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# Steroid-Induced Sex Determination at Incubation Temperatures Producing Mixed Sex Ratios in a Turtle with TSD

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Previous studies have shown that exogenous steroid hormones can affect sex determination in reptiles with temperature-dependent sex determination (TSD). These studies have also suggested that the sensitivity of TSD to exogenous steroids may vary with incubation temperature. The majority of these studies, however, have utilized incubation temperatures producing all males or all females in the control groups, rather than temperatures which produced mixed sex ratios in control groups. The goals of the current study were to examine the effects of steroids on sex determination in a turtle (*Trachemys scripta*) at temperatures which produced mixed sex ratios in the control groups. Collectively, the results of single-treatment experiments indicate that at incubation temperatures producing mixed sex ratios in control groups, (1) estradiol-17$\beta$, tamoxifen, norethindrone, and testosterone all showed a similar "type" of effect (i.e., feminizing) as in previous studies utilizing male-producing temperatures, (2) sex determination has significantly increased sensitivity to estradiol-17$\beta$ in comparison to its effect at temperatures producing all males, and (3) sex determination is sensitive to the masculinizing effects of dihydrotestosterone (DHT) (in previous studies utilizing female-producing temperature DHT did not affect sex determination). Last, a set of double-treatment experiments was performed in which eggs received both estradiol-17$\beta$ and DHT treatments. No significant increases in the production of males were detected. Significant increases in the production of females were detected, but only in the groups receiving the highest dosage of estradiol-17$\beta$ (1.0 $\mu$g). This contrasts the results of the single-treatment experiments in which lower dosages of estradiol-17$\beta$ were effective (0.1 and 0.01 $\mu$g), thus suggesting that DHT in some way decreases the effectiveness of estradiol-17$\beta$. Further, a number of hatchlings in the double-treatment experiments developed intersex gonads (i.e., the gonads had well-developed medullary and cortical regions), suggesting that cortical and medullary development of the gonads are not mutually exclusive. 

A variety of past studies have shown that estrogen, estrogen-related compounds, and testosterone can induce female sex determination at male-producing temperatures in reptiles with temperature-dependent sex determination (TSD) (Pieau, 1974; Raynaud and Pieau, 1985; Gutzke and Bull, 1986; Bull *et al.*, 1988; Crews *et al.*, 1989, 1991; Dorizzi *et al.*, 1991; Lance and Bogart, 1991, 1992; Wibbels and Crews, 1992, 1994). It has been hypothesized that steroid-induced sex determination is an estrogen-specific event and the feminizing effect of testosterone may be due to its aromatization to estrogen (Crews *et al.*, 1989; Dorizzi *et al.*, 1991; Desvages and Pieau, 1991, 1992a,b; Wibbels and Crews, 1992; Wibbels *et al.*, 1994). This hypothesis is supported by recent studies showing that aromatase inhibitors can induce male sex determination in a turtle with TSD (Wibbels and Crews, 1994; Crews and Bergeron, 1994) and have disrupted ovarian development in alligators (Lance and Bogart, 1992). Several studies have also indicated that putative estrogen antagonists such as tamoxifen and norethindrone can act as estrogen agonists in this sex determination system (Lance and Bogart, 1991; Wibbels and Crews, 1992). Further, one study suggests that the sensitivity of sex determination to estradiol-17$\beta$ varies with incubation temperature (Wibbels *et al.*, 1991b), with

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the greatest sensitivity occurring at temperatures which produced mixed sex ratios in control groups. This may also be the case with the putative masculinizing effect of the nonaromatizable androgen dihydrotestosterone (DHT), which has been reported to induce male sex determination when utilizing an incubation regimen in which eggs were shifted from male-producing to female-producing temperatures late in the temperature-sensitive period, resulting in a 1:1 sex ratio in control groups (Wibbels *et al.,* 1992). However, DHT does not appear to affect sex determination at incubation temperatures which produce all females (Crews *et al.,* 1989; Wibbels and Crews, 1992). Thus, the effects of steroids on sex determination can significantly vary depending upon the incubation temperature utilized. Considering these latter findings and the fact that the great majority of past studies have utilized temperatures producing either all males or all females in control groups, there is a need for studies that examine the effects of steroids at temperatures producing mixed sex ratios.

The current study addresses several of the hypotheses reviewed above by examining (1) if sex determination is sensitive to DHT at constant incubation temperatures which produce mixed sex ratios in control groups, (2) if the feminizing effects of estradiol-17$\beta$ increase at incubation temperatures which produce mixed sex ratios in control groups, and (3) if several reputed estrogen antagonists and the androgen testosterone show similar effects at incubation temperatures which produce mixed sex ratios in control groups in comparison to their previously described effects at male-producing temperatures (i.e., they exhibit feminizing effects at male-producing temperatures). Last, the current study also includes a double-treatment experiment which examines if masculinizing effects of DHT and feminizing effects of estradiol-17$\beta$ (respectively) can be simultaneously stimulated at a mixed sex ratio incubation temperature.

## MATERIALS AND METHODS

Freshly laid eggs from the red-eared slider, *Trachemys scripta,* were obtained commercially (Robert Kliebert, Hammond, LA). After transport to our laboratory, they were placed in containers with moistened vermiculite (vermiculite:water, 1:2) which were placed in incubators set at 28.6, 29.0, or 29.2°. Temperatures set points were based on thermometers which had been calibrated against an NIST-traceable thermometer. Temperatures were taken a minimum of twice daily. Based on the twice-daily readings, typical temperature variations around a given set point were approximately ±0.15° (standard deviation).

Previous studies indicated that continuous incubation at 31° produces all female hatchlings, whereas 26° produces all male hatchlings and incubation temperatures near 29.0° produce mixed sex ratios (Bull *et al.,* 1982; Crews *et al.,* 1991; Wibbels *et al.,* 1991a; Wibbels, Bull, and Crews, unpublished data). As such, the three temperatures used in the current experiment (28.6, 29.0, and 29.2°) were chosen in an effort to obtain mixed sex ratios in the control groups.

Embryonic development was monitored by candling eggs and by dissecting two to four eggs approximately twice a week to verify specific developmental stages, based on criteria described by Yntema (1968). When embryos reached stage 17–18, a time period within the temperature-sensitive window in this species (Wibbels *et al.,* 1991a), the eggs were randomized into control and experimental groups. A series of single-treatment and double-treatment experiments were performed. In single-treatment experiments, eggs from experimental groups received a single treatment of a specific ligand (i.e., hormone, agonist, or antagonist) suspended in 5 $\mu$l 95% ethanol. Specifically, the effects of estradiol-17$\beta$ (dosages = 0.001, 0.01, 0.1, and 1.0 $\mu$g) and dihydrotestosterone (i.e., 5$\alpha$-androstan-17$\beta$-ol-3-one or DHT; 1.0, 10.0, 100.0, 200.0 $\mu$g) were examined at all three temperatures. Furthermore, three additional ligands were examined at 29.2° (the temperature which produced an approximate 1:1 sex ratio): tamoxifen at dosages of 10.0 and 100.0 $\mu$g, norethindrone at dosages of 1.0, 10.0, and 100.0 $\mu$g, and testosterone at dosages of 10.0, 100.0, and 200.0 $\mu$g. All ligands were obtained from Sigma (St. Louis, MO). The dosages chosen for each ligand were based on previous studies with turtles (Gutzke and Bull, 1986; Bull *et al.,* 1988; Crews *et al.,* 1989, 1991; Wibbels and Crews, 1992). Eggs in control groups received a single treatment consisting of 5 $\mu$l 95% ethanol. A series of double-treatment experiments were carried out at a single incubation temperature (29.0°) in which eggs received various dosages of both estradiol-17$\beta$ and DHT (Table 1). Previous studies suggest that a female-producing temperature can negate any possible masculinizing effect of DHT (Crews *et al.,* 1989; Wibbels and Crews, 1992; Wibbels *et al.,* 1992); therefore, a temperature producing a male bias in control groups (29.0°) was utilized. Each of the two treatments was delivered in 5 $\mu$l ethanol as described above and control eggs received two 5-$\mu$l treatments of ethanol.

All treatments were applied topically to the vascularized portion of the upper shell (Crews *et al.,* 1991). Groups of approximately 30 eggs were used for each treatment group. After receiving treatments, all eggs were placed back into their respective incubators until they hatched. Turtles were euthanized approximately 2–4 weeks after hatching. Gonadal sex was assessed by examination of the reproductive

tracts under a dissection microscope. In the great majority of cases the gonads of hatchling *T. scripta* were well differentiated and appeared distinctly testicular or ovarian when viewed under a dissection microscope (Crews *et al.*, 1991; Wibbels *et al.*, 1991a). Ovaries are long and flat whereas testes are, relative to the ovaries, shorter, round, and have visible seminiferous tubules (see Crews *et al.*, 1991). In cases where the gonads did not appear distinctly male or female, gonads were examined histologically. Histological analysis included fixation in Bouin's solution and paraffin embedding, followed by 8.0-$\mu$m sectioning and hematoxylin/PAS staining (Humason, 1972).

The sex ratios from the dosage groups within each treatment were initially pooled at each temperature and compared to the respective control group using Fisher exact tests. If significance was detected, Fisher exact tests were then used to compare the sex ratio of each dosage of a particular treatment to the respective control group. Additionally, Fisher exact tests were used to compare dosages within a treatment group to one another to determine if certain dosages were more effective than others. Logistic regression of sex ratios from the single-treatment experiments was used to compare the effects of given hormones at the different temperatures (Chatterjee and Price, 1977; Gabriel, 1978; Sokal and Rolf, 1981; see detailed example by Crews *et al.*, 1995). Specifically, the effect of each hormone (estradiol-17$\beta$ or DHT) within each temperature group was determined using polynomial logistic regression. Regression coefficients from the different temperatures were then compared to coefficients by computing lower and upper comparison limits (Gabriel, 1978). That is, coefficients were significantly different if their comparison limits did not overlap. These analyses can reveal a synergism between temperature and hormone treatment if the $b_1$ regression coefficients at different temperatures are significantly different from one another.

## RESULTS

The results of the single-treatment experiments are sown in Figs. 1 and 2. The pooled sex ratios from the estradiol-17$\beta$ dosages at each temperature were significantly different from their respective control groups ($P < 0.001$). A comparison of each dosage group to the control groups revealed that at the 28.6° incubation temperature each of the three highest dosages of estradiol-17$\beta$ (0.01, 0.1, and 1.0 $\mu$g) resulted in the production of significantly more females than in the control groups (Fisher exact tests, $P < 0.01$), whereas the lowest dosage (0.001 $\mu$g) did not significantly induce female sex determination ($P > 0.05$). At 28.6° a distinct dose response was evident with each dosage of estradiol-17$\beta$ producing significantly more females

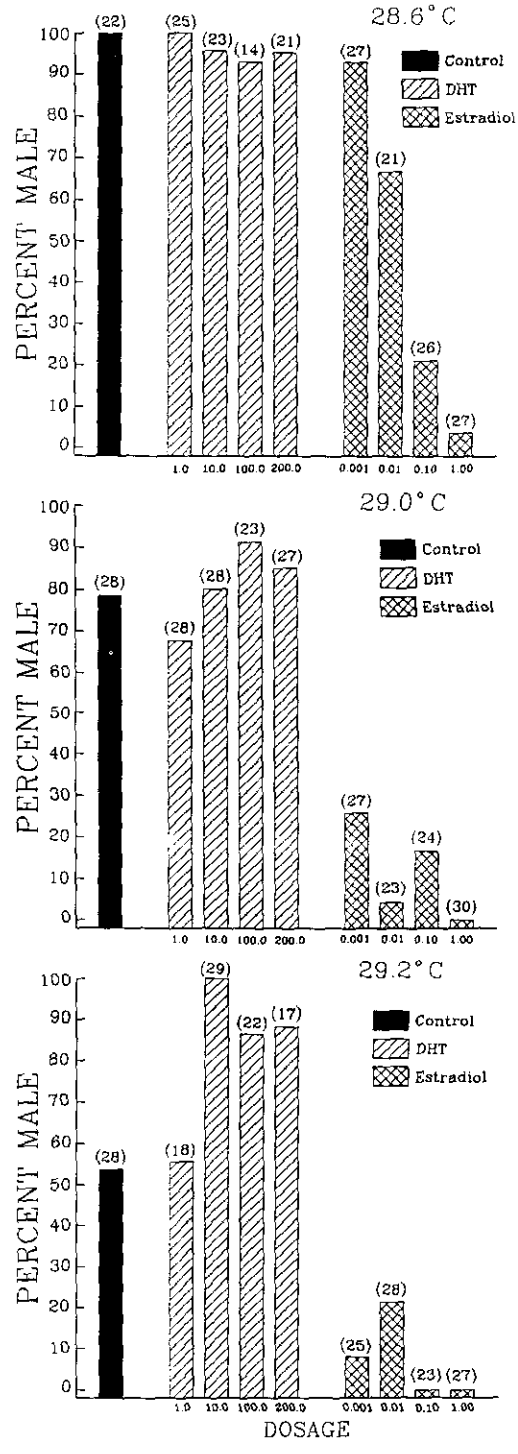


FIG. 1. Effects of single treatments of DHT or estradiol-17$\beta$ on an embryo incubated at 28.6, 29.0, or 29.2°. The numbers in parentheses above each bar indicate the number of individuals in each group. Dosages in micrograms are shown under each bar. Controls received 5 $\mu$l of ethanol.

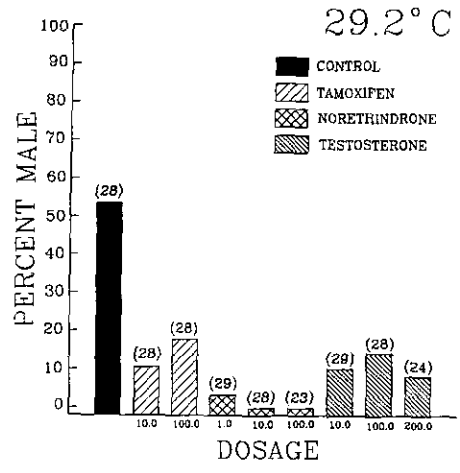


FIG. 2. Effects of single treatments of tamoxifen, norethindrone, or testosterone on an embryo incubated at 29.2°. The numbers in parentheses above each bar indicate the number of individuals in each group. Dosages in micrograms are shown under each bar. Controls received 5 μl of ethanol.

than the next lower dosage ($P < 0.05$). At the 29.0 and 29.2° incubation temperatures, each of the four dosages of estradiol-17β resulted in the production of significantly more females than in the control groups (Fisher exact tests, $P < 0.01$). Comparisons of sex ratios from different dosages from those two temperatures did not reveal any clear dose–response patterns. Logistic regression analysis revealed a syngeristic effect of temperature and estradiol-17β, with estradiol-17β exerting a significantly greater effect on sex determination at 29.0 and 29.2° in comparison to the results at 28.4° (see Table 2). Additionally, pooled sex ratios from the different dosage groups as well as the individual sex ratios from each dosage group of tamoxifen, norethindrone, and testosterone at the 29.2° incubation temperature (Fig. 2) produced significantly more females than in the control groups (Fisher exact tests, $P < 0.01$). At the 28.6 and 29.0° incubation temperatures, no significant differences were detected between the sex ratios of the control groups and the pooled DHT-treated groups from each temperature (Fisher exact tests, $P > 0.05$), whereas at 29.2° the pooled DHT-treated groups produced significantly more males than the control groups ($P = 0.001$). A comparison of the sex ratio of each dosage group to the controls revealed that each of the three highest dosages of DHT resulted in the production of significantly more males that in the control groups (Fisher exact tests, $P < 0.01$). Comparison of individual dosages of DHT at 29.2° indicated that each of the three highest dosages (10, 100, and 200 μg) produced significantly more males than the lowest DHT dosage (1.0 μg). The sex ratios of the three highest dosages of DHT at 29.2° were not significantly different from one another ($P > 0.05$). Logistic regression revealed no synergistic effect of DHT and temperature ($P > 0.05$).

TABLE 1

SUMMARY OF DOUBLE-TREATMENT EXPERIMENTS IN WHICH EGGS RECEIVED ESTRADIOL-17β AND DIHYDROTESTOSTERONE (DHT) TREATMENTS

| Group | DHT (μg) | Estradiol (μg) | n | ♂ | ♀ | ♂/♀ | % ♂ | FET |
|---|---|---|---|---|---|---|---|---|
| Control | — | — | 26 | 21 | 5 | 0 | 80.7 | — |
| 1 | 1.0 | 0.01 | 25 | 21 | 4 | 0 | 84.0 | ns |
| 2 | 1.0 | 0.10 | 29 | 21 | 8 | 0 | 72.4 | ns |
| 3 | 1.0 | 1.00 | 28 | 10 | 17 | 1 | 35.7 | ** |
| 4 | 10.0 | 0.01 | 22 | 22 | 0 | 0 | 100.0 | ns |
| 5 | 10.0 | 0.10 | 26 | 23 | 3 | 0 | 88.5 | ns |
| 6 | 10.0 | 1.00 | 26 | 13 | 13 | 0 | 50.0 | * |
| 7 | 100.0 | 0.01 | 29 | 28 | 1 | 0 | 96.5 | ns |
| 8 | 100.0 | 0.10 | 27 | 19 | 6 | 2 | 70.4 | ns |
| 9 | 100.0 | 1.00 | 28 | 6 | 15 | 7 | 20.7 | *** |

*Note.* Incubation temperature was maintained at 29.0°. ♂/♀, intersex gonads; FET, Fisher exact test comparing sex ratio of each group to control; ns, nonsignificant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

TABLE 2

SEPARATE REGRESSION RESULTS (IN COLUMNS) FOR THE EFFECTS OF INCREASING ESTRADIOL-17β CONCENTRATIONS ON HATCHLING SEX RATIOS AT THREE DIFFERENT INCUBATION TEMPERATURES

| Regression coefficients | Incubation temperature | | |
|---|---|---|---|
| | 28.6° | 29.0° | 29.2° |
| $B_0$ | $-3.38 \pm 0.79^a$ | $-1.30 \pm 0.46^{a,b}$ | $-0.14 \pm 0.38^b$ |
| | ($LR\chi^2 = 18.21$, $P < 0.001$) | ($LR\chi^2 = 7.96$, $P = 0.005$) | ($LR\chi^2 = 0.14$, $P = 0.71$) |
| | [$l = -4.73$, $u = -2.03$] | [$l = -2.09$, $u = -0.51$] | [$l = -0.79$, $u = 0.51$] |
| $B_1$ | $298 \pm 104^a$ | $2584 \pm 711^b$ | $2887 \pm 929^b$ |
| | ($LR\chi^2 = 8.23$, $P = 0.004$) | ($LR\chi^2 = 13.22$, $P < 0.001$) | ($LR\chi^2 = 9.65$, $P = 0.002$) |
| | [$l = 120$, $u = 475$] | [$l = 1367$, $u = 3801$] | [$l = 1297$, $u = 4477$] |
| $B_2$ | $-2767 \pm 1068^a$ | $-237{,}631 \pm 76{,}363^b$ | $-304{,}414 \pm 101{,}462^b$ |
| | ($LR\chi^2 = 6.72$, $P = 0.009$) | ($LR\chi^2 = 9.68$, $P = 0.002$) | ($LR\chi^2 = 9.0$, $P = 0.003$) |
| | [$l = -4594$, $u = -940$] | [$l = -368{,}288$, $u = -106{,}974$] | [$l = -478{,}015$, $u = -130{,}813$] |
| $B_3$ | $2476 \pm 965^a$ | $2{,}330{,}304 \pm 762{,}527^b$ | $3{,}042{,}939 \pm 1{,}031{,}432^b$ |
| | ($LR\chi^2 = 6.59$, $P = 0.01$) | ($LR\chi^2 = 9.34$, $P = 0.002$) | ($LR\chi^2 = 8.7$, $P = 0.003$) |
| | [$l = 825$, $u = 4127$] | [$l = 1{,}025{,}620$, $u = 3{,}634{,}988$] | [$l = 1{,}278{,}159$, $u = 4{,}807{,}719$] |
| $B_4$ | ns | $-2{,}095{,}250 \pm 686{,}874^b$ | $-2{,}741{,}399 \pm 932{,}462^b$ |
| | | ($LR\chi^2 = 9.31$, $P = 0.002$) | ($LR\chi^2 = 8.64$, $P = 0.003$) |
| | | [$l = -3{,}270{,}491$, $u = -920{,}009$] | [$l = -4{,}336{,}841$, $u = -1{,}145{,}957$] |

*Note.* Regression coefficients ± 1 standard error with $\chi^2$ and probability values in parentheses; there is 1 df for each regression coefficient. Regression coefficients with different superscripted letters are significantly different at a level of $\alpha = 0.05$ for different incubation temperatures. The coefficients are significantly different if their comparison limits in brackets do not overlap. $B_0$ represents the effect of temperature. Synergism of temperature and estradiol-17β is present if $B_1$ is significantly greater at the intermediate temperatures (29.0 and 29.2°) in comparison to the baseline temperature (28.6°). ns, not significant.

In the double-treatment experiment (Table 1) the highest dosage of estradiol-17β (1.0 μg) was combined with one of the three dosages of DHT, resulting in the production of significantly more females than in the control groups (Fisher exact tests, see Table 1). The two lower dosages of estradiol-17β (0.01 and 0.10 μg) did not significantly increase the production of females when combined with any of the DHT dosages (see Table 1). A number of intersex gonads resulted from the double-treatment experiments, including 7 of 28 from the group receiving 1 μg of estradiol-17β and 100 μg DHT. A longitudinal section of an intersex gonad from that treatment group is shown in Fig. 3. The gonad has both a distinct cortex and distinct seminiferous tubules in the medullary region of the gonad. Normal male hatchlings lack a cortex and normal female hatchlings lack developing seminiferous tubules in the medullary region of the gonad (see Wibbels *et al.*, 1991a, for detail photographs of normal hatchlings).

## DISCUSSION

The results from the current study support and extend several of the hypotheses concerning the effects of steroids on TSD. First, comparisons of the sex ratios produced at all three temperatures indicate that the sensitivity of sex determination to estradiol-17β is significantly greater at the temperatures producing mixed sex ratios in control groups in comparison to the temperature which produced all males in the control group. In fact, the effects of estradiol-17β and temperature were shown to exert a synergism on sex determination at the temperatures producing mixed sex ratios in the control groups. These findings are consistent with the results of a past study comparing the effects of estradiol-17β at a cooler male-producing temperature (26°) to a higher temperature which produced primarily males in the control group (28.2°, sex ratio = 1 female:30 males) (Wibbels *et al.*, 1991b). Additionally, sex determination

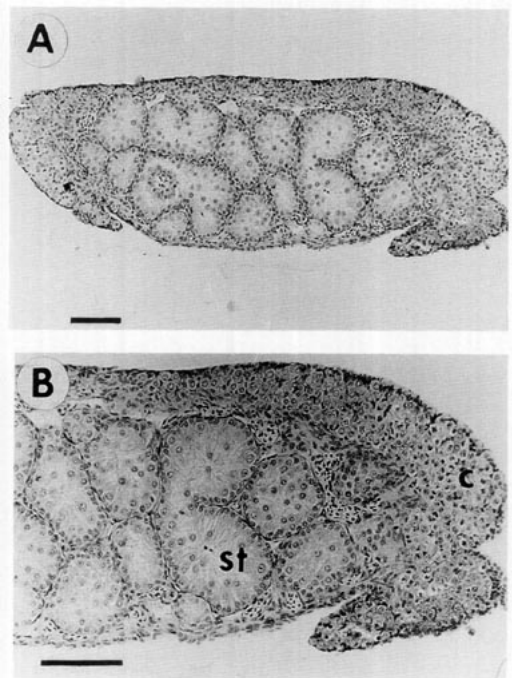


FIG. 3. (A) Longitudinal section through an intersex gonad treated with 100.0 μg DHT and 1.0 μg estradiol-17β. (B) Higher magnification photo of the same gonad showing well-developed cortical and medullary regions. See Results section for a description of the gonads from normal male and female hatchlings. The scale bar in the lower left corner of each photograph represents 100 μm. c, cortex; st, seminiferous tubule.

was shown to be sensitive to as little as 0.001 μg topically applied to the eggshell when eggs are incubated at temperatures producing mixed sex ratios. The sensitivity of sex determination to estradiol-17β together with the ability of temperature and estradiol-17β to exert a synergistic effect on sex determination is consistent with the hypothesis that the exogenous estrogen used in these experiments may simply be mimicking the endogenous production of estrogen during sex determination. A variety of past studies examining endogenous estrogen levels as well as aromatase activity support the hypothesis that incubation temperature may affect sex determination through the production of endogenous estrogen (see reviews by Wibbels *et al.*, 1994; Crews *et al.*, 1994).

The results of the tamoxifen, norethindrone, and testosterone treatments are consistent with the hypothesis that steroid-induced female sex determination is an estrogen-specific event and that the effects of testosterone may be mediated via aromatization to estrogen (Crews *et al.*, 1989; Dorizzi *et al.*, 1991; Wibbels and Crews, 1992, 1994; Wibbels *et al.*, 1994; Crews and Bergeron, 1994; Crews *et al.*, 1995). Tamoxifen and norethindrone induced female sex determination in the current study at the temperature producing a near 1:1 sex ratio (29.2°). These data are similar to those of past studies using male-producing temperatures (Crews *et al.*, 1989; Lance and Bogart, 1991; Wibbels and Crews, 1992), thus indicating that estrogen-related compounds (including some compounds reputed to be estrogen antagonists) consistently induce female sex determination at temperatures producing mixed sex ratios in control groups as well as at temperatures which produce all males in control groups. Additionally, the aromatizable androgen testosterone induced female sex determination, whereas the nonaromatizable androgen DHT induced male sex determination.

The results of the DHT treatments support the hypothesis that DHT can have a masculinizing effect on sex determination at temperatures which produce mixed sex ratios. DHT was shown to significantly increased the production of males in the current study at 29.2°. In previous studies utilizing female-producing incubation temperatures, DHT did not affect sex determination (Crews *et al.*, 1989; Wibbels and Crews, 1992). The masculinizing effect of DHT on sex determination has been reported in a previous study which utilized a temperature shift regimen that resulted in an approximate 1:1 sex ratio in control groups (Wibbels *et al.*, 1992); however, the current study is the first to show this masculinizing effect at a constant incubation temperature. Interestingly, the masculinizing ability of DHT does not appear as robust as the feminizing ability of estrogen, since temperature regimes resulting in mixed sex ratios coupled with relatively high dosages of DHT must be used. Further, regardless of the DHT dosage it is difficult to predictably obtain

masculinization of all embryos in a treatment group.

The results of the double-treatment experiments lend support the hypothesis that estradiol-17$\beta$ and DHT have opposite effects on sex determination. Although no significant production of males was detected, it is of particular interest that the lower dosages of estradiol-17$\beta$ (0.01 and 0.1 $\mu$g) given in combination with various dosages of DHT did not significantly induce female sex determination. This contrasts the results of the single-treatment estradiol-17$\beta$ experiments at 29.0° in which the lower dosages (0.01 and 0.1 $\mu$g) were effective and suggests that DHT decreases the effectiveness of estradiol-17$\beta$. However, caution should be used in comparing these two experiments, since they were conducted independently (although the control groups were similar, 21 males:5 females versus 22 males:6 females). Last, it is noteworthy that the group receiving the highest dosages of both DHT and estradiol-17$\beta$ had a relatively large number of intersex gonads (7 of 28) with distinct cortical as well as medullary development. Intersex gonads have been reported previously in embryonic reptiles treated with estradiol-17$\beta$ (Pieau, 1974; Raynaud and Pieau, 1985; Dorizzi *et al.*, 1991) or with tamoxifen and estradiol-17$\beta$ (Dorizzi *et al.*, 1991) and such findings suggest that cortical and medullary development are not mutually exclusive.

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