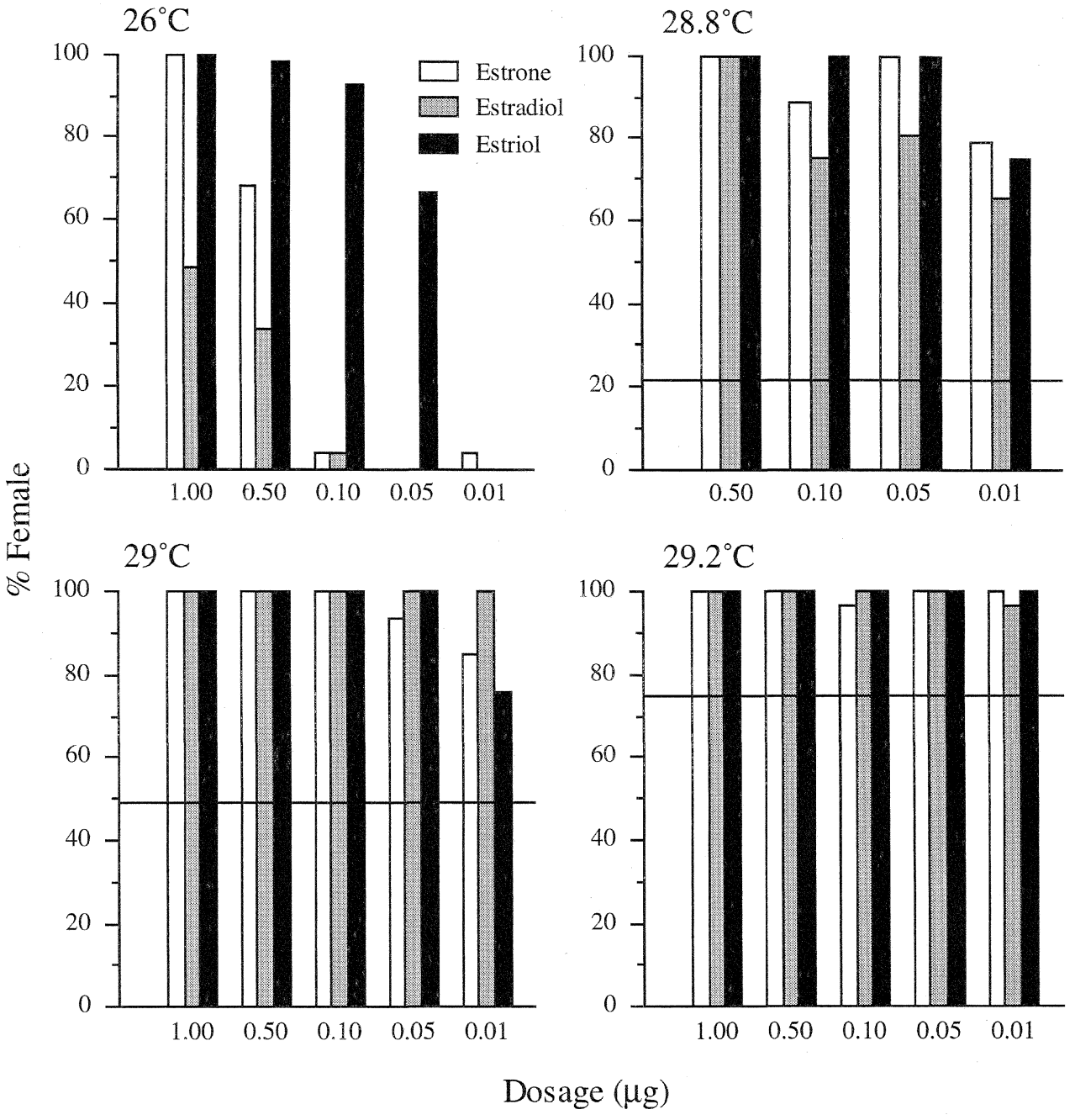


Fig. 1. Effect of different dosages of exogenous estrone, estradiol-17β, and estriol on sex determination in the red-eared slider turtle (*Trachemys scripta*). Eggs were incubated at four different temperatures: all-male producing, male-biased, threshold (that incubation temperature that produces a 1:1 sex ratio), and female-biased. Shown is the percentage of female hatchlings produced at each incubation temperature. Mean percentage of female hatchlings from eggs treated with alcohol alone (control) is represented by the horizontal bar. From Crews *et al.*, 1996b.

lead to a sterile adult. Estrogen-determined females are similar endocrinologically (Rhen *et al.*, 1995) and are as fecund (Crews *et al.*, 1994) as temperature-determined females.

NON-AROMATIZABLE ANDROGENS ARE THE PHYSIOLOGICAL EQUIVALENT OF A MALE-PRODUCING INCUBATION TEMPERATURE

Although exogenous estradiol-17β overcomes the effects of a male-producing temperature and induces female development, neither exogenous testosterone nor dihydrotestosterone can induce testicular development if administered at an incubation temperature that produces all females. However, if eggs incubating at a threshold temperature receive exogenous dihydrotestosterone, most or all of the offspring will be male (Crews and Bergeron, 1994; Wibbels and Crews, 1995: Wibbels *et al.*, 1992). As with exogenous estrogen, this is an "all-or-none" effect in individu-

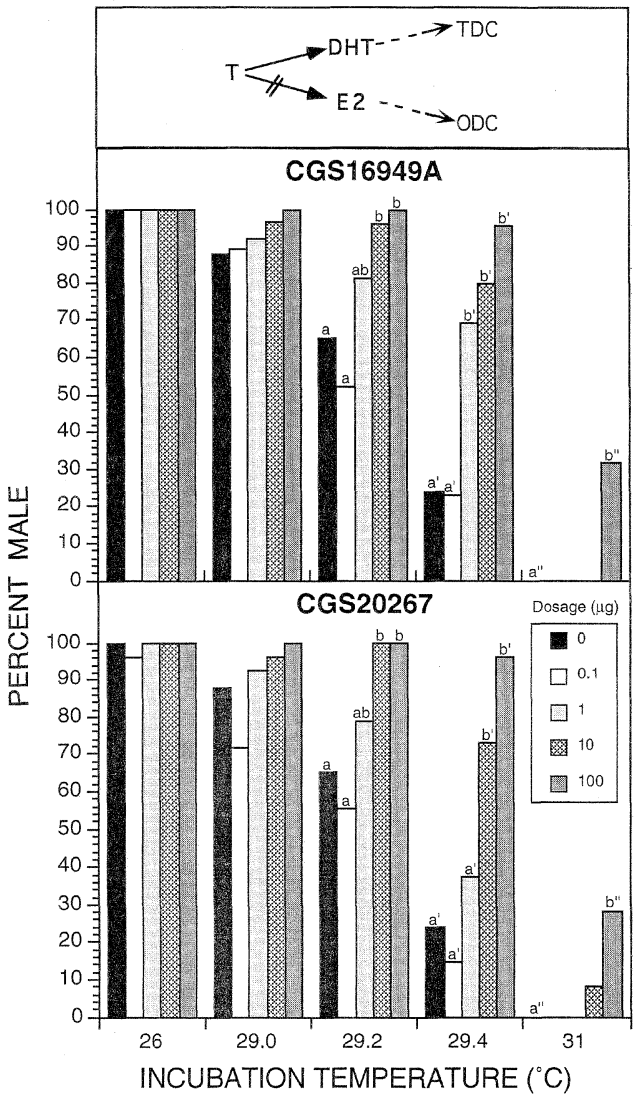


Fig. 2. Administration of aromatase inhibitor induces male development in the red-eared slider (*Trachemys scripta*), a turtle with temperature-dependent sex determination. Metabolism of testosterone to dihydrotestosterone and estradiol-17β by reductase and aromatase enzymes, respectively, depicted at top, with consequent hypothesized effect on testis determining cascade (TDC) and ovary determining cascade (ODC). Double hatched line denotes putative inhibitory action of the aromatase inhibitor. Top panel depicts results with Ciba Geigy 16949A and bottom panel depicts results with Ciba Geigy 20267. Eggs were treated with ethanol (control) or different dosages of the inhibitor at each incubation temperature. Sample sizes in text. Comparisons within each incubation temperature relative to control; values without the same letter are significantly different at a level of at least p=.05. Adapted from Crews and Bergeron, 1994.

als and hermaphrodites are not produced. This suggests that hormone-induced masculinization is specific to non-aromatizable androgens that probably is mediated via AR and, further, that dihydrotestosterone is the physiological equivalent of temperature in the male sex determination cascade.

The same strategy employed in the research with estrogen has been used to determine when dihydrotestosterone exerts its action, the hormone-specificity of male determination, and the relationship between incubation temperature and dihydrotestosterone. The following facts about the role of androgens in male sex determination have been established recently.

(i) Dihydrotestosterone dose-response. Unlike estrogen, administration of dihydrotestosterone to embryos incubated at the threshold temperature does not vary continuously in a dose-dependent fashion (Wibbels and Crews, 1995).

(ii) Critical period. Dihydrotestosterone acts during the temperature-sensitive window. Like estradiol-17β, the ability of dihydrotestosterone to induce male development corresponds to the temperature-sensitive window (Fig. 3) (Crews *et al.*, 1996a). Overall, embryos exhibit a slightly narrower period of "sensitivity" to a low dosage of dihydrotestosterone (1 μg), ranging from Stages 15–21, as opposed to Stages 13–21 at a high dosage (10 μg). At the threshold temperature the range of sensitivity at both dosages ends at Stage 19. There also is an interaction between dihydrotestosterone and the embryonic stage that treatment occurs, reflecting the significant effect of dihydrotestosterone on sex ratio during the temperature-sensitive period but not at the beginning or after this window of development.

(iii) Specificity of androgen in male sex determination. To study androgen-specificity, the following hormonal treatments have been administered to eggs: dihydrotestosterone (as a hormone control), androsterone, R1881, flutamide, progesterone, corticosterone, and 3α- and 3β-androstanediol (Fig. 4) (Crews *et al.*, 1989, 1996a). The percentage of males produced at both dosages is significantly greater than in the corresponding ethanol control group at temperatures that produce mixed sex ratios, indicating the compounds had male-determining effects (Crews *et al.*, 1989, 1996a; Wibbels and Crews, 1992). Taken together this suggests that non-aromatizable androgens are involved in the intiation of male sex determination whereas estrogens and their aromatizable androgen precursors are involved in the initiation of female sex determination.

(iv) Reductase inhibitors block male development. Administration of reductase inhibitor to incubating eggs prevents male development at both threshold and male-biased incubation temperatures (Fig. 5) (Crews and Bergeron, 1994). Administration of testosterone and reductase inhibitor to eggs incubating at a male-producing temperature results in the production of female hatchlings, suggesting that conversion of the precursor (testosterone or androstenedione) to estrogen is favored under these conditions.

(v) Equivalence of dihydrotestosterone and temperature. Unlike the synergism between estradiol-17β and incubation temperature, there is no apparent graded relationship between the dosage of exogenous dihydrotestosterone administered and incubation temperature (Wibbels and Crews, 1995; Crews *et al.*, 1996a).

Taken together, the experiments in which exogenous steroid hormones and their agonists and antagonists, as well

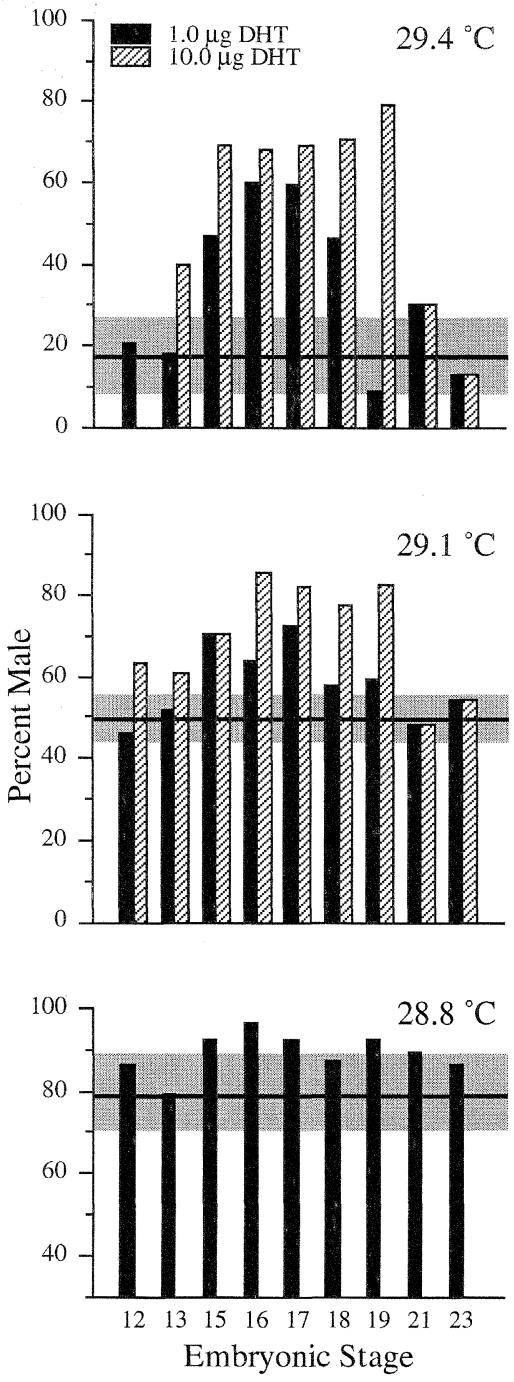


Fig. 3. Effect of different dosages of exogenous dihydrotestosterone administered at different embryonic stages on sex determination in the red-eared slider turtle (*Trachemys scripta*). Three different incubation temperatures are represented: male-biased, threshold (that incubation temperature that produces a 1:1 sex ratio), and female-biased. Shown is the percentage of male hatchlings produced at each incubation temperature. Mean percentage of male hatchlings from eggs treated with alcohol alone (control) at the same embryonic stages are represented by the horizontal bar. Shading indicates the 99% confidence interval; hence, any value lying outside the shaded area is statistically significant at the .01 confidence limit. Stages 15–21 correspond to the temperature-sensitive window. From Crews *et al.*, 1996a.

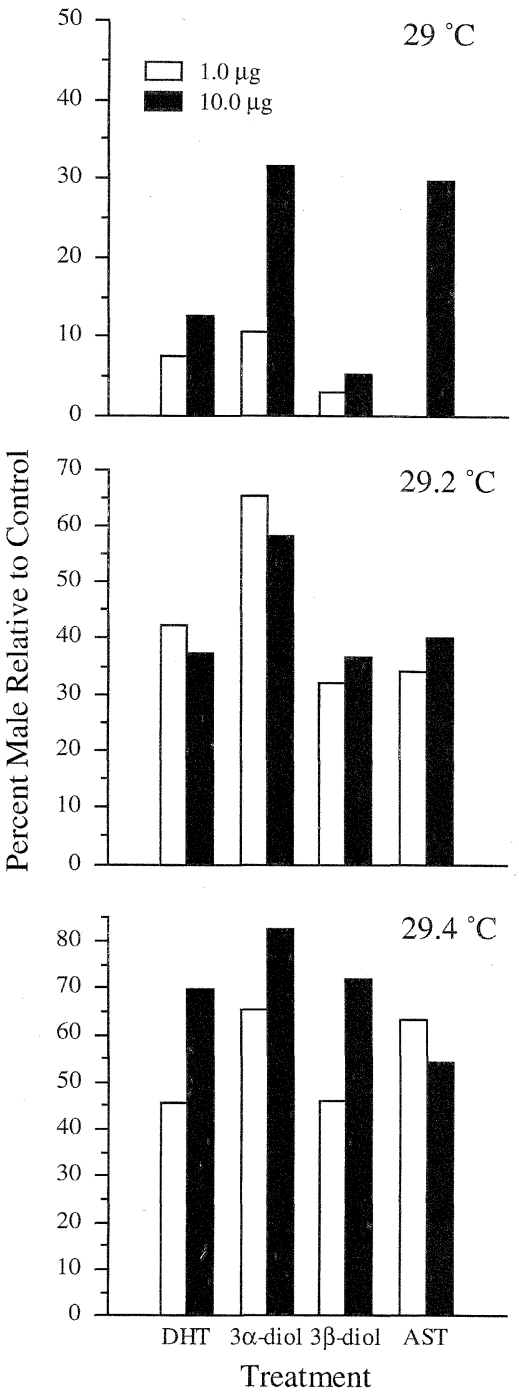


Fig. 4. Effect of dihydrotestosterone (DHT) and its metabolites 3α-androstanediol (3α-diol) and 3β-androstanediol (3β-diol), and androsterone (AST) on male sex determination in the red-eared slider turtle (*Trachemys scripta*). Represented is the effect of two dosages (1.0 and 10.0 μg) of DHT, 3α-diol, 3β-diol, AST compared to the sex ratio produced in the ethanol control (equivalent to zero baseline) at each incubation temperature. From Crews *et al.*, 1996a.

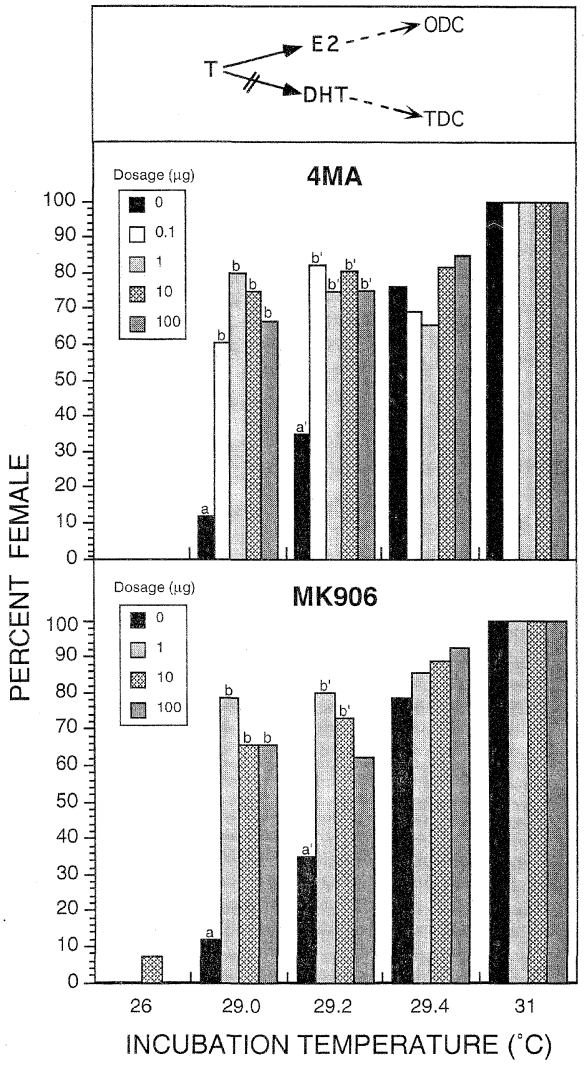


Fig. 5. Administration of reductase inhibitor induces female development in the red-eared slider (*Trachemys scripta*), a turtle with temperature-dependent sex determination. Metabolism of testosterone to estradiol-17β and dihydrotestosterone by aromatase and reductase enzymes, respectively, depicted at top, with consequent hypothesized effect on ovary determining cascade (ODC) and testis determining cascade (TDC). Double hatched line denotes putative inhibitory action of the reductase inhibitor. Top panel depicts results with 4MA and bottom panel depicts results with MK906. Eggs were treated with ethanol (control) or different dosages of the inhibitor at each incubation temperature. Sample sizes in text. Comparisons within each incubation temperature relative to control; values without the same letter are significantly different at a level of at least p=.05. Adapted from Crews and Bergeron, 1994.

as steroidogenic enzyme blockers, are administered to eggs during the temperature sensitive period, have resulted in the hypothesis that estrogens and aromatizable androgens are the physiological equivalent of a female-producing incubation temperature, whereas non-aromatizable androgens are the physiological equivalent of a male-producing incubation temperature (Fig. 6). If incubation temperature creates a temperature-specific steroid hormone milieau within the embryo, how does this occur?

STEROIDOGENIC ENZYMES AND INCUBATION TEMPERATURE

I predict that incubation temperature modulates the activity of genes expressing steroidogenic enzymes and hormone receptors. Since testosterone is converted to dihydrotestosterone by reductase and to estradiol-17β by aromatase, information on these enzymes is critical to understanding temperature-dependent sex determination. In addition to histochemical studies localizing hydroxysteroid dehydrogenase enzymes (Thomas *et al.*, 1992), we have studied the levels of aromatase and 5α-reductase activity in the developing embryo. Tritiated water assays were designed to maximize enzyme levels *in vitro*, measuring enzyme capacity in both fresh intact and frozen homogenate tissue. Kinetic studies established the optimal buffer and incubation temperature conditions. Incubations for various periods (.5, 1, 2, 4 hrs) indicate that in the AKG tissue reductase levels are initially high and then decline at I hr and beyond, perhaps due to depletion of substrate or co-factor; aromatase continues to increase, reaching a plateau at 2 hr. Adding estradiol-17β to the incubation reaction in varying ratios to the substrate testosterone has no apparent effect on aromatase or reductase activity. Thus, if the product of aromatase plays a feedback activation or inhibition role in the cascade of sex determination, then it would have to be at the gene level. This hypothesis is presently being tested by assaying embryos which have had exogenous estradiol-17β administered to eggs during the temperature-sensitive window while incubating at a male-producing temperature to insure female development. Addition of aromatase inhibitor (CGS 20267) to the incubation reaction effectively reduces estrogen production in AKG incubates. Incubation of fresh intact AKG tissue from embryos at the beginning, during, and after the temperature-sensitive window indicate that the ratio of reductase to aromatase is greater at the male-producing temperature than at the female-producing temperature (Fig. 7).

INCUBATION TEMPERATURE INFLUENCES GENE TRANSCRIPTION

Hormones have no biological function unless there are receptors to detect their presence. The existence of estrogen-concentrating tissues during the critical period for sex determination supports the hypothesis that estrogen-ER dependent mechanisms may have a role in female sex determination. It becomes important, therefore, to identify, locate, and quantify ER and AR in the embryo. The DNA- and the steroid-binding domains for each type of steroid receptor have been highly conserved at the amino acid level during vertebrate evolution (Evans, 1988). For example, published sequences of the ER of trout (Pakdel *et al.*, 1989), frog (Weiler *et al.*, 1987), chicken (Krust *et al.*, 1986; Maxwell *et al.*, 1987),

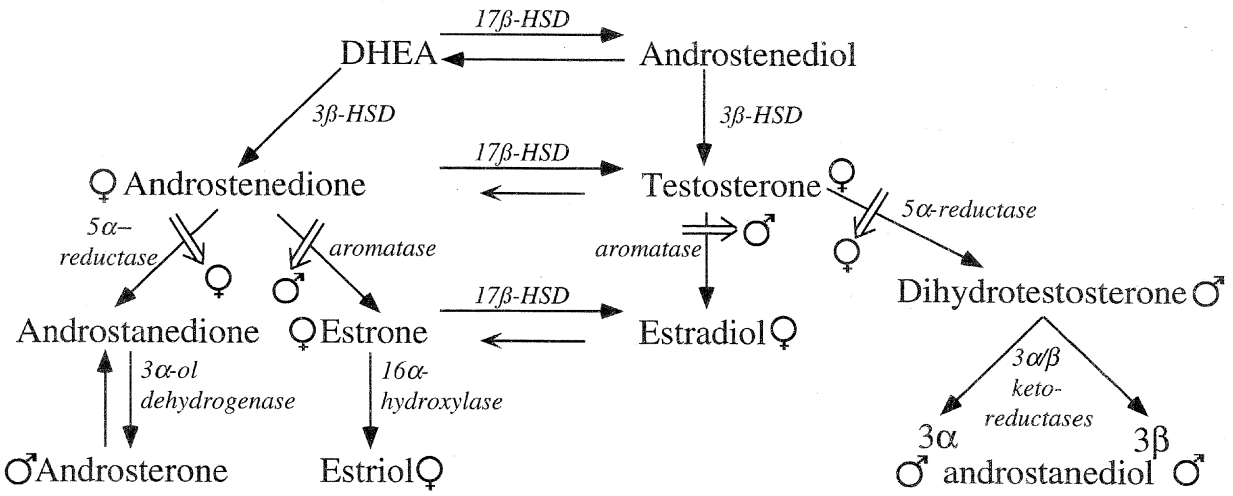


Fig. 6. Effects of various hormones and enzyme inhibitors on sex determination in the red-eared slider turtle (*Trachemys scripta*). Note that aromatizable androgens are female-determining, whereas non-aromatizable androgens are male-determining. Illustrated is the process of steroid metabolism of androgens and estrogens with steroidogenic enzymes in italics. The cytochrome P450 17α-hydroxylase/C_{17-20} lyase is involved in formation of androstenedione, the immediate precursor of testosterone. Androstenedione in turn is converted by aromatase to estrone and by 17β-hydroxysteroid dehydrogenase to testosterone. Testosterone is converted by the cytochrome P450 enzyme aromatase to estradiol-17β whereas estrone is converted by 17β-hydroxysteroid dehydrogenase to estradiol-17β or by 16α-hydroxylase to estriol. Symbol indicates male-determining or female-determining effect of the ligand on sex determination. Break symbols indicate the effect of enzyme inhibitors on sex determination. From Crews *et al.*, 1996b.

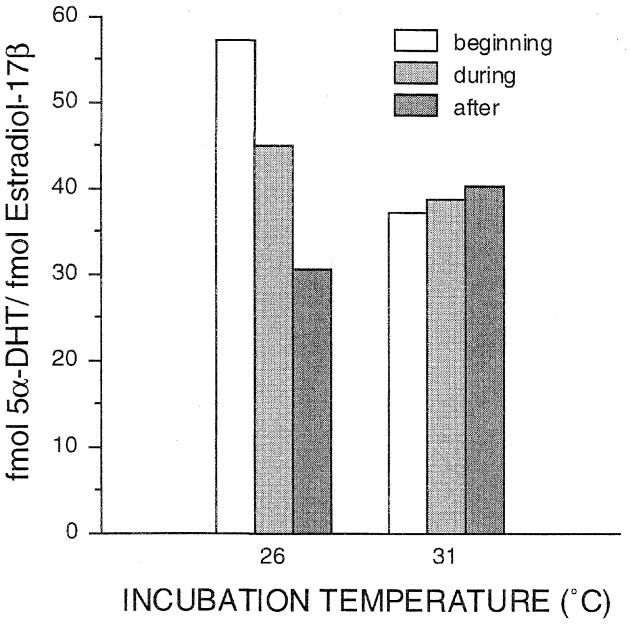


Fig. 7. Enzyme ratios (5α-reductase/aromatase) in adrenal-kidney-gonad tissue from embryos incubating at a male-producing versus a female-producing temperature at the beginning, during, and after the temperature-sensitive window. Determinations made on same tissue and expressed per mg protein.

rat (Koike *et al.*, 1987), and human (Green *et al.*, 1986; Greene *et al.*, 1986) have virtually identical amino acid sequences in these areas. Because of such conservation in sequence in other vertebrates, DNA primers designed from these regions have been very successful in producing turtle cDNA clones of these receptors and steroidogenic enzymes by RT-PCR.

(i) Estrogen and androgen receptor. Messenger RNA was isolated from oviducts of adult female turtles (for ER) and from AKG complex of hatchling turtles incubated at a male-producing temperature (for AR). Using these primers and RNA preparations in reverse transcriptase-polymerase chain reactions, cDNA products of the predicted sizes for both ER and AR were obtained. These cDNAs were cloned into pCRII vector (Invitrogen). Three independent clones spanning 0.9kb were obtained for ER. Six independent clones spanning 0.39 kb were obtained for AR. Sequencing of the clones showed that both ER and AR had the characteristic conservation of amino acid sequence in the DNA-(100%) and steroid-binding (>90%) domains, but had many differences in the poorly conserved hinge region (Fig. 8).

(ii) Aromatase gene. Clones of the aromatase cDNA were also obtained by the RT-PCR approach using tissue from Stage 19 embryos (during the temperature-sensitive window) incubated at a female-producing temperature. DNA primers were designed from conserved heme-binding and aromatic regions deduced by comparison of published aromatase sequences of channel catfish (Trant, 1994), trout (Tanaka *et al.*, 1992), diamondback terrapin (Jeyasuria *et al.*, 1994), chicken (McPhaul *et al.*, 1988), rat (Hickey *et al.*, 1990), cow (Hinshelwood *et al.*, 1993), and human (Corbin *et al.*, 1988; Harada, 1988). A nested primer strategy was used wherein the PCR products from the first round of PCR served as template for a second round of PCR using primers internal to those used in the first round. With the assistance of Y.

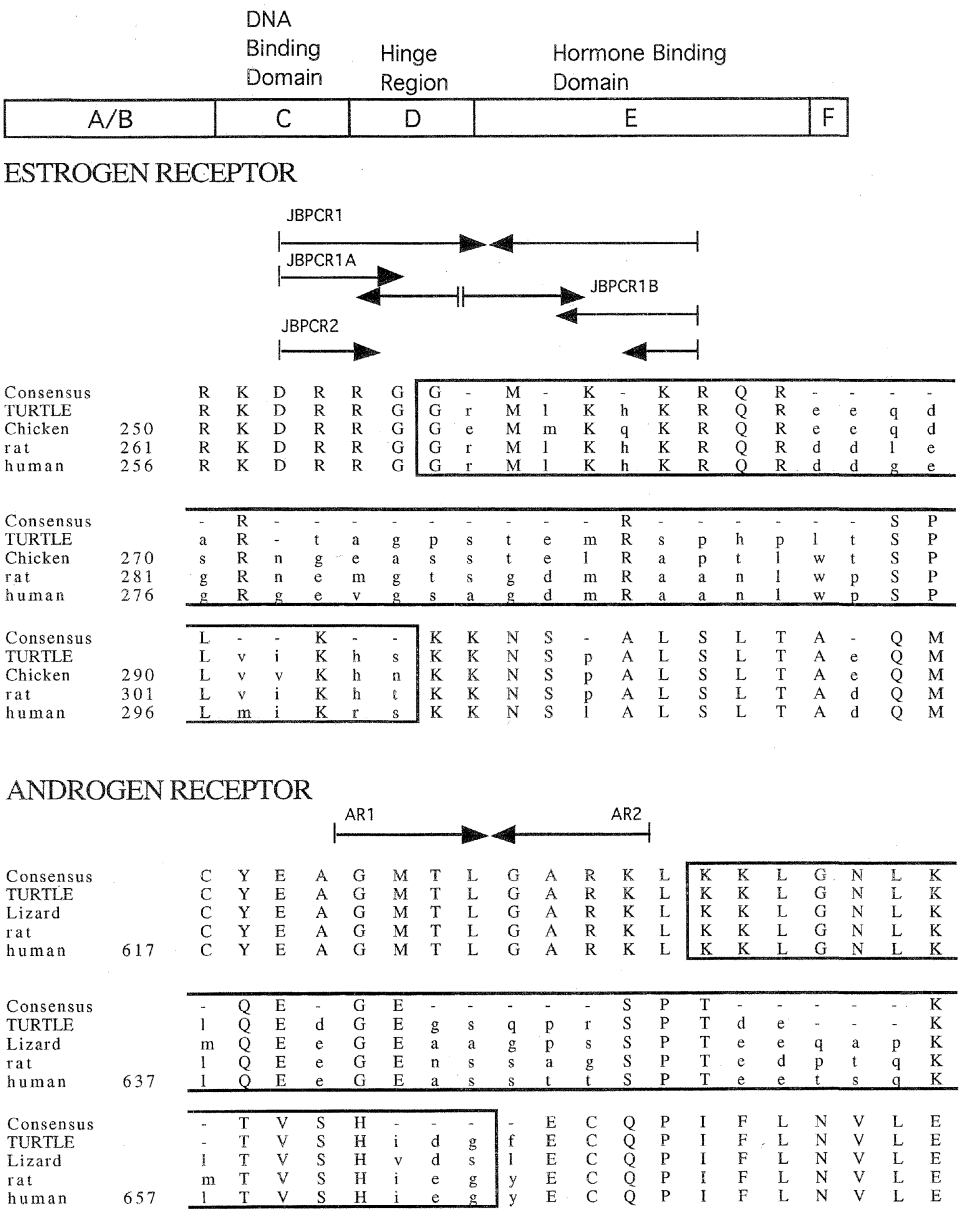
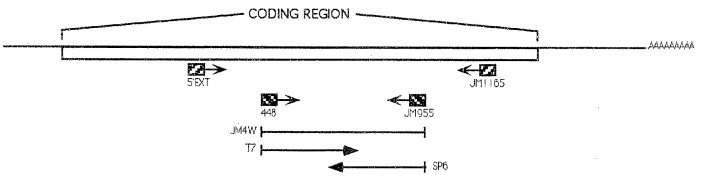


Fig. 8. Schematic of turtle estrogen receptor (ER) and androgen receptor (AR) gene. Top portion shows functional domains of steroid hormone receptors with subclones indicating regions sequenced below. The lower portion presents consensus sequence showing a portion of the deduced amino acid sequence for the turtle ER and AR and corresponding regions of published sequences for these hormone receptors of other species. Boxed segment of the sequence signifies the hinge region. Dashes in the consensus sequence indicate non-conserved amino acids.

Nagahama and M. Tanaka, a cDNA library has been constructed from mRNA extracted from brains of male and female hatchlings. Screening this library should provide us with full-length cDNA clones, particularly the region which covers exon I. This provides an opportunity to screen genomic libraries for 5'-flanking regions of the genes of interest. Cloning the 5' flanking regions will be necessary to study the transcription factors and enhancer sequences involved in the regulation of expression of the genes involved in temperature-dependent sex determination. The cDNA generated by the second round was the expected size for aromatase (0.56 kb) and cloning and sequencing of three independent clones confirmed their identity. Figure 9 presents a comparison of the deduced amino acid sequence of the aromatase sequence with aromatase clones of other species. A similar strategy is being undertaken for reductase and several independent clones have been obtained.

Another important question concerns the mechanism whereby temperature and steroids regulate the expression of steroid hormone receptor genes. This follows from the fact that steroid hormones will have no function unless there are specialized receptors to detect their presence.



TURTLE	S	N	K	L	F	L	G	I	P	L	D	E	N	S	I	V	L	K	I	Q
trout	-	-	R	-	-	-	-	V	-	-	N	-	K	E	L	L	Q	-	-	-
chicken	-	-	-	-	-	-	-	V	-	-	-	-	S	A	-	-	-	-	-	-
rat	-	-	T	-	-	-	-	-	-	-	-	-	S	A	-	-	K	-	-	-
human	-	-	T	-	-	-	R	-	-	-	-	-	S	A	-	-	V	-	-	-
TURTLE	N	Y	F	D	A	W	Q	A	L	L	L	K	P	D	I	F	F	K	I	S
trout	K	-	-	-	T	-	-	T	V	-	I	-	-	-	V	Y	-	-	L	D
chicken	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
rat	G	-	-	N	-	-	-	-	-	-	I	-	-	N	-	-	-	-	-	-
human	G	-	-	-	-	-	-	-	-	-	I	-	-	-	-	-	-	-	-	-
TURTLE	W	L	Y	K	K	Y	E	K	S	V	K	D	L	K	E	A	I	E	V	L
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rat	-	-	-	R	-	-	-	R	-	-	-	-	-	-	D	E	-	-	I	-
human	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D	-	-	-	-	-
TURTLE	I	E	Q	K	R	Q	R	L	S	T	V	E	K	L	E	E	H	M	D	F
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chicken	-	-	-	-	-	-	K	-	-	-	-	-	-	-	D	-	-	-	-	-
rat	V	-	K	-	-	-	K	V	-	S	A	-	-	-	-	D	C	-	-	-
human	-	A	E	-	-	R	-	I	-	-	E	-	-	-	-	-	C	-	-	-
TURTLE	A	S	Q	L	I	F	A	Q	S	R	G	D	L	T	G	E	N	V	N	Q
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chicken	-	-	-	-	-	-	-	-	N	-	-	-	-	-	A	-	-	-	-	-
rat	-	T	D	-	-	-	-	E	R	-	-	-	-	-	K	-	-	-	-	-
human	-	T	E	-	-	L	-	E	K	-	-	-	-	-	R	-	-	-	-	-
TURTLE	C	V	L	E	M	M	I	A	A	P	D	T	L	S	V	T	L	F	F	M
trout	-	-	-	-	-	V	-	-	-	-	-	-	-	-	I	S	-	-	-	-
chicken	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	-
rat	-	I	-	-	-	L	-	-	-	-	-	-	M	-	-	-	-	Y	V	-
human	-	I	-	-	-	L	-	-	-	-	-	-	M	-	-	S	-	-	-	-
TURTLE	L	V	L	I	A	E	H	P	K	V	E	E	D	M	M	K	E	I	Q	A
trout	-	L	-	L	K	Q	N	-	D	-	-	L	Q	L	L	E	-	-	D	T
chicken	-	L	-	-	-	D	D	-	T	-	-	-	K	-	-	R	-	-	E	T
rat	-	L	-	-	-	-	Y	-	E	-	-	T	A	I	L	-	-	-	H	T
human	-	F	-	-	-	K	-	-	N	-	-	-	A	I	I	-	-	-	-	T
TURTLE	V	I	G	D	R	D	V	Q	S	N	D	M	S	N	L	K	V	V	E	N
trout	A	-	-	-	-	E	L	H	N	S	-	L	Q	-	-	R	-	L	-	S
chicken	-	M	-	H	-	E	-	-	-	D	-	-	P	-	-	-	I	-	-	-
rat	-	V	-	-	-	-	I	R	I	G	-	V	Q	-	-	-	-	-	-	-
human	-	-	-	E	-	-	I	K	I	D	-	I	Q	K	-	-	-	M	-	-
TURTLE	F	I	N	E	S	M	R	Y	Q	P	V	V	D	L	V	M	R	K	A	L
trout	-	-	-	-	-	L	-	F	H	-	-	-	-	F	T	-	-	R	-	-
chicken	-	-	Y	-	-	-	-	-	-	-	-	-	-	-	I	-	-	-	-	-
rat	-	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	R	-	-
human	-	-	Y	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TURTLE	Q	D	D	V	I	D	G													
trout	S	-	-	-	-	S	-													
chicken	-	-	-	-	-	-	-													
rat	E	-	-	-	-	-	-													
human	E	-	-	-	-	-	-													

Fig. 9. Schematic of turtle aromatase gene. Top portion shows region which has been characterized for the red-eared slider and location of primers used in RT-PCR. Bottom portion represents consensus sequence showing amino acid homology comparison between a portion of the 0.56 kb clone of turtle and published sequences of other species. A dash indicates identity to turtle amino acid; highly conserved areas are shown by box (Ozols peptide region) and shading (aromatic region).

(iii) Localization and quantification of transcription of hormone receptor and steroidogenic enzyme genes during embryogenesis using *in situ* hybridization. Since estrogen uptake is similar at male- and female-producing temperatures, and preliminary results indicate that ER-mRNA appears to be expressed in both tissues (albeit with different localization, see Fig. 7), the presence of ER would not be a limiting factor for determining the development in the male or the female direction. Indeed, in the mouse as in the turtle, ER is present in the testes prior to phenotypic differentiation of the gonad (Greco *et al.*, 1992) and probably plays an important role in male sex determination (Lubahn *et al.*, 1993). Interestingly,

ER-mRNA continues to be present in the differentiated testis *after* the embryo loses its sensitivity to exogenous estrogen treatment. Thus, temperature could alter the level of ER-mediated gene expression by (i) altering the availability of estrogens in the embryo (e.g., by changing the activity of estrogen delivery systems) and hence altering of the activity of the ER, (ii) activating other transcription factors that act in concert with ER during sex determination, and/or (iii) post-translation processing.

In situ hybridization enables both the localization and quantification of the message produced during the temperature-sensitive window. Future questions to be addressed include: (i) Are the mRNAs of hormone receptors and steroidogenic enzymes synthesized in the same cells? (ii) When are these genes expressed during development? (iii) Do steroids modulate expression of their receptors and steroidogenic enzymes?

The cDNA clones have generated species-specific nucleic acid probes for *in situ* hybridization to identify ER-, AR-, and aromatase-mRNA at tissue and cellular levels. Consecutive frozen sections are being utilized for *in situ* hybridization using various probes. Present efforts are focused on quantifying the amount of ER-mRNA in different tissues and within cells in these tissues in embryos from the various times and conditions. Figure 10 illustrates the localization of ER-mRNA in the beginning, during, and after the temperature-sensitive window in embryos incubating at male- and female-producing temperatures, and Figure 11 presents initial data on the abundance of message at the different Stages. It is evident that at the beginning the temperature-sensitive window, when only a gonadal ridge exists, ER-mRNA is evenly distributed throughout the structure. During the window, when the gonad has distinct medullary and cortical compartments, transcript is localized in the cortex in embryos incubating at a female-producing temperature and in the remnants of the cortex (arrow) and in whorls in the dominant medullary component in embryos incubating at a male-producing temperature. Following the window the gonad is fully formed and ER-mRNA is localized throughout the ovary or in the sex cords of the testis.

Exogenous estrogen is capable of overriding the effect of a male-producing temperature at the beginning and during the temperature-sensitive window. Therefore, it is important to identify the gonadal cells that are competent to respond to estrogen by virtue of the presence of the ER in these cells. This can be done at two molecular levels: at the mRNA level using *in situ* hybridization and at the protein level using ER-specific antibodies. The presence of estrogens and ER in the differentiated testis in both newborn turtles and mammals implies additional physiological roles for estrogens apart from switching differentiation from a male to a female pathway. The latter approach will involve producing antibodies through cloned expression vectors. This is necessary because available monoclonal and polyclonal antibodies do not show immunoreactivity in turtles (Gahr and Crews, 1996). The ER and AR clones (which include the nonconserved regions) will be subcloned into the pET-family of bacterial expression vectors (Rosenberg *et al.*, 1987) to produce large quantities of fusion protein. These fusion proteins will be purified by conventional biochemical techniques and used to raise polyclonal antibodies to ER and AR in rabbits. A potential drawback of this approach is that there can be great variation in antigenicity both within and between rabbits. Further, polyclonal antibodies may cross-react with other proteins. To reduce nonspecific binding with other proteins, the antibodies will be affinity-purified using the expressed ER (and AR) proteins coupled to cyanogen bromide sepharose.

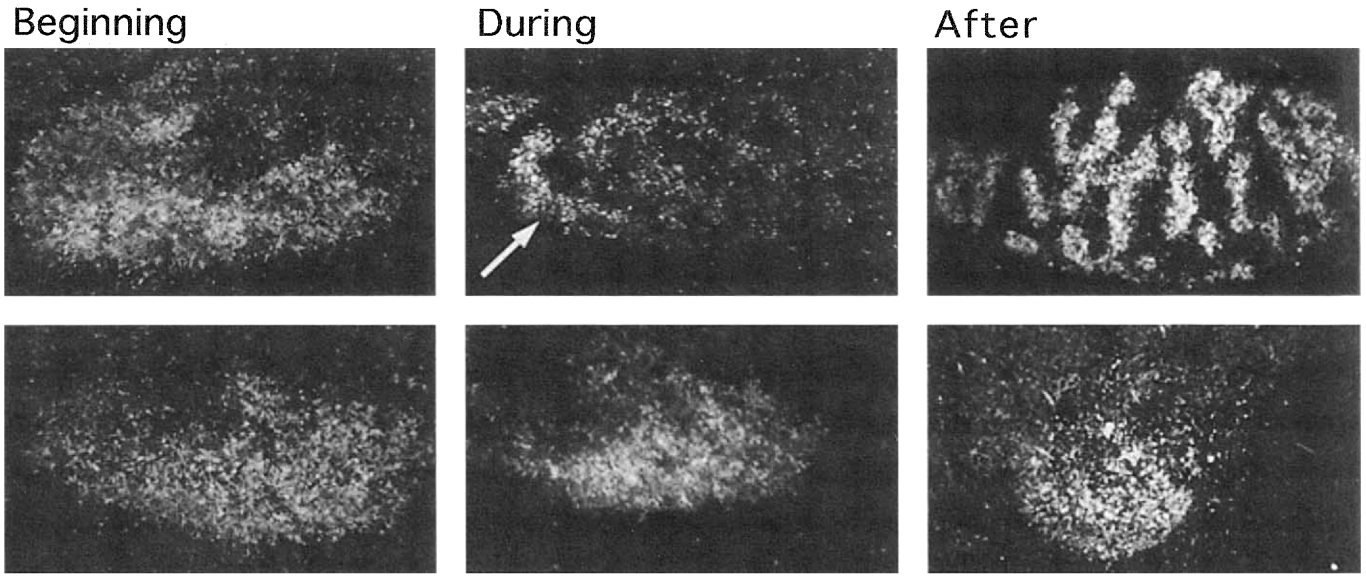

Fig. 10. Dark-field images of *in situ* hybridization of ER-mRNA in the gonad at the beginning (left, 400×), during (middle, 200×), and after (right, 100×) the temperature-sensitive window in embryos incubating at a male- (top) and a female-producing (bottom) temperature.

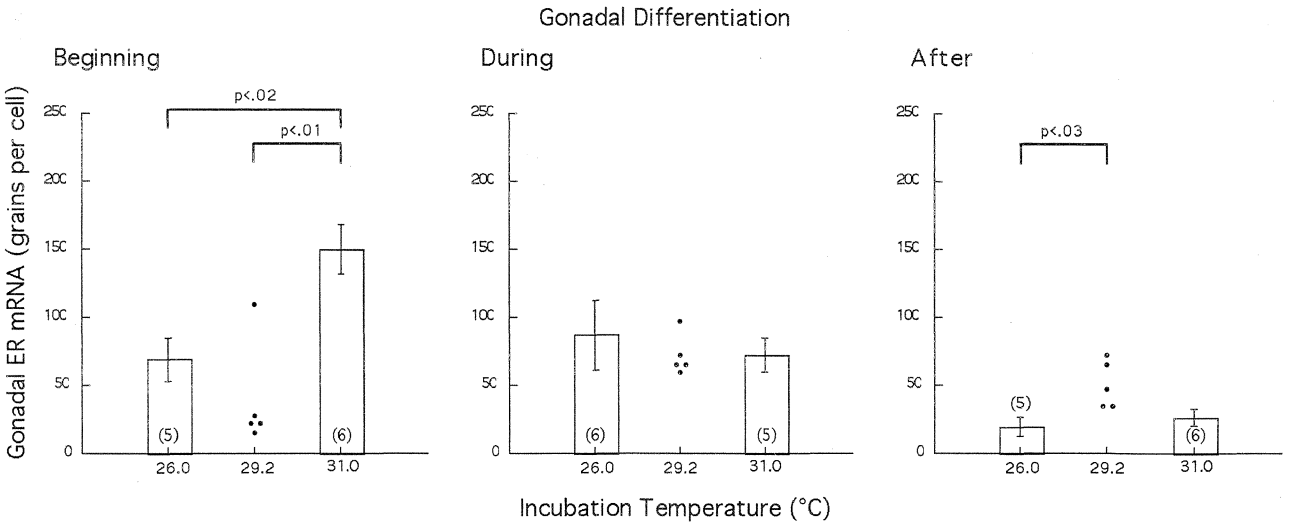

Fig. 11. Quantitative *in situ* hybridization of ER-mRNA in the gonad at the beginning (Stage 15), during (Stage 19), and after (Stage 23) the temperature-sensitive window in embryos incubating at a male-producing, threshold, and a female-producing temperature. Distribution of values for the threshold incubation temperature, which produces equal numbers of both sexes, illustrates presumptive male and female embryos. Sample sizes shown in parentheses.

CONCLUSION

In many egg-laying reptiles the incubation temperature of the egg, not sex chromosomes, determines the sex of the offspring. In temperature-dependent sex determination, sex determination is an "all-or-none" process and intersexes are rarely formed. How is the external signal of temperature transduced into a genetic cascade that determines gonadal sex and channels sexual development? Evidence indicates that incubation temperature modifies the endocrine microenvironment of the embryo and that steroid hormones serve as the proximate trigger for sex determination. Using the red-eared slider turtle as an animal model system, my laboratory has demonstrated that both male and female development are active processes requiring the simultaneous activation of one sex determining cascade and suppression of the other cascade rather than the organized/default system characteristic of mammals with genotypic sex determination.

The ability to manipulate sex in species with temperature-dependent sex determination by incubation temperature and/or exogenous hormones and other agents provides unparalleled experimental control, thereby enabling more detailed analysis of the normal pattern of gene expression during sex determination than is possible with other amniote vertebrate species having heritable sex chromosomes. Indeed, the cascade leading to sex determination and sexual differentiation appear similar in amniote vertebrates having sex chromosomes or temperature-dependent sex determination. The only exception appears to be the initiating trigger; in the former it is the presence or absence of a testis-determining gene that determines sex and in the latter, it is incubation temperature.

Further research on temperature-dependent sex determination is important for several reasons (Crews, 1994). First, it draws attention to the fact that temperature and steroid hormones can play a pivotal role in sexual development in an amniote vertebrate. Temperature has not been adequately investigated as a factor in steroid hormone action in homeotherms despite numerous studies documenting how both hormone responsiveness and hormone action are markedly dependent on temperature. Also, it has long been thought that in sex determination by sex chromosomes, steroid hormones of maternal or embryonic origin are not involved on gonad formation. The work with reptiles with temperature-dependent sex determination indicates that this conclusion may be premature. Second, since temperature-dependent sex determination represents the evolutionary precursor to sex chromosomes, temperature and steroid effects in sex determination may be partly or wholly masked in homeotherms. Third, this work speaks directly to the issue of female sex determination. Research on temperature-dependent sex determination provides support for the existence of specific ovary-determining gene(s) just as there exists testis-determining gene(s). This is in contrast to most effort today in vertebrate sex determination research which focuses on elucidating the mechanisms underlying male development while relegating female development to a passive or default state.

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