GENERAL AND COMPARATIVE ENDOCRINOLOGY 81, 357–364 (1991)

Estrogen and Sex Reversal in Turtles: A Dose-Dependent Phenomenon

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Accepted March 12, 1990

Exogenous estradiol benzoate (EB) or estradiol-17β (E2) caused dose-dependent gonadal feminization of slider turtle (*Trachemys scripta*) embryos incubated at a male-producing temperature (26°), suggesting that sex reversal requires a threshold dosage of these hormones. Even at dosages resulting in mixtures of males and females, nearly all hatchlings had normal-appearing ovaries or testes. Only 7 of 241 hatchlings had gonads that had not differentiated fully into ovaries or testes. Thus, with rare exception, estrogens exerted an "all or none" effect. The transport of hormone into the embryo varied with mode of administration and E2 was more effective than was EB at the lowest dosage used. These studies suggest either that exogenous estrogen is capable of coordinating cortical/medullary development in the gonad so that intersexes are prevented or that estrogen acts "upstream" of the developmental processes responsible for coordinating gonadal development.

Exposure of embryos to estrogens has long been known to feminize the gonads of certain reptiles (Dantchakoff, 1937). More recently, estradiol benzoate (EB) has been shown to feminize the developing gonads of a variety of reptiles, including species with temperature-dependent sex determination (TSD) as well as some with genotypic sex determination (GSD) (Raynaud, 1965; Pieau, 1974; Gutzke and Bull, 1986; Bull *et al.*, 1988; Crews *et al.*, 1989). Although a consensus is emerging with respect to the feminizing effect of estrogen, a related topic lacks consensus and has not been a focus of these studies: Do low dosages of estrogen produce gonadal intermediates? In the latter three studies cited, the dosages administered produced nearly all females; hence, the data did not address this question. Pieau's (1974) results suggested that a correlation existed between estrogen dosage and the proliferation or regression of the cortical and medullary regions of the gonads. He reported treatment dosages ranging from 5 to 80 μg which produced either ovaries or "bisexual" gonads in embryos of the European pond turtle, *Emys orbicularis* (Pieau, 1974). In this instance, bisexual referred to embryonic gonads with relatively well-developed cortical and medullary regions. Similar results were reported for embryonic *Lacerta viridis* following injections ranging from 20 to 50 μg of EB per egg (Raynaud, 1965). The present study examined in the red-eared slider turtle (*Trachemys scripta*), a turtle with TSD, (i) the threshold dosage for estrogen-induced feminization, (ii) the transport of steroid hormone into the egg, and (iii) the relative effectiveness of natural and synthetic estrogens.

MATERIALS AND METHODS

Eggs and embryos. Eggs were obtained commercially from R. Kliebert (Hammond, LA) in May–July 1989 and incubated on moist vermiculite at 26° (±1°), a temperature producing only male hatchlings in this species; higher temperatures such as 31° produce only

0016-6480/91 $1.50

female hatchlings (Bull *et al.*, 1982, and unpublished data). Intersexes or individuals capable of producing both eggs and sperm, or hatchlings with both cortical and medullary development, have never been reported in this species (Bull *et al.*, 1982).

Shortly after the egg is laid, the embryo attaches to the upper portion of the eggshell, and the yolk forms a layer on the bottom of the egg with the albumen on top (and nearest the embryo). The first visible sign of development is a blood ring forming around the embryo on the inner surface of the eggshell (3–6 days after oviposition, depending on temperature). The diameter of this ring expands as the embryo grows, and the embryo can be "candled" to reveal its size and developmental stage. At about embryonic stage 13 (Yntema, 1968), the embryo begins to sink into the center of the egg and is no longer visible, but expansion of the vascularized region continues. Hatching is defined as stage 26 by Yntema's criteria.

Hormone administration. Hormones consisted of β-estradiol 3-benzoate (Sigma, catalog No. E-9000) or estradiol-17β (Sigma, catalog No. E-8875) dissolved in 95% ethanol at the specified concentration per 5 μl. Two basic methods of hormone administration were employed: (i) injection of hormone into the egg, and (ii) topical application of hormone onto the eggshell. In both methods, a 5-μl bolus was administered at stage 17 (±1 stage) using a Hamilton microliter syringe (catalog No. 725LT) and a 30-gauge needle. Controls received 5 μl of vehicle only. For injections, the needle was inserted through the shell and approximately 2 cm into the (upper) "albumen" layer of the egg (as in Bull *et al.*, 1988; Crews *et al.*, 1989). For topical application of hormone, a 5-μl bolus was deposited on the upper portion of the eggshell, either on the region directly above the vascularized area or over a region lacking obvious vascularization.

To evaluate how rapidly the injected or topically applied estrogen reached the embryo, [^{3}H]estradiol-17β (^{3}H-E2; New Englander Nuclear) was administered to eggs via injection or topical application as described above (40 eggs per administration technique). Each egg received approximately 10^6 cpm. Embryos were processed at 2, 25, 50, and 216 hr following hormone administration (10 eggs/time period/administration technique). During processing, embryos were killed by cervical dislocation, their surfaces were washed with 95% EtOH, and they were frozen at −80°. Embryos were later thawed, weighed, homogenized in 4 ml of ethyl acetate (Fisher Scientific, HPLC grade), allowed to stand for 2 hr at 4°, and then centrifuged at 2000*g* for 10 min. The supernatant was decanted into scintillation vials and dried under N_2. The pellet was resuspended in 4 ml of ethyl acetate and the extraction procedure was repeated. After centrifugation, the supernatant was added to the original supernatant and dried under N_2. Scintillation fluid (2.5 ml, Omniflour, NEN) was added to the scintillation vials and radioactivity was measured on a β-scintillation spectrometer (Beckman LS5800). To monitor extraction efficiencies, parallel homogenizations were conducted on embryos that had been frozen, but had not been exposed previously to ^{3}H-E2. After being thawed, embryos were processed as described above, except that 20,000 cpm of ^{3}H-E2 in 95% EtOH was applied to the surface of each embryo prior to the addition of the first 4-ml aliquot of ethyl acetate. Extraction efficiencies in those tests were always higher than 89%.

In a separate study, the relatively high mortality associated with egg injections was investigated. In this study, 5 μl of either 95% ethanol or sterile water was injected through either the vascularized or the nonvascularized portion of the upper-half of the shell; molten paraffin was also applied to the injection site in some instances.

Sex diagnosis. Sex was diagnosed in hatchlings using two methods. First, hatchlings were decapitated and dissected, and the gonads were visually inspected using stereomicroscopy. Sex was diagnosed as in Bull and Vogt (1979), Bull (1987), Bull *et al.* (1988), and Crews *et al.* (1989). Briefly, a hatchling was considered a female if each gonad was long and thin with a well-developed cortex and minimal vascularization restricted to a regressed medullary region. A hatchling was considered male if each gonad was short and well vascularized, with visible seminiferous tubules in a prominent medullary region and a reduced or absent cortex. Sex was diagnosed independently by at least two of the authors. The second method was histological examination. At least one gonad from each turtle was examined histologically; in the case of dosages that produced both males and females, both gonads were examined (see Crews *et al.*, 1989, for histological procedures). Diagnosis was made after examination at several levels of the gonad.

RESULTS

Dose dependency. For both hormones and both modes of administration, the sex ratio (proportion female) varied significantly with dosage, with the higher dosages producing higher sex ratio (χ^2 heterogeneity test, $P < 10^{-3}$ for all four tests; Fig. 1). Seven of 241 hatchlings that received hormone treatment had gonads that had not differentiated fully into testes or ovaries by the time of hatchling. That is, the gonads still possessed both medullary and cortical components. They were not true intersexes as defined by a gonad capable of producing

both egg and sperm. Male-typical and female-typical characteristics were as described by Pieau (1974) for embryos of the European pond turtle, *E. orbicularis*. The other 234 hatchlings had normal-appearing ovaries or testes, even at dosages that produced mixtures of males and females (Fig. 2). Macroscopic diagnosis was confirmed in all cases when analyzed histologically.

Hormones and mode of administration. Overall, feminizing ability did not differ significantly between EB and E2 (χ^2_7 heterogeneity test, $0.1 > P > 0.05$). However, topical application of 0.1 μg E2 produced significantly more females than the same dose of EB (Fisher's exact test, two-tailed, $P < 0.05$); the effectiveness of the two hormones was not significantly different at the 1.0-μg dosage ($P > 0.05$). The mode of administration significantly affected the feminizing ability of these estrogens (χ^2_7 heterogeneity test, $P < 10^{-3}$), with the greatest variation being detected for 1.0 μg of EB (Fisher's exact test, two-tailed, $P < 10^{-4}$) and 0.1 μg of E2 (Fisher's exact test, two-tailed, $P < 10^{-2}$).

The site of placement of the hormone on the eggshell did not appear to affect sex determination in topical administration. Both sexes were obtained whether the hormone was placed over the embryo or away from the embryo.

Tritiated E2 was detectable within embryos 2 hr following injection or topical administration (Fig. 3). The amount of tritiated hormone in embryos varied significantly relative to time and method of hormone administration (two-way ANOVA of log cpm values: by time, $F = 17.4$, $P < 10^{-4}$; by method of administration, $F = 20.0$, $P < 10^{-4}$; interaction between time and method, $F = 53.56$, $P < 10^{-4}$). In com-

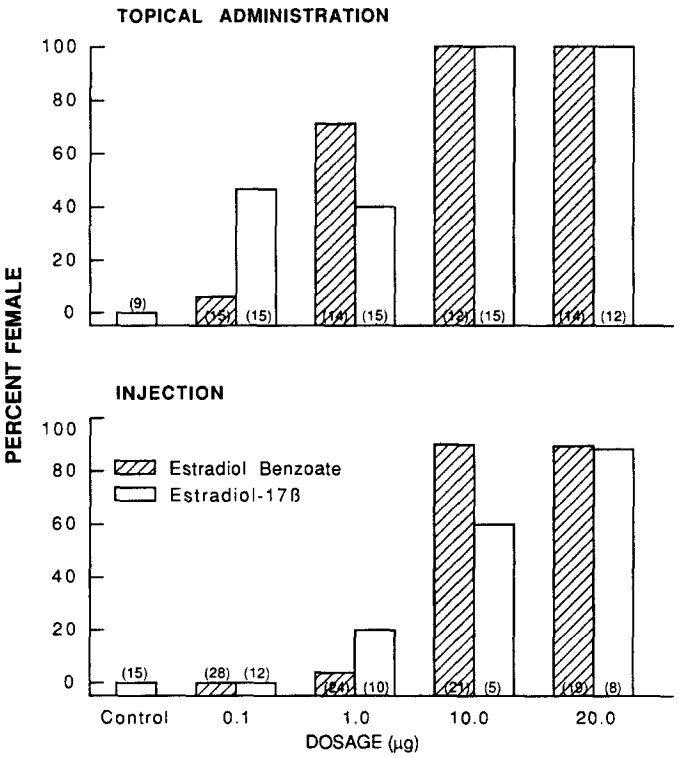

FIG. 1. Effect of exogenous estradiol-17β and estradiol benzoate on feminizing hatchling *Trachemys scripta* incubated at a male-producing temperature. Sample sizes are in parentheses.

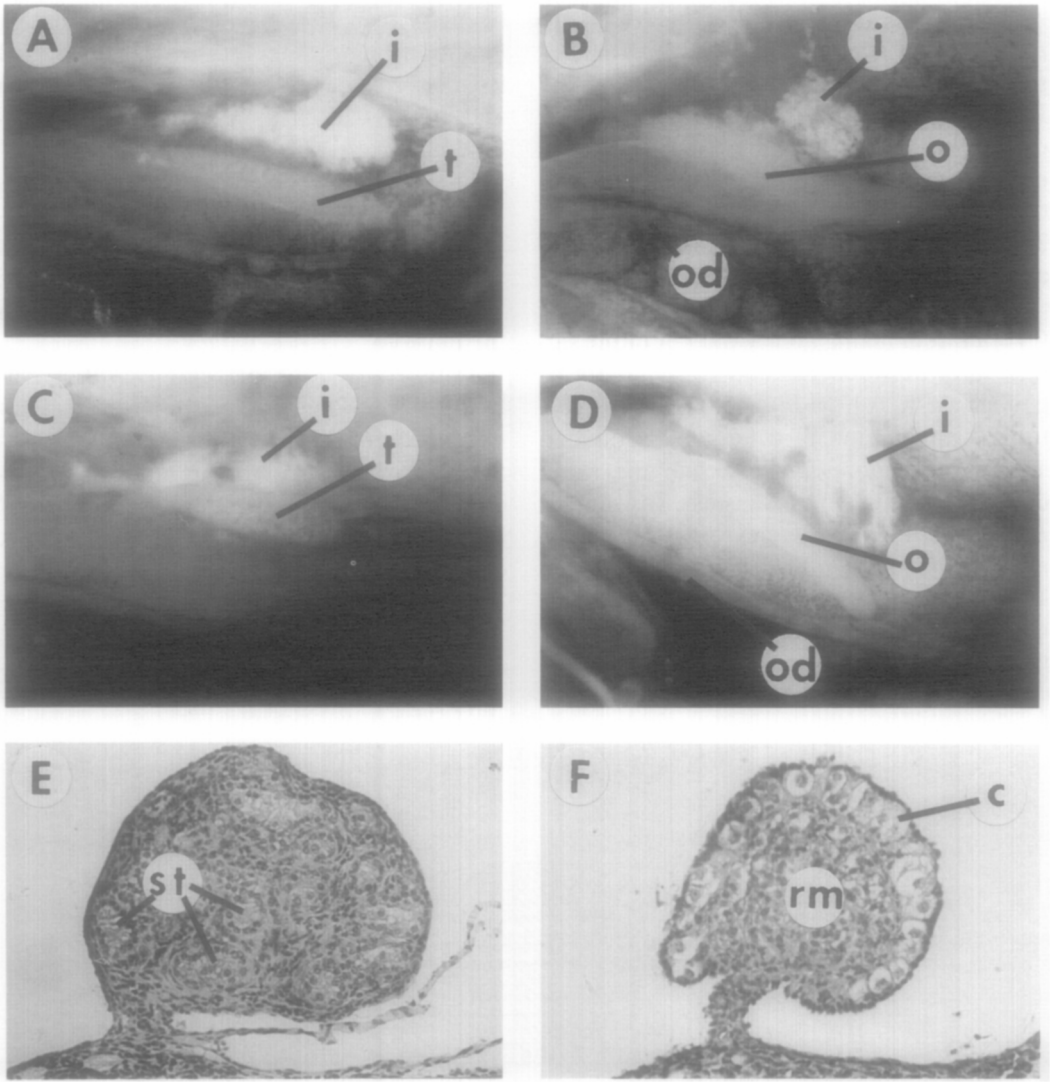

FIG. 2. Macroscopic views of preserved gonads: (A) control testis, (B) control ovary, (C) testis from hatchling that received 0.1 μg of estradiol benzoate (EB), and (D) ovary from a hatchling that received 1.0 μg EB. Histological sections are of (E) a testis and (F) an ovary from gonads that received 0.1 μg EB. In all instances, hormones were administered topically. Histological sections were stained with PAS/hematoxylin.

parison to the topical administration method, the injection method resulted in significantly more hormone in the embryos 2 hr after it was administered (t test of log cpm values; $t = 12.2$, $P < 10^{-4}$). However, 50 hr after hormone administration, significantly more counts per minute were detected in embryos from eggs that had received topical administration of hormone (t test; $t = 18.0$, $P < 10^{-3}$). Both hormone administration methods resulted in significant changes in the counts per minute content of embryos over time (Multiple t test analyses, $P < 10^{-4}$) with the highest counts per minute values being detected in embryos 2 hr after injection or 25 hr after topical administration.

Mortality. Injection of hormones resulted

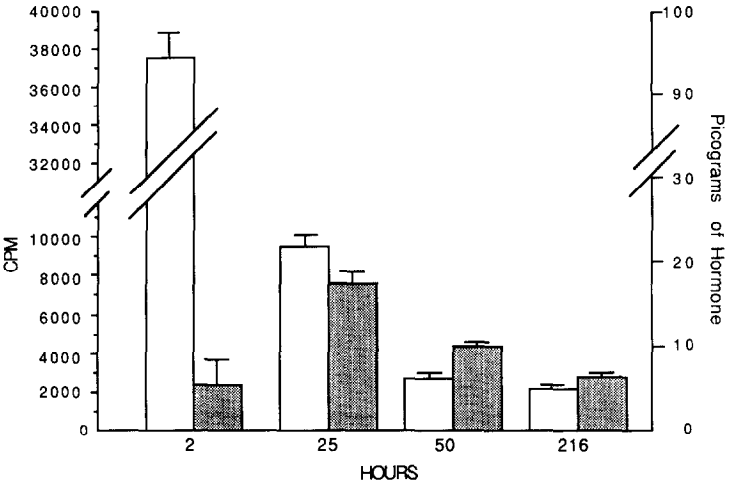

FIG. 3. Amount (in picograms) of tritiated hormone obtained from extractions of whole embryo homogenates that had received either injections or topical administration of [^{3}H]estradiol-17β. Means with standard errors are shown. Sample sizes are in parentheses.

in significantly lower survival than topical application (Fisher's exact test, one-tailed, $P < 10^{-6}$). The mean percentages of individuals per hormone group surviving after injection were EB = 56.6 (5.0) and E2 = 43.3 (7.6) (standard error in parentheses); after topical administration, the corresponding values were EB = 93.3 (2.7) and E2 = 96.7 (3.3). The low survival of injected eggs was consistent with previous studies (Gutzke and Bull, 1986; Bull *et al.*, 1988) and was probably due to infection. The site of injection was also important. Eggs injected with ETOH in the vascular area had significantly higher survival compared to eggs injected with ETOH in the nonvascular area (80% vs 50%; Fisher's exact test, two-tailed, $P < 0.05$).

DISCUSSION

We have shown that in the red-eared slider (*T. scripta*) (i) both EB and E2, applied topically or injected, cause female development at incubation temperatures that would otherwise have produced only males, (ii) the percentage of feminization was greater at the higher dosages, (iii) E2 is more effective at the lowest dosage used (0.1 μg) than EB, and (iv) undifferentiated gonads were rare, even at dosages that produced both sexes.

In contrast to injection, topical application of estrogen yielded much higher survival and resulted in female determination at lower dosages. It is interesting, and potentially useful for future studies, that feminization can be induced with administration of estrogen on the eggshell. Topical application of hormones has been shown previously to influence development, but sex reversals were not observed in those studies (Burns, 1961; Crews *et al.*, 1983; Billy and Crews, 1986). The greater sensitivity may be due to the active transport of hormone (topical application) through the eggshell versus passive transport of hormone into the egg (injection).

The sex ratio of hatchlings was dose-dependent, with the lowest dosages producing significantly fewer females than the higher dosages (0.1 and 1.0 μg vs 10.0 and 20.0 μg). This aspect of the result is consistent with the hypothesis that a "threshold" amount of exogenous estrogen is required to facilitate ovarian differentiation and inhibit testicular differentiation at male-producing temperatures. It is significant that the individual response to estrogen did

not appear graded. A few hatchlings (7/241 or 2.9%) had gonads that had not differentiated fully into testes or ovaries. The majority of hatchlings (97.1%) had either testes or ovaries, indicating that, with rare exception, exogenous estrogens produced an "all or none" effect. Even at dosages that produced a mixture of males and females, the gonads were normal-appearing testes or ovaries. It is possible that the few gonads that had not fully differentiated would have continued to differentiate in post-hatchlings, developing into either ovaries or, as in the European pond turtle having "bisexual" gonads, testes (C. Pieau, personal communication). Two possible explanations could account for why our results are not similar to those of Pieau (1974) and Raynaud (1965). First, different stages of development were compared (embryo vs hatchling). Second, these differences reflect species differences.

Steroid hormones have been shown to affect sex determination in a variety of vertebrates. It has long been known that sex steroid hormones will cause functional sex reversal of many fishes and amphibians (Witschi, 1939; Witschi and Dale, 1962; Yamamoto, 1962). Cortisone and deoxycortisone have also been shown to transform amphibian ovaries into testes (Witschi and Chang, 1950). Although steroid hormones influence gonadal differentiation in many amniote vertebrates, the permanence of their effects varies phylogenetically. Administration of estrogen to reptiles with TSD will cause embryos incubating at a male-producing temperature to become females (Raynaud, 1965; Pieau, 1974; Raynaud and Pieau, 1985; Gutzke and Bull, 1986; Gutzke and Chymiy, 1988; Bull *et al.*, 1988). Estradiol will also feminize reptiles with GSD (Bull *et al.*, 1988) but not apparently birds and mammals. In birds, it has been claimed that genetic male chickens hatched from eggs immersed in an estradiol emulsion laid eggs as adults (Seltzer, 1956), but attempts to replicate such results have been unsuccessful (e.g., van Tienhoven, 1957). A variety of hormonal and surgical manipulations with chickens result in masculinization of ovaries, but none has resulted in full sex reversal (Benoit, 1923, 1950; Domm, 1924, 1939; Maraud *et al.*, 1987). In some marsupial mammals, estradiol can partially feminize male gonads (Burns, 1955, 1956; Fadem and Tesoriero, 1986), whereas in other marsupials the effects of steroids on the gonad are not as dramatic (Shaw *et al.*, 1988, 1990). In eutherian mammals, administration of exogenous steroid hormones does not appear to affect gonadal differentiation (Jost, 1970).

It is significant that in past studies administration of testosterone to reptilian eggs incubated at a female-producing temperature did not result in males. Instead, one-half of the embryos given testosterone at male-producing temperatures developed ovaries (Pieau, 1974; Raynaud and Pieau, 1985; Gutzke and Bull, 1986; Crews *et al.*, 1989). This suggests a role of aromatase enzymes in sex determination. This even distribution suggests also that there may be an organizing (versus a neutral) sex in species with TSD. In this instance, the inability of testosterone to reverse the effects of a female-producing temperature, compared with the 100% reversal of sex in eggs incubated at a male-producing temperature using estrogens, suggests that the female may be the "organizing" sex.

The findings of this study indicate that early estrogen treatment results in the coordinated development of cortical and medullary components of the gonad. Two classes of explanation are tenable: (i) estrogen may itself influence both cortex and medulla to produce the coordination directly, or (ii) gonadal development may normally involve a mechanism for cortical–medullary coordination, and estrogen merely acts at an earlier developmental stage. In fact, given the sensitivity of sex determination to incubation temperature and the virtually infinite range of incubation

conditions encountered in nature, it is not implausible that species with TSD would have evolved mechanisms to limit the development of functional intersexes.

ACKNOWLEDGMENTS

We thank A. Alexander, S. Sinclair, L. Riemenschnieder, and P. Kellog for technical assistance. This research was supported by a grant from NICHD 24976 and NIMH Research Scientist Award 00135 to D.C. and a grant from the Texas Higher Education Coordinating Board to J.J.B.

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