Role of reductase and aromatase in sex determination in the red-eared slider (*Trachemys scripta*), a turtle with temperature-dependent sex determination

D Crews and **J M Bergeron**

Institute of Reproductive Biology, Department of Zoology, University of Texas at Austin, Austin, Texas 78712, USA

(Requests for offprints should be addressed to D Crews)

Abstract

In many turtles the temperature during the middle of incubation determines the gonadal sex of the hatchling. In the red-eared slider turtle (*Trachemys scripta*), an incubation temperature of 26 °C results in all male offspring, whereas an incubation temperature of 31 °C results in all female offspring; at temperatures intermediate to these (e.g. 29, 29·2, 29·4 °C) a mixed sex ratio is obtained. Administration of exogenous oestrogens will overcome the effects of an all-male producing incubation temperature to cause female sex determination, whereas administration of exogenous dihydrotestosterone (DHT) or testosterone to eggs incubating at an all-female temperature will have no discernible effect. Administration of DHT will cause male sex determination only if administered at intermediate incubation temperatures whereas administration of testosterone to eggs incubating at all male-producing and male-biased intermediate temperatures results in a significant number of female offspring, an effect presumably due to aromatization of testosterone to oestradiol (OE_2). Since testosterone serves as the precursor to both DHT and OE_2, being metabolized by reductase and aromatase respectively, three experiments were conducted to determine whether various putative reductase and aromatase inhibitors would overcome the effect of incubation temperature. First, while administration of testosterone to eggs incubating at all male-producing and male-biased intermediate temperatures produced females in a dose- and temperature-dependent manner, significant numbers of intersex individuals resulted from high dosage testosterone treatment to eggs incubating at a female-biased intermediate temperature. The reductase inhibitors 4MA and MK906 were capable of producing female offspring if administered at intermediate temperatures, but not in a dose-dependent fashion. Administration of the aromatase inhibitors CGS16949A and CGS20267 resulted in male offspring at both female-biased intermediate and at all female-producing temperatures in a dose-dependent fashion. Second, similar findings were obtained with combined doses of testosterone and reductase or aromatase inhibitors. Combined treatment of eggs at male-biased intermediate incubation temperatures with testosterone and reductase inhibitor resulted in female hatchlings, whereas combined treatment of testosterone and aromatase inhibitor at both female-biased intermediate and at all female-producing temperatures resulted in male hatchlings. Finally, treatment with reductase inhibitor and aromatase inhibitor combined resulted in only male offspring at all incubation temperatures with the exception of the all-female incubation temperature; in the latter instance almost all offspring were female. These studies indicate that in the red-eared slider turtle (i) male and female sex determination are independent cascades residing equally in each individual and regulated by incubation temperature, (ii) steroid hormones are involved in temperature-dependent sex determination, and (iii) testosterone plays a pivotal role in this process. The data also suggest that aromatase and oestrogen receptors may be involved in the initiation of an ovary determining cascade and that reductase and androgen receptors may be involved in the initiation of a testis determining cascade.

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Introduction

Temperature-dependent sex determination (TSD) is well established in many reptiles. Depending upon the species, reptiles with TSD may exhibit one of several different patterns: low incubation temperatures producing males, high incubation temperatures producing females, and intermediate incubation temperatures producing males with females being produced at both extremes (Bull 1980). Hormonal involvement in sex determination has been demonstrated in all three reptilian orders (reviewed by Raynaud & Pieau 1985, Janzen & Paukstis 1991, Crews *et al.* 1994, Pieau *et al.* 1994, Wibbels *et al.* 1994), including the red-eared slider turtle (*Trachemys scripta*)

(Crews *et al.* 1991, Wibbels *et al.* 1992, Wibbels & Crews 1992). For example, treatment of eggs with exogenous oestrogen will overcome the effects of a male-producing incubation temperature to result in female hatchlings in representatives of all three reptile orders demonstrating TSD (Bull *et al.* 1988). Furthermore, in the European pond turtle (*Emys orbicularis*) administration of tamoxifen, an oestrogen receptor (ER) antagonist, to eggs incubating at a female-producing incubation temperature results in intersexes; simultaneous administration of tamoxifen and oestradiol (OE_2) to eggs incubating at a male-producing incubation temperature blocks oestrogen-induced female development (Dorizzi *et al.* 1991). However, there may be important species differences as tamoxifen administered to eggs incubating at a female-producing incubation temperature failed to disrupt normal female development in the red-eared slider (Wibbels & Crews 1992) and in the American alligator (*Alligator mississippiensis*; Lance & Bogart 1991).

Unlike oestrogens, androgens are not capable of overcoming an incubation temperature that produces only females. If administered to eggs incubated at a female-producing temperature, neither testosterone or dihydrotestosterone (DHT) have any discernible effect on gonadal sex (Gutzke & Bull 1986, Crews *et al.* 1989). Administration of testosterone, but not DHT, to eggs incubated at an all male-producing incubation temperature results in approximately half of the hatchlings being female; this 'paradoxical' feminization may be the result of conversion of testosterone to OE_2 by the enzyme aromatase (Gutzke & Bull 1986, Crews *et al.* 1989, Wibbels & Crews 1992). Only when utilizing incubation regimens which result in mixed sex ratios is a sensitivity to exogenous androgen revealed; under these conditions exogenous DHT will result in male offspring (Wibbels *et al.* 1992, Wibbels & Crews 1993).

Steroidogenic enzymes are influenced by incubation temperature in turtles with TSD. Pieau (1973, 1974) demonstrated 3β-hydroxysteroid dehydrogenase (HSDH) activity in the undifferentiated gonads of the European pond turtle. Merchant-Larios *et al.* (1989) detected significant 3β-HSDH activity in the adrenals, but not in the undifferentiated gonad of the Olive Ridley sea turtle (*Lepidochelys olivacea*). Similar results were reported for 3α-, 3β-, and 17β-HSDH in the red-eared slider (Thomas *et al.* 1992). Aromatase enzyme levels increase at the end of the temperature-sensitive window in embryos of the European pond turtle incubating at female-producing incubation temperatures, but not in embryos incubating at a male-producing incubation temperature (Desvages & Pieau 1992a). This increase does not appear to be due to temperature modulation of aromatase activity, but rather to a temperature-induced increase in aromatase production. Shifting eggs from a male-producing to a female-producing incubation temperature greatly increases aromatase activity, but a complementary shift from a

female-producing to a male-producing incubation temperature only gradually decreases aromatase activity (Desvages & Pieau 1992b; see also Desvages *et al.* 1993).

The demonstration that exogenous androgens and oestrogens can determine sex in the red-eared slider turtle, and the fact that reductase and aromatase are key regulatory enzymes in the production of DHT and OE_2, respectively, from the precursor testosterone, suggests that these enzymes may play a pivotal role in TSD. The present investigation explores this possibility in the red-eared slider turtle. In addition to the suggestion of Dorizzi *et al.* (1991) that aromatase is involved in determination of females, our hypothesis includes a model in which regulation of reductase gene(s) may be critical to a testis-determining cascade. Because testosterone serves as the precursor to both DHT and OE_2 via the actions of the enzymes reductase and aromatase, respectively, we hypothesize that incubation temperature acts by modifying the metabolism of testosterone. In the first series of experiments chemicals known to be reductase or aromatase inhibitors in mammalian and avian systems were administered to eggs incubating at different temperatures. The second series of experiments examined the effects of simultaneous administration of testosterone and these putative enzyme inhibitors at different incubation temperatures. In the final series of experiments the effect of combined dosages of these putative reductase and aromatase inhibitors on sex determination was examined at different incubation temperatures. The results indicate (i) that TSD can be reversed by the administration of either reductase or aromatase inhibitors and that the inhibitors probably act on the enzymes themselves, rather than through the steroid receptors, (ii) that the feminizing effect of testosterone when administered to eggs incubating at a male-producing temperature is due to the aromatization of testosterone to OE_2, and (iii) that the action of aromatase is more potent than that of reductase in TSD.

Materials and Methods

Eggs and incubation

Approximately 4000 freshly-laid eggs were obtained commercially (Robert Kliebert, Hammond, LA, USA). After transport to our laboratory, eggs were held at room temperature until viability was established by candling. They were then placed in containers with moistened vermiculite (vermiculite:water, 1:1) and the containers placed in reach-in incubators (Precision, Chicago, IL, USA) programmed to provide a constant temperature of 26, 29, 29·2, 29·4, or 31 °C. Previous studies with this species indicate that a continuous incubation temperature of 26 °C produces all male hatchlings whereas an incubation temperature of 31 °C produces all female hatchlings. Temperatures intermediate to these result in different sex ratios of gonadal males and females rather

TABLE 1. Experimental protocol utilized in experiment one. Eggs from the red-eared slider turtle (*Trachemys scripta*) were incubated at one of five incubation temperatures (26–31 °C) and treated with reductase inhibitor (4MA or MK906) or aromatase inhibitor (CGS16949A or CGS20267) at stage 17, the midpoint in the temperature-sensitive window. Controls consisted of administration of ethanol vehicle only

	Dose (μg)	Putative action	Number of eggs treated				
			26 °C	29·0 °C	29·2 °C	29·4 °C	31 °C
Chemical							
4MA	0·1	Reductase inhibitor	28	28	28	26	26
	1·0		28	30	28	26	28
	10·0		28	28	26	22	27
	100·0		0	27	28	27	26
MK906	1·0	Reductase inhibitor	27	23	30	28	29
	10·0		28	29	28	27	27
	100·0		26	29	29	26	25
CGS16949A	0·1	Aromatase inhibitor	27	28	25	26	25
	1·0		22	25	28	26	25
	10·0		28	28	27	25	21
	100·0		25	28	24	22	22
CGS20267	0·1	Aromatase inhibitor	26	28	29	27	24
	1·0		27	27	27	24	30
	10·0		28	25	27	26	25
	100·0		26	21	24	26	25

than in intersex individuals: 29 °C is a male-biased incubation temperature, 29·2 °C is the threshold temperature typically producing equal numbers of males and females, and 29·4 °C is a female-biased incubation temperature (Crews *et al.* 1991, 1994, Wibbels *et al.* 1991*a*, Wibbels & Crews 1993). After receiving treatments, all eggs were returned to their respective incubators until they hatched.

Embryonic development was monitored by candling eggs and by dissecting 2–4 eggs approximately twice a week to verify specific developmental stages, based on criteria described by Yntema (1968). All eggs were incubated until stage 17, the approximate midpoint in the temperature-sensitive period in this species (Wibbels *et al.* 1991*a*). Eggs were then randomized into control and experimental treatments.

Enzyme inhibitors

Three series of experiments were conducted. In all experiments, eggs in control groups at each incubation temperature received a single treatment consisting of 5 μl 95% ethanol; inhibitor and/or ligand was also dissolved in 5 μl 95% ethanol.

In the first experiment, eggs in the experimental groups received a single treatment of a specific enzyme inhibitor (Table 1). Two reductase inhibitors, 17β-(N, N-diethyl) carbomoyl-4-methyl-4-aza-5α-androstane-3-one (4MA) and 17β-(N,t-butyl) carbomoyl-4-aza-5α-androst-1-en-3-one (MK906), were used; both compounds inhibit 5α-reductase (Russell & Wilson 1994). Two aromatase inhibitors, 4-(5,6,7,8,-tetrahydroimidazo [1,5-α] pyridin-5-y1) benzonitrile monohydrochloride (CGS16949A) and 4-[1-(cyanophenyl)-1-(1,2,4-triazollyl) methyl] benzo-nitril (CGS20267) were used; both compounds are effective in a variety of mammalian and avian species (Steele *et al.* 1987, Santen *et al.* 1990, Benoit *et al.* 1992, Bonsall *et al.* 1992, Elbrecht & Smith 1992, Wozniak *et al.* 1992, Wozniak & Hutchison 1993). The doses of reductase inhibitors were 0·1, 1·0, 10, or 100 μg/egg for 4MA and 1·0, 10, or 100 μg/egg for MK906. The doses for both aromatase inhibitors were 0·1, 1·0, 10, or 100 μg/egg. Mean sample sizes were as follows. 4MA: 26 individuals, ranging from 22–30, and totalling 642 animals. MK906: 26 individuals, ranging from 22–29, and totalling 529 animals. CGS16949A: 25 individuals, ranging from 21–28 and totalling 623 animals. CGS20267: 26 individuals, ranging from 21–30, and totalling 638 animals.

In the second experiment, eggs in the experimental groups received combined treatments of testosterone and an enzyme inhibitor (Table 2). To determine the effect of testosterone plus reductase inhibitor, eggs were incubated at all five temperatures and each received 100 μg testosterone and 50 μg 4MA, or 75 μg testosterone and 50 μg MK906. As treatment controls some eggs received testosterone alone. Thus, other eggs received 50 or 100 μg of testosterone (Sigma Chemical Co., St Louis, MO, USA). In addition, the sex ratio resulting from the administration of high doses of inhibitor alone was used as a comparison with that resulting from the application of precursor and inhibitor in combination.

TABLE 2. Experimental protocol utilized in experiments two and three. Eggs from the red-eared slider turtle (*Trachemys scripta*) were incubated at one of five incubation temperatures (26–31 °C) and received a combined treatment of testosterone (T) and reductase inhibitor (RI; 4MA or MK906) or aromatase inhibitor (AI; CGS16949A or CGS20267). Controls consisted of administration of ethanol vehicle alone or T alone

		Number of eggs treated				
	Dose (µg)	26 °C	29·0 °C	29·2 °C	29·4 °C	31 °C
Chemical						
Experiment 2						
T	10·0	24	26	29	24	28
	50·0	27	28	27	28	29
	100·0	25	30	28	20	25
RI+T	50·0 4MA + 100·0 T	24	26	22	29	25
	50·0 MK906 + 75·0 T	26	23	23	26	30
AI+T	100·0 CGS16949A + 10·0 T	24	26	28	26	28
	100·0 CGS16949A + 100·0 T	24	29	22	28	24
	100·0 CGS20267 + 10·0 T	19	26	23	21	28
	100·0 CGS20267 + 100·0 T	24	27	27	25	25
Experiment 3						
RI+AI	50·0 4MA + 50·0 CGS16949A	19	27	25	27	28
	50·0 MK906 + 50·0 CGS16949A	23	27	25	29	23

To determine the effect of testosterone plus aromatase inhibitor, eggs were also incubated at all five temperatures and each received 10 or 100 µg testosterone with 100 µg CGS16949A or CGS20267. As treatment controls some eggs received testosterone alone. Thus, other eggs received 10 or 100 µg testosterone. In addition, the sex ratio resulting from the administration of high doses of inhibitor alone was used as a comparison with that resulting from the application of precursor and inhibitor in combination.

In a third experiment, eggs in the experimental groups received combined treatments of both reductase and aromatase inhibitor (Table 2). Eggs were incubated at 26·0, 29·0, 29·2, 29·4, or 31 °C and received simultaneously 50 µg CGS16949A and either 50 µg 4MA or 50 µg MK906.

Sex diagnosis

Turtles were killed within two weeks of hatching. Gonadal sex and developmental status of the Müllerian ducts were 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.* 1991*a*). Ovaries are long and flat whereas testes are shorter, rounder, and have visible seminiferous tubules (see Crews *et al.* 1991). The developmental status of both the cranial and the caudal halves of the Müllerian ducts were examined and scored as either absent, regressed but visible, normal (as in a typical female hatchling), or hypertrophied. A phallus was also noted if present. 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.

Statistical analysis

In the first series of experiments, the effects of each dose of different enzyme inhibitor was evaluated versus control using Fisher's Exact test (two-tailed). To determine effect of incubation temperature on sensitivity to the different enzyme inhibitors, inhibitors were examined for effect within each incubation temperature using Pearson Chi-square for difference between doses and Fisher's Exact test for low dose of inhibitor versus control. In the second series of experiments focusing on the combination of testosterone and enzyme inhibitor, the effect of testosterone or enzyme inhibitor alone compared to control was evaluated using Fisher's Exact test (two-tailed). Combination treatments were compared to ethanol control, testosterone alone, or enzyme inhibitor alone using Fisher's Exact test (two-tailed). The third series of experiments examining the effects of combined doses of enzyme inhibitors was evaluated within each incubation temperature using Fisher's Exact test (two-tailed).

To determine the interaction of incubation temperature and experimental treatment, the hatchling sex ratio obtained at the different incubation temperatures for the varying doses of inhibitor was compared to that obtained with the ethanol control at that temperature, as in Wibbels *et al.* (1991*b*) with slight modifications. Here, the significance of synergism was calculated as follows. First, maximum-likelihood estimates of the following

probabilities were calculated, under the assumption that synergism did not exist: that of producing one sex at a baseline temperature, that of producing one sex at the second temperature, that of producing one sex at the base-line temperature in the presence of the compound, that of producing one sex at the second temperature in the presence of the compound, and finally, the probability that the increases in the proportion of one sex were due to the compound and temperature increment (measured at the baseline temperature) respectively (Wibbels *et al.* 1991*b*). These estimates were then used to calculate expected sex ratios for each of the four treatments (temperature × inhibitor), again under the hypothesis that synergism did not exist. A trial consisting of simulated samples was then generated as follows: (*a*) n_i uniform, random variables were chosen, where n_i was the total number of males plus females obtained in the experiment for treatment *i*, and (*b*) the simulated number of females in treatment *i* was calculated as the number of the n_i random variables whose value was less than the sex ratio expected for treatment *i* under the null hypothesis (the number of males was n_i minus the number of females). When numbers of males and females had been simulated for all four treatments, a synergism value was calculated for the trial. Ten thousand simulated synergisms were calculated, and the significance of the synergism in the actual data was taken as the proportion of these random samples in which the synergism equalled or was more extreme than the synergism in the data.

Results

Ethanol (control) treatment had no significant effect on the hatchling sex ratio as previously established for the red-eared slider turtle (Wibbels *et al.* 1991*a*); an incubation temperature of 26 °C produced all males (male:female sex ratio=100:0), 31 °C produced all females (sex ratio=0:100), whereas 29 °C produced a 88:12 sex ratio, 29·2 °C produced a 65:35 sex ratio, and 29·4 °C produced a 24:76 sex ratio.

The first experiment focused on the effects of enzyme inhibitors at incubation temperatures that yielded different sex ratios. Administration of the reductase inhibitors 4MA (Fig. 1*a*) or MK906 (Fig. 1*b*) to eggs incubating at 26 °C, an incubation temperature that produces only males, failed to reverse gonadal sex (4MA, P=0·405; MK906, P=0·497). Female hatchlings did result at 29 °C, an incubation temperature that produces approximately 90% male development (4MA, P<0·0001; MK906, P<0·0001) as well as at 29·2 °C, an incubation temperature that results in 65% male development (4MA, P<0·001; MK906, P<0·002). Differences were not statistically significant at 29·4 °C, an incubation temperature that results in a female-biased sex ratio (24% male) (4MA, P=0·746; MK906, P=0·714). At the 31 °C incubation temperature,
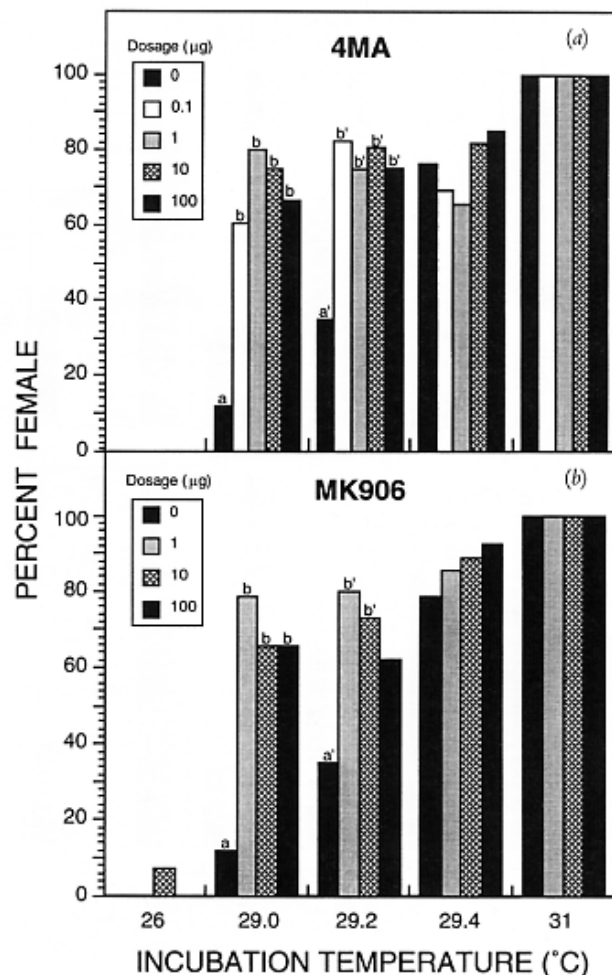

FIGURE 1. Administration of reductase inhibitor induces female development in the red-eared slider (*Trachemys scripta*). (*a*) results with 4MA; (*b*) results with MK906. Eggs were treated with ethanol (control) or different doses of the inhibitor at each incubation temperature. Sample sizes are given in the text. Comparisons are made within each incubation temperature relative to control; values without the same letter are significantly different at a level of at least P<0·05.

which produces normally only females, all hatchlings were diagnosed as female. There was no evidence of a dose-response relationship with the different doses of 4MA or MK906 at any incubation temperature (4MA: 26 °C, P=0·143; 29 °C, P=0·381; 29·2 °C, P=0·853; 29·4 °C, P=0·283. MK906: 26 °C, P=0·124; 29 °C, P=0·534; 29·2 °C, P=0·226; 29·4 °C, P=0·509). The effect of the reductase inhibitor compared to that of the ethanol control was not temperature-sensitive; that is, the medium dosage of either 4MA or MK906 did not produce greater numbers of females at more female-producing incubation temperatures (1 μg vs. 10 μg 4MA: 29·0 °C vs. 29·2 °C,
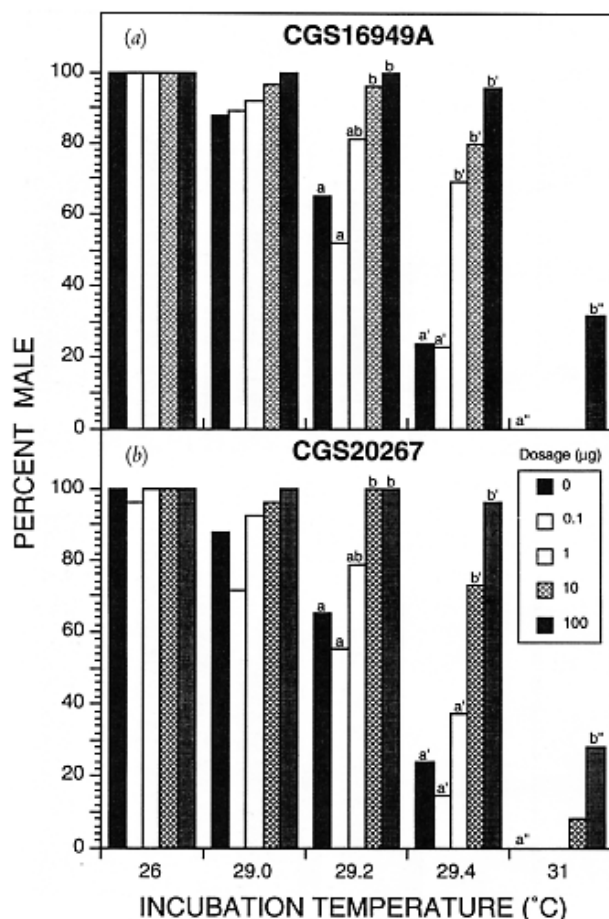
FIGURE 2. Administration of aromatase inhibitor induces male development in the red-eared slider (*Trachemys scripta*). (*a*) results with CGS16949A; (*b*) results with CGS20267. Eggs were treated with ethanol (control) or different doses of the inhibitor at each incubation temperature. Sample sizes are given in the text. Comparisons are made within each incubation temperature relative to control; values without the same letter are significantly different at a level of at least $P<0.05$.

$P=0.241$; 1 µg vs. 10 µg MK906: 29·0 °C vs. 29·2 °C, $P=0.260$).

Administration of the aromatase inhibitors CGS16949A (Fig. 2*a*) or CGS20267 (Fig. 2*b*) to eggs incubating at 31 °C, an incubation temperature that produces only females, resulted in male hatchlings (CGS16949A at 100 µg, $P<0.002$; CGS20267 at 100 µg, $P<0.004$). At the 26 °C incubation temperature, which produces normally only males, all hatchlings were diagnosed as male. Differences were statistically significant at 29·4 °C (CGS16949A at 100 µg, $P<0.0001$; CGS20267 at 100 µg, $P<0.0001$) and at 29·2 °C (CGS16949A at 100 µg, $P<0.002$; CGS20267 at 100 µg, $P<0.002$), but not statistically significant at 29 °C (CGS16949A at 100 µg $P=0.098$;

CGS20267 at 100 µg $P=0.239$). Intersex gonads were occasionally observed (1–2 individuals in the hatchlings treated with CGS20267 from the 29·0, 29·2 and 29·4 °C incubation temperatures). There was evidence of a dose-response relationship with the different doses of CGS16949A (29·2 °C, $P=0.0001$; 29·4 °C, $P=0.0001$) and CGS20267 (29·2 °C, $P=0.0001$; 29·4 °C, $P=0.0001$) at the intermediate incubation temperatures. The effect of both aromatase inhibitors was temperature-sensitive; that is, the medium dose compared to the ethanol control produced greater numbers of males at more male-producing incubation temperatures (1 µg vs. 10 µg CGS16949A, 29·4 °C vs. 29·2 °C, $P=0.028$; 1 µg vs. 10 µg CGS20267, 29·4 °C vs. 29·2 °C, $P=0.064$).

The second experiment utilized combinations of precursor (testosterone) and enzyme inhibitor. Since statistically significant differences were obtained only in the 26 °C and the 29 °C groups, only these are represented graphically (Fig. 3). The effect of testosterone alone was temperature-sensitive (Pearson Chi-square test within dosage: temperature × sex, $P=0.005$ for 100 µg and $P=0.0001$ for 50 µg). At incubation temperatures of 26·0 °C and 29 °C (Fig. 3*b*), the number of females resulting from administration of either 50 or 100 µg testosterone was significantly greater than control ($P=0.0001$ for both); the sex ratio produced at 29 °C compared to 26 °C (Fig. 3*a*) was statistically significant at the 50 µg dose ($P=0.0001$) but not at the 100 µg dose ($P=0.088$). At 29·2 °C, both doses were significantly different from control ($P=0.0001$ for both). At 29·4 °C, the effects of treatment with 50 µg and 100 µg testosterone were not statistically different from control ($P=0.470$ and $P=0.505$ respectively). At 31 °C all hatchlings were female in both testosterone treatment and ethanol (temperature) control groups. However, it is perhaps significant that intersex gonads were observed only at the 29·4 °C incubation temperature in the testosterone treatment groups. Such cases were rare (1–2 individuals) except at the 100 µg dose, where there were eight intersex individuals, seven males and 13 females; many of the intersex individuals having large phalluses.

As shown in the first experiment, administration of either reductase inhibitor alone was without effect at 26 °C (P(4MA)=0.405; P(MK906)=0.497), but both were effective compared to control at 29 °C (P(4MA)=0.0001; P(MK906)=0.0001). Administration of combined treatments of testosterone and reductase inhibitor (both 4MA and MK906) to eggs incubating at 26 °C resulted in a significant number of females compared to control (P(4MA)=0.0001; P(MK906)=0.0001), but this was not significantly different from that of testosterone alone (P(4MA)=1.0; P(MK906)=0.160).

The complementary experiment evaluated the effects of aromatase inhibitor. Since statistically significant differences were obtained only in the 29·4 °C and the 31 °C groups, only these are represented graphically
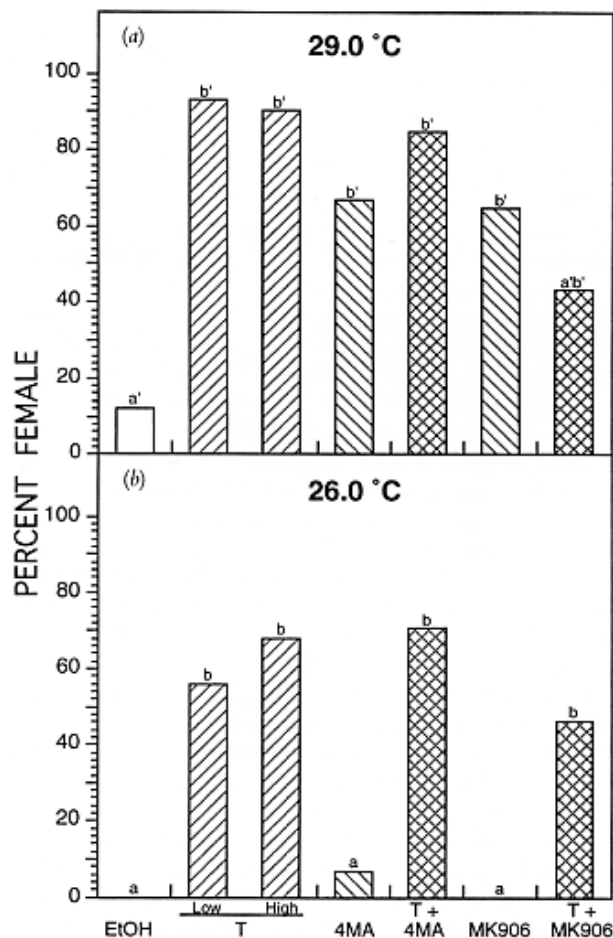
FIGURE 3. Effect of combined treatments of testosterone and reductase inhibitor at two incubation temperatures on male sex determination in the red-eared slider turtle (*Trachemys scripta*). Eggs were treated with ethanol (EtOH; control) or different doses of testosterone alone (T, low dose=50 µg; high dose=100 µg), reductase inhibitor alone (4MA, 50 µg at 26 °C and 100 µg at 29 °C; and MK906, 100 µg at both 26 °C and 29 °C), or testosterone plus reductase inhibitor (T+4MA, 100 µg T+50 µg 4MA at both 26 °C and at 29 °C; T+MK906, 75 µg T+50 µg MK906 at both 26 °C and 29 °C). Sample sizes are given in the text. Values without the same letter are significantly different at a level of at least $P<0.05$.

(Fig. 4). There was no statistical difference in the sex ratio produced between the ethanol control group and the testosterone alone group at an incubation temperature of 29·4 °C (Fig. 4*a*) or 31 °C (Fig. 4*b*) at either the 10 or 100 µg dose (29·4 °C: $P=0.713$ and 0.505, respectively; 31 °C: $P=1.0$ for both doses); administration of 10 µg at 26 °C also had no effect on the hatchling sex ratio compared to control ($P=0.490$). However, administration of 100 µg at 26 °C did produce a significant number of female hatchlings ($P=0.0001$). This was also true for both doses at 29 °C and 29·2 °C ($P=0.0001$ for all groups).
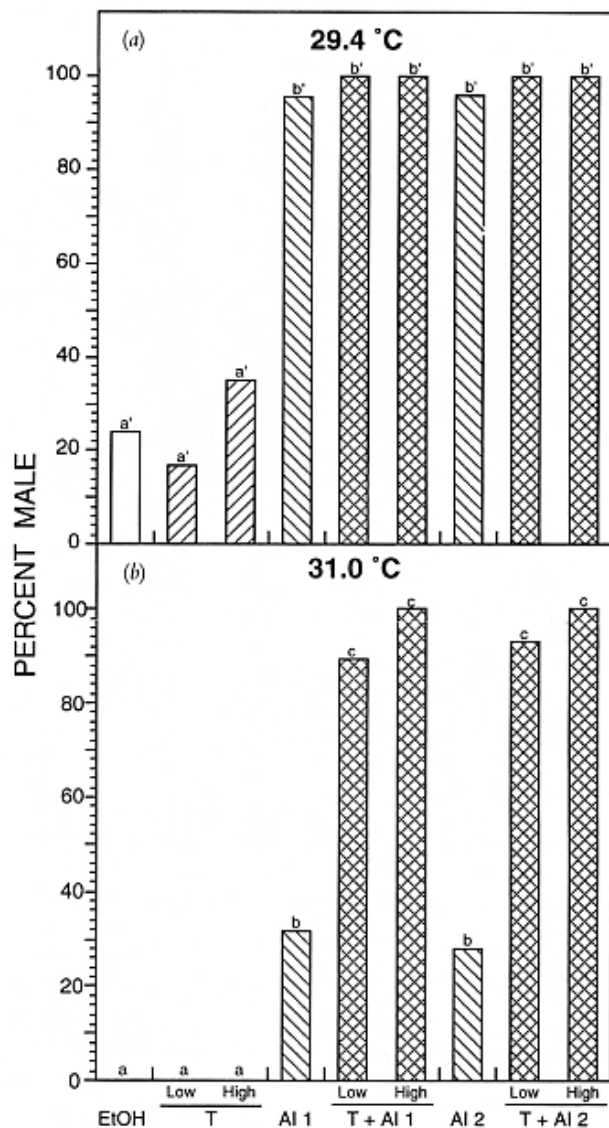

FIGURE 4. Effect of combined treatments of testosterone and aromatase inhibitor at two incubation temperatures on female sex determination in the red-eared slider turtle (*Trachemys scripta*). Eggs were treated with ethanol (EtOH; control) or different doses of testosterone alone (T), aromatase inhibitor alone (AI 1, CGS16949A; AI 2, CGS20267), or testosterone plus aromatase inhibitor (T+AI 1, T+AI 2) at each incubation temperature. 'Low' and 'high' refer to testosterone doses of 10 µg and 100 µg respectively; AI 1 and AI 2 were administered in 100 µg doses in all cases. Sample sizes are given in the text. Values without the same letter are significantly different at a level of at least $P<0.05$.

As shown in the first experiment, administration of either aromatase inhibitor alone was without effect at 26·0 and 29 °C; the highest dose (100 µg) resulted in a significantly more male-biased sex ratio than control groups at
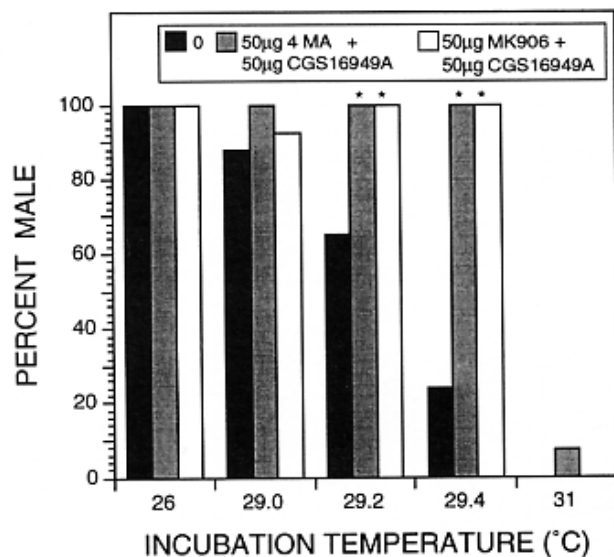
FIGURE 5. Effect of combined treatments of reductase and aromatase inhibitor at different incubation temperatures on the sex ratio of the red-eared slider turtle (*Trachemys scripta*). Sample sizes are given in the text. Asterisk indicates a significant difference from the ethanol control within each incubation temperature; $P<0.05–P<0.001$.

29·2, 29·4, and 31 °C (for all temperatures CGS16949A: $P=0.0001$; CGS20267: $P=0.0001$). Only at 29·4 and 31 °C did combined treatments show an effect over the testosterone and ethanol controls ($P=0.05–0.0001$ as illustrated in Fig. 4). Administration of combined treatments of testosterone and aromatase inhibitor to eggs incubating at 29·4 °C and 31 °C resulted in 100% of the hatchlings being male; there was no significant difference between doses (29·4 °C, $P=1.0$; 31 °C, $P=0.678$) or temperature (P(CGS16949A)$=0.115$; P(CGS20267)$=0.497$) for combined treatments.

The third experiment focused on the effects of simultaneous administration of reductase and aromatase inhibitor (Fig. 5). At all incubation temperatures, with the exception of 31 °C, experimentally-treated animals were mostly or all male; at 29·2 °C and 29·4 °C this was significantly different from the ethanol-treated controls (29·2 °C: 4MA plus CGS16949A, $P=0.001$; MK906 plus CGS16949A, $P=0.001$; 29·4 °C: 4MA plus CGS16949A, $P=0.0001$; MK906 plus CGS16949A, $P=0.0001$). At a 31 °C incubation temperature all offspring were female except for the few males obtained from the 4MA plus CGS16949A treatment group.

Discussion

Steroid hormone-induced sex determination occurs in fish (Hunter & Donaldson 1983), amphibians (Burns 1961)

and reptiles (Crews *et al.* 1994, Pieau *et al.* 1994). In birds, administration of exogenous steroid hormones to embryos will feminize the gonads, creating an ovotestis in genetic males but this does not usually persist into adulthood (Scheib 1983). In metatherian mammals administration of exogenous oestrogen will sex-reverse embryos (reviewed by Burns 1961), but in eutherian mammals such treatment has no apparent effect on genetic males, indicating that in these mammals testicular differentiation is not influenced by steroid hormones (see George & Wilson 1994 for review). However, there is evidence of a vestigial sensitivity to steroid hormones in fetal ovarian development. For example, transplanted primordial gonadal tissue from genetic female mice placed under the kidney capsules of adult males will develop into ovotestes (Taketo *et al.* 1984, see also George & Wilson 1994 for review). Finally, recent studies with both mammalian and avian embryos suggest that genes or gene products such as the testis determining factor at the sex-determining region of the Y chromosome or Müllerian inhibiting substance (MIS) can affect the cytochrome P450 aromatase system and indirectly influence sex determination (Vigier *et al.* 1989, Haqq *et al.* 1993).

Steroid hormones are not gene products, so if steroids are important in TSD as has been suggested (Crews *et al.* 1989, 1991, Dorizzi *et al.* 1991, Pieau *et al.* 1994), the mechanism of action might involve differential response to temperature of steroid-converting enzymes, which are gene products. Because testosterone serves as the precursor to both DHT and OE_2 via the actions of the enzymes reductase and aromatase respectively, it is attractive to suggest that temperature acts by modifying the metabolism of testosterone. Previous studies have demonstrated the role of the cytochrome P450 aromatase in female sex determination in the European pond turtle (Dorizzi *et al.* 1991). The present study with the red-eared slider turtle is the first to suggest that reductase enzymes may be similarly involved in male sex determination. At an incubation temperature that normally produces only male offspring, we found that reductase inhibitors were without discernible effects on the hatchling sex ratio. This is an indication the reductase inhibitors are not acting through ERs, since OE_2 administration at 26 °C does lead to female determination. However, at intermediate male-biased incubation temperatures, administration of reductase inhibitor to incubating eggs prevented male development and resulted in significant numbers of female hatchlings. In experiment one, MK906 had a negligible feminizing effect, whereas 4MA had no apparent effect at 26 °C (Fig. 1). In experiment two the opposite pattern was shown, with 4MA having a slightly feminizing effect at 26 °C, whereas MK906 had none. This apparent discrepancy may be attributed to individual differences in sensitivity to these chemicals. Finally, combined administration of testosterone and reductase inhibitor to eggs incubating at a male-producing temperature also resulted in the

production of female hatchlings. These findings support the hypothesis of the role of reductase enzymes in male sex determination. When considered with the previous finding that exogenous DHT will induce male development when administered to eggs incubating at a threshold temperature – the temperature that yields a 50:50 sex ratio (Wibbels & Crews 1993) – the present results suggest that male development may be mediated by an androgen receptor (AR).

Previous experiments have demonstrated that the aromatase inhibitor CGS16949A will interfere with temperature-induced female sex determination in the alligator (Lance & Bogart 1992) and the red-eared slider turtle (Wibbels & Crews 1994). The present study indicates that the aromatase inhibitor CGS20267 will also cause male development if administered at either intermediate or all female-producing incubation temperatures. In mammalian systems CGS20267 is more potent than CGS16949A, but there was no difference detected in the hatchling sex ratio or in the histology of the gonads for the effective doses in the present study. Similarly, administration of CGS16949A during early development causes genetic female chickens to develop as gonadal males. Interestingly, another aromatase inhibitor, 1,4,6-androstatrien-3,17-dione (ATD), is not effective in this regard (Elbrecht & Smith 1992; see also Wartenberg *et al.* 1992). As in the chicken, neither ATD nor 4-androsten-4-ol-3,17-dione are effective in reversing temperature-induced female sexual development in the red-eared slider (Wibbels & Crews 1992) or alligator (Lance & Bogart 1992). The fact that aromatase inhibitors produced more males at 29·4 °C compared to 31 °C is indirect evidence that aromatase production is temperature-sensitive, with more aromatase being produced at more female-biased temperatures. Interestingly, a similar synergism does not appear to occur with temperature and either reductase inhibitor.

In the present study administration of testosterone to eggs incubating at male-biased intermediate temperatures produced females in a dose- and temperature-dependent manner. This effect was blocked by administration of testosterone and aromatase inhibitor. It is perhaps significant that treatment of eggs incubating at a female-biased intermediate temperature with a high dose of testosterone resulted in a significant number of intersex individuals, a finding similar to that of the increased number of intersexes produced upon administration of both DHT and OE_2 (Wibbels & Crews 1993). This is a further indication that an excess of precursor (testosterone) may lead to simultaneous activation of the ovary- and testis-determining cascades. Finally, the observation that the combined application of testosterone and aromatase inhibitor produced males at an all female-producing incubation temperature as well as at a female-biased intermediate incubation temperature compared to administration of either compound alone supports the hypothesis that (i)

there is a testis-determining cascade independent of an ovary-determining cascade, and (ii) the metabolism of testosterone to OE_2 via aromatization is critical to female sex determination; that is, blocking aromatase activity and providing excess precursor testosterone results in the production of males, presumably through the metabolism of testosterone to DHT via reductase. Again, the significant increase in the number of males produced with testosterone and aromatase inhibitor compared to aromatase inhibitor alone indicates that the aromatase inhibitor is not acting through an AR.

Combined treatment with both aromatase inhibitor and reductase inhibitor resulted in male development, suggesting that aromatase inhibitor is more potent than reductase inhibitor. Alternatively, testosterone may not be metabolized to DHT or to OE_2 and could act through ARs which may be mediating male sexual development. Finally, the fact that administration of both reductase and aromatase inhibitors simultaneously to eggs incubating at 31 °C resulted in only (4MA) or mostly (MK906) female hatchlings suggests a nonsteroidal temperature effect, perhaps a temperature-induced upregulation of steroid receptor.

How then might temperature be acting to determine sex in the red-eared slider turtle? In reptiles with TSD it would appear that female and male sex determination are separate processes that are differentially affected by incubation temperature rather than the organization/default system characteristic of genotypic sex determination (Jost 1961). Thus, females may result from the activation of an ovary-determining cascade and inhibition of a testis-determining cascade; conversely, males may result from the activation of a testis-determining cascade and inhibition of an ovary-determining cascade. Central to the steroid hormone-mediation hypothesis (Crews *et al.* 1989, 1991, Dorizzi *et al.* 1991, Pieau *et al.* 1994) is the role of testosterone as a precursor molecule destined for conversion to DHT (via reductase) or OE_2 (via aromatase). One hypothetical model 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 OE_2), resulting in increased OE_2 production. For example, Claude Pieau and colleagues (reviewed by Pieau *et al.* 1994) have documented a positive feedback relationship between OE_2 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 OE_2 may exist between DHT and reductase. An alternative model to account for the finding of a lack of a dose-response in the effect of the reductase inhibitor, as well as the absence

of a synergism between DHT sensitivity and incubation temperature as occurs between OE_2 sensitivity and incubation temperature, indicates that the gene(s) for reductase are constitutively expressed at the various incubation temperatures. Thus, incubation temperature would act only indirectly via the testis-determining gene(s), having a negative feedback on the regulation of aromatase gene expression, leading to suppression of OE_2. 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. In both models incubation temperature would modulate the activity of at least four distinct genes; the genes encoding for aromatase, ERs, reductase and ARs. Further details of this model are provided by Crews (1994).

We propose further that incubation temperature activates the gene(s) encoding for steroid hormone receptors (e.g., male-producing temperature upregulating ARs and female-producing temperature upregulating ERs). Whatever the actual enzymatic regulation, the resulting hormonal milieu would lead to the binding of DHT and OE_2 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. (i) The effect of temperature or exogenous steroids is all-or-none; that is, individuals are either male or female. (ii) Incubation at a threshold temperature results in a 50:50 sex ratio, rather than intersexes being formed. (iii) Intersexes can be formed experimentally by the simultaneous administration of DHT and OE_2 to eggs incubating at a threshold incubation temperature or by high doses of testosterone at female-biased incubation temperatures. (iv) Exogenous OE_2 will overcome the effects of male-producing temperatures, and there is a correlation between oestrogen-sensitivity and temperature-sensitivity. (v) 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 synergism between incubation temperature and sensitivity to DHT. (vi) Steroid-induced gonadal feminization is specific to ER-responsive oestrogens whereas gonadal masculinization appears to be specific to AR-responsive androgens. (vii) 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 develop-

ment, in eggs incubating at intermediate incubation temperatures.

Acknowledgements

References

Benoit AM, Inskeep EK & Dailey RA 1992 Effect of a nonsteroidal aromatase inhibitor on *in vitro* and *in vivo* secretion of estradiol and on the estrous cycle in ewes. *Domestic Animal Endocrinology* **9** 313–327.

Bonsall RW, Clancy AN & Michael RP 1992 Effects of the nonsteroidal aromatase inhibitor, fadrozole, on sexual behavior in male rats. *Hormones and Behavior* **26** 240–254.

Bull JJ 1980 Sex determination in reptiles. *Quarterly Review of Biology* **55** 3–21.

Bull JJ, Gutzke WHN & Crews D 1988 Sex reversal in three reptilian orders. *General and Comparative Endocrinology* **70** 425–428.

Burns RK 1961 Role of hormones in the differentiation of sex. In *Sex and Internal Secretions*, Vol 1, pp 76–156. Ed WC Young. Baltimore: Williams and Wilkins.

Crews D 1994 Temperature, steroid and sex determination. *Journal of Endocrinology* **142** 1–8.

Crews D, Bergeron JM, Bull JJ, Flores D, Tousignant A, Skipper JK & Wibbels T 1994 Temperature-dependent sex determination in reptiles: proximate mechanisms, ultimate outcomes, and practical applications. *Developmental Genetics* **15** 297–312.

Crews D, Bull JJ & Wibbels T 1991 Oestrogen and sex reversal in turtles: a dose-dependent phenomenon. *General and Comparative Endocrinology* **81** 357–364.

Crews D, Wibbels T & Gutzke WHN 1989 Action of sex steroid hormones on temperature-induced sex determination in the snapping turtle (*Chelydra serpentina*). *General and Comparative Endocrinology* **76** 159–166.

Desvages G, Girondot M & Pieau C 1993 Sensitive stages for the effects of temperature on gonadal aromatase activity in embryos of the marine turtle *Dermochelys coriacea*. *General and Comparative Endocrinology* **92** 54–61.

Desvages G & Pieau C 1992*a* Aromatase activity in gonads of turtle embryos as a function of the incubation temperature of the egg. *Journal of Steroid Metabolism, Biochemistry and Molecular Biology* **41** 851–853.

Desvages G & Pieau C 1992*b* Time required for temperature-induced changes in gonadal aromatase activity and related gonadal structures in turtle embryos. *Differentiation* **52** 13–18.

Dorizzi M, Mignot T-M, Guichard A, Desvages G & Pieau C 1991 Involvement of oestrogens in sexual differentiation of gonads as a function of temperature in turtles. *Differentiation* **47** 9–17.

Elbrecht A & Smith RG 1992 Aromatase enzyme activity and sex determination in chickens. *Science* **255** 467–470.

George FW & Wilson JD 1994 Sex determination and differentiation. In *The Physiology of Reproduction*, ed 2, pp 3–28. Eds E Knobil & J Neill. New York: Raven Press.

Gutzke WHN & Bull JJ 1986 Steroid hormones sex reverse turtles. *General and Comparative Endocrinology* 64 368–372.

Haqq CM, King C-Y, Donahoe PA & Weiss MA 1993 SRY recognizes conserved DNA sites in sex-specific promoters. *Proceedings of the National Academy of Sciences of the USA* 90 1097–1101.

Hunter GA & Donaldson EM 1983 Hormonal sex control and its application to fish culture. In *Fish Physiology*, pp 223–303. Eds WS Hoar, DJ Randall & EM Donaldson. New York: Academic Press.

Janzen F & Paukstis GL 1991 Environmental sex determination in reptiles: ecology, evolution, and experimental design. *Quarterly Review of Biology* 66 149–179.

Jost A 1961 The role of fetal hormones in prenatal development. *Harvey Lectures* 55 201–226.

Lance VA & Bogart MH 1991 Tamoxifen sex reverses alligator embryos at male producing temperatures, but is an antioestrogen in female hatchlings. *Experientia* 47 263–266.

Lance VA & Bogart MH 1992 Disruption of ovarian development in alligator embryos treated with an aromatase inhibitor. *General and Comparative Endocrinology* 86 59–71.

Merchant-Larios H, Fierro IV & Urriza BC 1989 Gonadal morphogenesis under controlled temperature in the sea turtle *Lepidochelys olivacea*. *Herpetological Monographs* 3 43–61.

Pieau C 1973 Variation de l'activité enzymatique Δ5–3β-hydroxystéroide déshydrogénasique dans les glandes génitales d'embryons d'*Emys orbicularis* L (Chélonien) en fonction de la température d'incubation. *Comptes Rendus de l'Academie des Sciences, Paris, Serie D* 276 197–200.

Pieau C 1974 Différenciation du sexe en fonction de la température chez les embryons d'*Emys orbicularis* L (Chélonien); effets des hormones sexuelles. *Annales d'Embryologie et de Morphogenese* 7 365–394.

Pieau C, Girondot M, Desvages G, Dorrizzi M, Richard-Mercier N & Zaborski P 1994 Environmental control of gonadal differentiation. In *The Differences Between the Sexes*, pp 433–448. Eds RV Short & E Balaban. Cambridge, England: Cambridge University Press.

Raynaud A & Pieau C 1985 Embryonic development of the genital system. In *Biology of the Reptilia*, pp 149–300. Eds C Gans & F Billet. New York: Wiley.

Russell DW & Wilson JD 1994 Steroid 5α-reductase: two genes/two enzymes. *Annual Review of Biochemistry* 63 25–61.

Santen RJ, Langecker P, Santner SJ, Sikka S, Rajfer J & Swerdloff R 1990 Potency and specificity of CGS-16949A as an aromatase inhibitor. *Endocrinological Research* 16 77–91.

Scheib B 1983 Effects and roles of oestrogens in avian gonadal differentiation. *Differentiation* 23 (Suppl.) 587–592.

Steele RE, Mellor LB, Sawyer WK, Wasvary JM & Browne LJ 1987 *In vitro* and *in vivo* studies demonstrating potent and selective estrogen inhibitions with the nonsteroidal aromatase inhibitor CGS 16949A. *Steroids* 50 147–161.

Taketo T, Merchant-Larios H & Koide SS 1984 Induction of testicular differentiation in the fetal mouse ovary by transplantation into adult male mice. *Proceedings of the Society for Experimental Biology and Medicine* 176 148–153.

Thomas EO, Licht P, Wibbels T & Crews D 1992 Hydroxysteroid dehydrogenase activity associated with sexual differentiation in embryos of the turtle *Trachemys scripta*. *Biology of Reproduction* 46 140–145.

Vigier B, Forest MG, Eychenne B, Bezard J, Garrigou O, Robel P & Josso N 1989 Anti-Müllerian hormone produces endocrine sex reversal of fetal ovaries. *Proceedings of the National Academy of Sciences of the USA* 86 3684–3688.

Wartenberg H, Lenz E & Schweikert HU 1992 Sexual differentiation and the germ cell in sex reversed gonads after aromatase inhibition in the chicken embryo. *Andrologia* 24 1–6.

Wibbels T, Bull JJ & Crews D 1991a Chronology and morphology of temperature-dependent sex determination. *Journal of Experimental Zoology* 260 371–381.

Wibbels T, Bull JJ & Crews D 1991b Synergism of temperature and estradiol: a common pathway in sex determination? *Journal of Experimental Zoology* 260 130–134.

Wibbels T, Bull JJ & Crews D 1992 Steroid hormone-induced male sex determination in an amniotic vertebrate. *Journal of Experimental Zoology* 263 454–457.

Wibbels T, Bull JJ & Crews D 1994 Temperature-dependent sex determination: A mechanistic approach. *Journal of Experimental Zoology* (In Press).

Wibbels T & Crews D 1992 Specificity of steroid hormone-induced sex determination in a turtle. *Journal of Endocrinology* 133 121–129.

Wibbels T & Crews D 1993 Steroid-induced sex determination at a near-pivotal temperature in a turtle with TSD. *American Zoologist* 33 8A (Abstract).

Wibbels T & Crews D 1994 Putative aromatase inhibitor induces male sex determination in a female unisexual lizard and in a turtle with TSD. *Journal of Endocrinology* 141 295–299.

Wozniak A, Homan SD & Hutchison JB 1992 *In vitro* potency and selectivity of the non-steroidal androgen aromatase inhibitor CGS 16949A compared to steroidal inhibitors in the brain. *Journal of Steroid Biochemistry and Molecular Biology* 43 281–287.

Wozniak A & Hutchison JB 1993 Action of endogenous steroid inhibitors of brain aromatase relative to fadrozole. *Journal of Steroid Biochemistry and Molecular Biology* 44 641–645.

Yntema CL 1968 A series of stages in the embryonic development of *Chelydra serpentina*. *Journal of Morphology* 125 219–252.