Temperature and non-aromatizable androgens: a common pathway in male sex determination in a turtle with temperature-dependent sex determination?

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Abstract

This study addressed the hypothesis that, in the red-eared slider turtle, *Trachemys scripta*, non-aromatizable androgens are the physiological equivalent of temperature in determining male development. In the first experiment, eggs were treated in the middle of the temperature-sensitive period with 1·0 or 10·0 μg androsterone, 5α-dihydrotestosterone, 3α-androstanediol, or 3β-androstanediol, while at an all-male, male-biased, or one of two female-biased incubation temperatures. In the second experiment, eggs were treated with the same dosages of dihydrotestosterone at different stages of embryonic development while at a male-biased, threshold, or a female-biased incubation temperature. Results of experiment one indicated that hormone-induced masculinization is specific to non-aromatizable androgens. Results of experiment two indicated that the sensitivity to dihydrotestosterone corresponds to the temperature-sensitive window during development. Further, there is a dose–response relationship but no apparent synergism between exogenous dihydrotestosterone and incubation temperature. When considered with other research, it is suggested that non-aromatizable androgens and their products are involved in the initiation of male sex determination whereas oestrogens and their aromatizable androgen precursors are involved in the initiation of female sex determination.

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Introduction

In many turtles, the temperature of the incubating egg determines the sex of the hatchling. The mechanism of action of temperature is not well understood, although steroid hormones appear to be involved. There is general agreement that, in female sex determination, oestrogen formation is the physiological equivalent of incubation temperature. Experiments with the red-eared slider (*Trachemys scripta*) indicate the following. (i) Exogenous oestrogen can over-ride the effects of a male-producing incubation temperature (Gutzke & Bull 1986, Crews *et al.* 1991). (ii) The temperature-sensitive window is in the middle third of development (Wibbels *et al.* 1991*a*) and corresponds to the oestrogen-sensitive window (Gutzke & Chymiy 1988, Wibbels *et al.* 1991*b*). (iii) Steroid-induced feminization is mediated via an oestrogen-specific receptor (Crews *et al.* 1989, Wibbels & Crews 1992). (iv) Oestrogen is concentrated primarily in the liver and the adrenal-kidney-gonad area (Gahr *et al.* 1992). (v) Administration of the aromatizable androgens testosterone or androstenedione to eggs incubating at a male-producing temperature results in female hatchlings, presumably through their metabolic conversion to oestrogen (Wibbels & Crews 1992, 1995, Crews & Bergeron 1994, Crews *et al.* 1995). (vi) Application of aromatase inhibitor to eggs incubating at a female-producing temperature results in male hatchlings (Crews & Bergeron 1994, Wibbels & Crews 1994). Administration of testosterone in combination with aromatase inhibitor at a male-producing incubation temperature blocks the testosterone-induced feminization and all offspring are male. Similarly, co-administration of testosterone and aromatase inhibitor to eggs incubating at a female-producing temperature results in all male offspring. (vii) There is a synergism between exogenous oestrogen and incubation temperature (Wibbels *et al.* 1991*b*). (viii) The morphological changes occurring in response to exogenous oestrogen are indistinguishable from the changes induced by incubation temperature (Wibbels *et al.* 1993). Taken together, these data are consistent with the hypothesis that temperature and oestrogen work in the same biochemical pathway for female sex determination.

Similarly, male sex determination appears to be an independent sex-determining cascade that is induced by non-aromatizable androgen (Crews *et al.* 1994). Although exogenous 5α-dihydrotestosterone (DHT) will not induce testicular development when administered at an all-female

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producing incubation temperature, if administered to eggs incubating at a threshold temperature (that temperature which produces equal numbers of males and females), most or all of the offspring will be male (Wibbels *et al.* 1992, Wibbels & Crews 1995). As with exogenous oestrogen, this is an 'all-or-none' effect and hermaphrodites are not produced. In addition, administration of a specific reductase inhibitor to incubating eggs prevents male development at both threshold and male-biased incubation temperatures (Crews & Bergeron 1994). Administration of both testosterone and reductase inhibitor to eggs at an all-male incubation temperature results in the production of female hatchlings, suggesting that conversion of testosterone to oestrogen is favoured under these conditions.

However, before the hypothesis that hormone-induced masculinization is androgen-specific and is mediated via androgen receptor and, further, that non-aromatizable androgens are the physiological equivalent of temperature in male sex determination, additional evidence is required. This study established that the effect is androgen-specific, that the sensitivity to androgen corresponds to the temperature-sensitive window during development, and that a dose–response relationship exists, yet there is no apparent synergism between exogenous DHT and incubation temperature.

Materials and Methods

Freshly laid eggs were obtained commercially (Robert Kliebert, Hammond, LA, USA). After transport to our laboratory, they were held at room temperature until viability was established by candling (Stage 12 based on criteria described by Wibbels *et al.* 1991*a*). They were then placed in containers with moistened vermiculite (vermiculite:water, 1:1) and the containers placed in reach-in incubators (Precision Scientific, Chicago, IL, USA) programmed to provide a constant temperature (± 0·1 °C). In addition to monitoring the digital readout of the chamber by members of the laboratory throughout the day, the temperature in each incubator was also checked daily by visual inspection of an internal certified calibrated thermometer as well as by a HOBO temperature logger (Onset Computer Corp., Pocasset, MA, USA) which sampled incubator temperature every 15 min.

Eggs were divided at random into control and experimental treatments. They were spotted with either 5 µl 95% ethanol (control) or steroid dissolved in 5 µl 95% ethanol using the topical administration method described in Crews *et al.* (1991). After receiving treatment, all eggs were returned to their respective incubators until they hatched. Embryonic development was monitored by candling eggs and by dissecting two to four eggs approximately twice a week to verify specific developmental stages.

Within 1 week after hatching, gonadal sex and developmental status of the Müllerian ducts were assessed macroscopically by examination of the reproductive tracts under a dissection microscope. The gonads of hatchling red-eared sliders are relatively well differentiated and, with rare exception, appear distinctly testicular or ovarian when viewed under a dissection microscope. Ovaries are long and flat whereas testes are shorter, more round, and have visible sex cords (Crews *et al.* 1991, Wibbels *et al.* 1991*a*). The gonads of three to five individuals from each experimental group were processed for histological examination of the gonad to confirm sex assignment. In all instances, the histological assessment of sex coincided with the macroscopic assignment of sex. The oviducts were examined and scored as either absent, regressed but visible, or normal (as in a typical female hatchling). The presence and development of the phallus was also noted. Normally, in the female hatchling, the cloaca is without pigment and the phallus development is rudimentary, if present at all, with the shaft posterior to the cloacal opening smooth and unmodified. In a hatchling male, the tissue surrounding the phallus is pigmented and the shaft has the beginnings of an elementary modification on the distal end. As found in previous studies (e.g. Wibbels *et al.* 1992), the administration of exogenous DHT has either an accelerated or extreme effect on phallus development. Accelerated development is indicated by a hypertrophied and more solid shaft with a well-developed caudal portion: the genital skin also has a greater amount of pigmentation than typically seen in a normal male and is visible through the cloacal wall. High dosages commonly lead to a greatly hypertrophied structure which has a swollen and misshapen appearance and is everted through the cloacal opening: the area is heavily pigmented.

Experiment one: specificity of androgen

To study androgen specificity, a total of 975 eggs were divided randomly into experimental and control groups incubated at one of four temperatures: an all-male incubation temperature (26·0 °C) which produced a 29:0 male:female sex ratio in the ethanol control, a male-biased incubation temperature (29·0 °C) which produced a 15:9 male:female sex ratio in the ethanol control, or one of two female-biased incubation temperatures (29·2 °C or 29·4 °C), which produced an 8:15 and a 4:25 male:female sex ratio in the ethanol control respectively. Experimental eggs (n=870) received either a low (1·0 µg) or a high (10·0 µg) dosage of steroid administered to the egg at Stage 17 of embryonic development. The following hormones were tested: DHT, 3α-androstanediol (3α-diol), 3β-androstanediol (3β-diol), or androsterone (AST). All hormones were from Sigma Chemical Co. Ltd, St Louis, MO, USA. Experimental eggs for the different incubation temperatures averaged 27 eggs per group at the low dosage (n=436) and averaged 27 eggs per group at

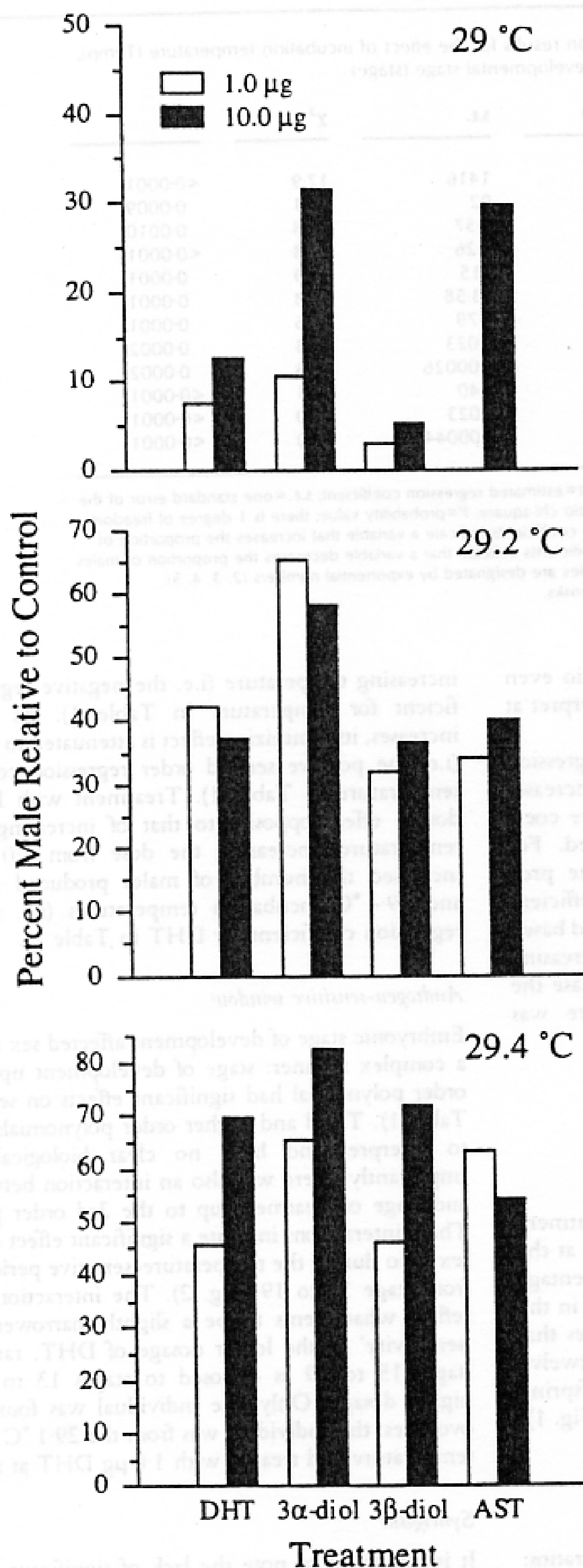


Figure 1 Effect of DHT and its metabolites 3α-diol and 3β-diol, and AST on male sex determination in the red-eared slider turtle (*Trachemys scripta*). Represented is the effect of two dosages (1·0 and 10·0 µg) of DHT, 3α-diol, 3β-diol, and AST compared with the sex ratio produced in the ethanol control at each incubation temperature (equivalent to zero baseline).

the high dosage (n=434). Control eggs (n=105) averaged 26 eggs per group.

Experiment two: androgen-sensitive period and synergism

To determine if the ability of DHT to induce male development has a critical period that corresponds to the temperature-sensitive window, a total of 3814 eggs were divided randomly into experimental and control groups. Experimental eggs (n=2582) received either a low (1·0 µg) or a high (10·0 µg) dosage of DHT administered at embryonic stage 12, 13, 15, 16, 17, 18 19, 21, or 23; control eggs (n=1232) received a 5 µl bolus of 95% ethanol at the same embryonic stage.

For the low dosage, eggs were incubated at either a male-biased incubation temperature (28·8 °C), a threshold incubation temperature producing a 50:50 sex ratio (29·1 °C), or a female-biased incubation temperature (29·4 °C). Sample sizes of the experimental groups at 28·8 and 29·4 °C averaged 27 per group at each of the nine developmental stages for a total of 242 and 240 eggs respectively. Control groups at 28·8 °C averaged 26 per group for a total of 232 eggs. Control groups at 29·4 °C averaged 28 per group for a total of 251 eggs. Sample sizes of the experimental groups at 29·1 °C averaged 26 per group with two groups at each developmental stage for a total of 473 eggs. Control groups at 29·1 °C consisted of 18 groups, two at each developmental stage, and averaged 28 eggs per group for a total of 473 eggs.

For the high dosage, eggs were incubated at either 29·1 or 29·4 °C. Sample sizes of the experimental groups at 29·1 °C averaged 27 per group with two groups at each developmental stage for a total of 456 eggs. Sample sizes of the experimental groups at 29·4 °C averaged 27 per group for a total of 215 eggs.

Sex ratio was tabulated as a nominal dependent variable. Analysis of sex ratios utilized multiple logistic regression; a backwards stepwise procedure was used to reduce the model to one including only significant independent variables (Chatterjee & Price 1977, Sokal& Rohlf 1981). Logistic regression is the most appropriate model to use for this experiment because incubation temperature, DHT dosage, and stage of development (which is a somewhat artificial division of a continuous process) are on a continuous scale of measurement and the dependent variable is frequency data. Independent variables were removed at a P>0·1 to arrive at the final model (in Table 1). Polynomials were included in the model if they explained

Table 1 Multiple logistic regression results for the effect of incubation temperature (Temp), dosage of DHT treatment, and developmental stage (stage)

Term	Coefficient	S.E.	χ^2	P
Intercept	+5894	1416	17·9	<0·0001
Temp	−305	92	11·1	0·0009
Temp^2	+5·16	1·57	10·8	0·0010
DHT	+10·53	2·26	21·8	<0·0001
Stage	−444	115	14·9	0·0001
Stage^2	+52·35	13·58	14·8	0·0001
Stage^3	−3·03	0·79	14·6	0·0001
Stage^4	+0·086	0·023	14·3	0·0002
Stage^5	−0·00097	0·00026	13·8	0·0002
Stage*DHT	−1·88	0·40	22·1	<0·0001
Stage^2*DHT	+0·111	0·023	23·0	<0·0001
Stage^3*DHT	−0·00214	0·00044	24·0	<0·0001

Term=independent variable; coefficient=estimated regression coefficient; S.E.=one standard error of the regression coefficient; χ^2=likelihood ratio chi-square; P=probability value; there is 1 degree of freedom for each regression coefficient. Positive coefficients indicate a variable that increases the proportion of males produced, whereas negative coefficients indicate that a variable decreases the proportion of males produced. Polynomial orders for variables are designated by exponential numbers (2, 3, 4, 5). Interaction effects are indicated by asterisks.

a significant proportion of the variation in sex ratio even though the basis of these effects are harder to interpret at a biological level.

The model was fitted such that positive regression coefficients indicate that an independent variable increases the number of males produced whereas a negative coefficient decreases the number of males produced. For example, if increasing DHT dosage increases the proportion of males produced, then its regression coefficient will be significantly positive (this result was expected based on previous studies with DHT). Conversely, increasing the incubation temperature was expected to decrease the proportion of males produced; thus temperature was expected to have a negative regression coefficient.

Results

Specificity of androgen

Only male hatchlings resulted from hormone treatment (DHT, 3α-diol, 3β-diol, and AST) at both dosages at the all-male producing temperature (26·0 °C). The percentage of males produced at both dosages was greater than in the corresponding ethanol control group at temperatures that normally produce mixed sex ratios. In nine of the twelve groups the higher dosage produced more male offspring relative to the lower dosage (χ^2=3·0, 0·1>P>.05) (Fig. 1). No intersex individuals were found.

Dose–response

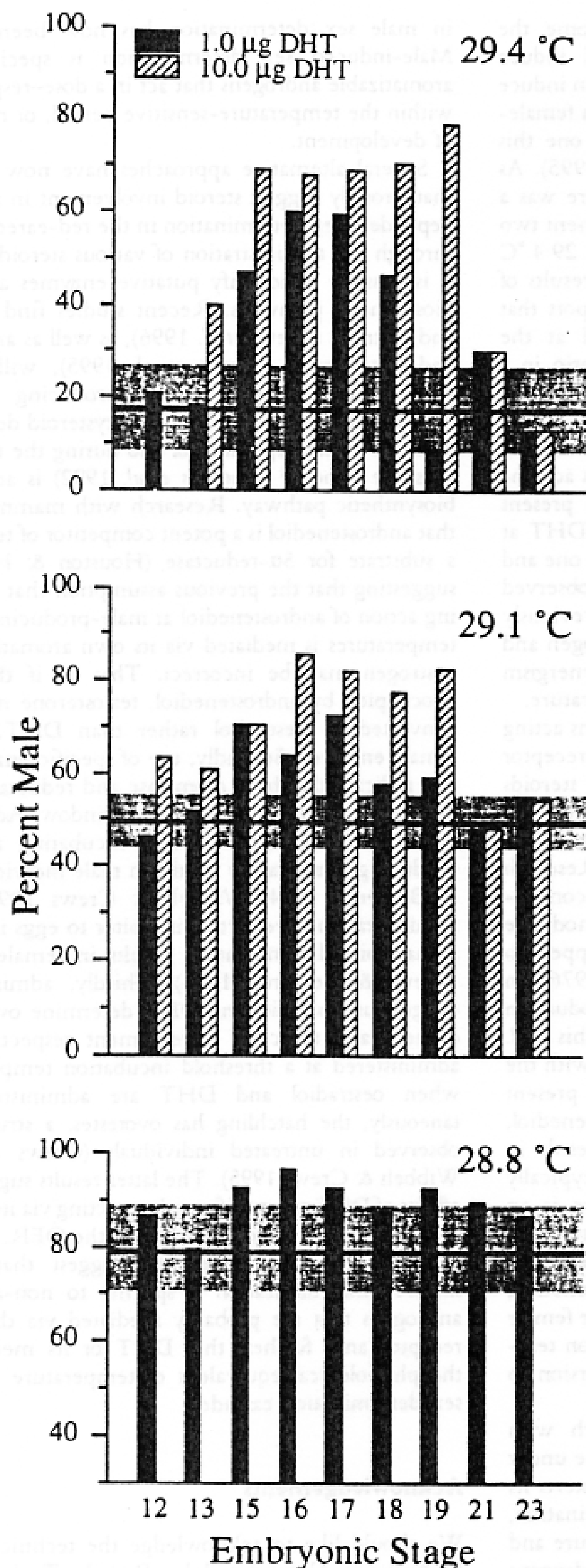
Incubation temperature significantly affected sex ratios: decreasing numbers of males were produced with increasing temperature (i.e. the negative regression coefficient for temperature in Table 1). As temperature increases, its feminizing effect is attenuated to some degree (i.e. the positive second order regression coefficient for temperature in Table 1). Treatment with DHT had a dosage effect opposite to that of increasing incubation temperature: increasing the dose from 1·0 to 10·0 µg increased the number of males produced at the 29·0 and 29·4 °C incubation temperatures (i.e. the positive regression coefficient for DHT in Table 1).

Androgen-sensitive window

Embryonic stage of development affected sex ratio, but in a complex manner: stage of development up to the 5th order polynomial had significant effects on sex ratio (see Table 1). Third and higher order polynomials are harder to interpret and have no clear biological meaning. Importantly, there was also an interaction between DHT and stage of treatment up to the 3rd order polynomial. These interactions indicate a significant effect of DHT on sex ratio during the temperature-sensitive period which is from stage 15 to 19 (Fig. 2). The interactions may also reflect what seems to be a slightly narrower period of 'sensitivity' to the lower dosage of DHT, ranging from stages 15 to 19 as opposed to stages 13 to 19 at the higher dosage. Only one individual was found to have ovotestes; this individual was from the 29·1 °C incubation temperature and treated with 1·0 µg DHT at stage 21.

Synergism

It is important to note the lack of significant interaction between incubation temperature and DHT or between



incubation temperature, DHT, and stage of development. Thus, there is no detectable synergism between temperature and DHT (see the similar sex ratios of DHT treatments at different temperatures in Fig. 1) or difference in the DHT-sensitive periods between incubation temperatures.

Discussion

The sex ratios for the different temperatures varied between experiments one and two. This is probably because the studies were done using eggs laid at different times of the breeding season. There is good evidence that in the map turtle and the snapping turtle individual clutches can display different sex ratio responses to temperature (Bull *et al.* 1982, Janzen 1992, Rhen & Lang 1994); a similar phenomenon probably occurs in the red-eared slider as well. However, thousands of freshly-laid red-eared slider eggs are available daily during the 3-month reproductive season (our supplier ships over 1·2 million hatchlings annually), so the clutches of many individuals are collected each day, thereby distributing any potential clutch effects.

In the red-eared slider, temperature-sensitivity of sex determination occurs from stage 15 through stage 21 or the middle third of development (Wibbels *et al.* 1991*a*). The present results suggest that the developmental window of DHT sensitivity for this species corresponds to this window of temperature sensitivity. However, such comparisons are necessarily superficial, because we do not know the period of exposure and activity for a given application of DHT in the way that we know the period of exposure for a controlled temperature. A previous study indicated that radiolabelled hormone rapidly enters the egg after being applied to the shell, and is still detectable in the embryo at least 9 days later (Crews *et al.* 1991). Thus, there is a long 'half-life' after being applied to the eggshell, which could be due to low levels of hormone metabolism in the egg and/or some hormone initially being sequestered in the egg shell or yolk and then gradually entering the embryo.

Figure 2 Effect of two dosages (1·0 and 10·0 μg) of exogenous DHT administered at different embryonic stages on sex determination in the red-eared slider turtle (*Trachemys scripta*). Three different incubation temperatures are represented: 28·8 °C (male-biased), 29·1 °C (threshold), and 29·4 °C (female-biased); only the low dosage (1·0 μg DHT) was administered at the male-biased incubation temperature. 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 0·01 confidence limit. Stages 15–21 correspond to the temperature-sensitive window.

Exogenous oestrogen can completely overcome the effects of a male-producing temperature and induce female development, whereas exogenous DHT can induce testicular development only in eggs incubating at a female-biased or a threshold temperature (experiment one this study; Wibbels *et al.* 1992, Wibbels & Crews 1995). As with oestrogen and incubation temperature, there was a consistent dose-dependent relationship in experiment two between the dosage of DHT at the 29·1 and 29·4 °C incubation temperatures. This is similar to the results of experiment one, but in contrast to an earlier report that administration of DHT to embryos incubated at the threshold temperature does not affect the sex ratio in a dose-dependent fashion (Wibbels & Crews 1995). The lack of a dose–response relationship between DHT dosage and incubation temperature may depend upon the stage of embryonic development at which the hormone is administered. For example, in experiment two of the present study, the relative effects of 1·0 and 10·0 μg of DHT at stage 17 (the embryonic stage used in experiment one and by Wibbels & Crews 1995) was less than that observed at stages 16, 18, and 19. As demonstrated previously (Wibbels & Crews 1995), and unlike with oestrogen and incubation temperature, there is no apparent synergism between exogenous DHT and incubation temperature.

Are DHT and other non-aromatizable androgens acting via the androgen receptor or via the oestrogen receptor (OER)? It is conceivable that the effects of these steroids are mediated through the OER system perhaps by occupying the OER and preventing temperature-induced oestrogen activation of female sex determination. Research with mammals indicates that while DHT in high concentrations can bind to the OER *in vitro* to modulate oestrogen-mediated responses, androgens do not appear to act directly via the OER *in vivo* (Schmidt *et al.* 1976). In hamster Harderian glands, the sexes vary in the production of the metabolites of DHT, and 3α/3β-diols (Vilchis *et al.* 1994) and, in rats, these metabolites may interact with the OER (Baum & Vreeburg 1976). However, the present results indicate that AST, a metabolite of androstenediol, and 3α/3β-diols, both metabolites of DHT, result in male-biased sex ratios even at temperatures that typically produce female-biased sex ratios. Thus, if there is an affinity of these metabolites with the OER in the turtle, it is not sufficient to initiate the female-determining cascade. This is in contrast with testosterone and androstenediol, both aromatizable androgens, which reliably induce female development even at all-male producing incubation temperatures, probably due to their metabolic conversion to oestrogens (Crews *et al.* 1995).

The same strategy employed in our research with oestrogen (Crews *et al.* 1994) was used 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. The following fact about the role of androgens in male sex determination has now been established. Male-induced sex determination is specific to non-aromatizable androgens that act in a dose–response fashion within the temperature-sensitive period, or mid-trimester of development.

Several alternative approaches have now yielded data that strongly suggest steroid involvement in temperature-dependent sex determination in the red-eared slider. First, through the administration of various steroid metabolites, it is possible to identify putative enzymes and preferred biosynthetic pathways. Recent studies find that oestriol and oestrone (Crews *et al.* 1996), as well as androstenediol and testosterone (Crews *et al.* 1995), will sex-reverse embryos incubating at a male-producing temperature. This indicates that the 17β-hydroxysteroid dehydrogenase detected at the beginning of and during the temperature-sensitive window (Thomas *et al.* 1992) is an operational biosynthetic pathway. Research with mammals indicates that androstenediol is a potent competitor of testosterone as a substrate for 5α-reductase (Houston & Habib 1988), suggesting that the previous assumption that the feminizing action of androstenediol at male-producing incubation temperatures is mediated via its own aromatization to an oestrogen may be incorrect. That is, if the reductase is occupied by androstenediol, testosterone must then be converted to oestradiol rather than DHT, leading to female embryos. Secondly, use of specific enzyme inhibitors indicate that both aromatase and reductase are active during the temperature-sensitive window. Administration of aromatase inhibitor to eggs incubating at a female-producing temperature results in male individuals (Crews & Bergeron 1994, Wibbels & Crews 1994), whereas administration of reductase inhibitor to eggs incubating at a male-biased temperature results in female individuals (Crews & Bergeron 1994). Thirdly, administration of exogenous oestradiol and DHT determine ovarian development and testicular development respectively, when administered at a threshold incubation temperature and, when oestradiol and DHT are administered simultaneously, the hatchling has ovotestes, a structure never observed in untreated individuals (Crews *et al.* 1994, Wibbels & Crews 1995). The latter results suggest that the effects of DHT are specific and not acting via its metabolites (3α/3β-androstanediols) binding to the OER.

Taken together, these data suggest that hormone-induced masculinization is specific to non-aromatizable androgens that are probably mediated via the androgen receptor and, further, that DHT or its metabolites are the physiological equivalent of temperature in the male sex-determination cascade.

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