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Effect of Exogenous Estradiol Applied at Different Embryonic Stages on Sex Determination, Growth, and Mortality in the Leopard Gecko (*Eublepharis macularius*)

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ABSTRACT Temperature-dependent sex determination (TSD) occurs in three orders of reptiles. Several studies have examined the ability of estradiol to produce female hatchlings incubated at a male-producing temperature. The results of these experiments support the idea that estradiol could be used as a powerful tool in the conservation of endangered species with TSD by manipulating hatchling sex ratios. However, these experiments have concentrated on the mechanism of determination. This experiment was designed to test the efficacy of various dosages of estradiol applied at two different stages to alter the hatchling sex ratio as well as determining the potential use of such manipulation for conservation efforts by monitoring egg mortality and hatchling growth. The leopard gecko (*Eublepharis macularius*) exhibits TSD and reaches reproductive maturity in less than one year, making it an excellent model for evaluating the long-term effects of estradiol. The results demonstrate that estradiol has a dose-dependent effect on the hatchling sex ratio while only high dosages applied at the later stage of development showed increased mortality. Estrogen-determined females grew at the same rate as temperature-determined females and have produced viable hatchlings. Estradiol treatment of eggs from endangered species may provide a method of insuring female offspring when the TSD pattern is unknown or equipment for controlled incubation is unavailable.

In reptiles with temperature-dependent sex determination (TSD), it is the temperature at which the egg incubates that determines the sex of the offspring. Administration of exogenous estrogen to an egg incubating at a male-producing temperature can counteract the effect of temperature and result in a female hatchling (Bull et al., '88; Crews et al., '91; Gutzke et al., '86; Raynaud and Pieau, '85; Wibbels et al., '91a,b). Use of estrogen to overcome the effects of temperature and insure female development promises to be a useful method by which female offspring can be obtained, particularly in those instances where the species in question is rare and/or endangered and the pattern of TSD is not known. Thus, it is important to know how much estrogen to administer, when to administer the hormone, the embryonic mortality associated with the administration of estrogen, and the long-term consequences of manipulating sex in this way. The purpose of this study was to evaluate the effects of various dosages of estradiol benzoate (EB) administered at specific stages of embryonic development in the leopard gecko (*Eublepharis macularius*).

MATERIALS AND METHODS

Eggs of the leopard gecko were collected on the day of laying from our captive breeding population. Female parentage was determined for all eggs through weekly visual determination of follicular stage of breeding females followed by visual inspection of the abdomen and weighing of all possible mothers whenever eggs were found. Only one male was present in each breeding cage. The leopard gecko generally lays a clutch of two eggs and, when this was the case, clutches were split between treatments and controls. Successive clutches of individual females were distributed across treatments.

A total of 107 eggs were weighed and incubated individually in moist vermiculite (1.5:1; H_2O: vermiculite) in polyethylene covered plastic cups in controlled temperature incubators (Precision Scientific, Inc.) set at 32.5°C (±0.2°C). The eggs remained at this temperature throughout the incubation period.

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All treatments were performed using the spotting technique of Crews et al. ('89). Treatments consisted of either 5 μl of 95% ethanol alone (control) or a solution of estradiol benzoate/ethanol (hormone) at final concentrations of 0.1 μg estradiol/5 μl ethanol, 1.0 μg/5 μl, or 10 μg/5μl. Treatment was applied to the eggshell surface at five (d5) or eleven (d11) days following oviposition. These treatment times correspond to approximately 15% and 30% of the total incubation period. Two embryos were killed at each stage and preserved in Bouin's fixative to histologically determine the stage of development at the time of spotting. External characters were used to stage the embryos according to the developmental stages described by Dufaure and Hubert ('61) for *Lacerta vivipara*.

Hatchlings were sexed using either histological methods for those animals killed shortly after hatching or by observing the development of secondary sexual characteristics in those animals allowed to grow. The growth of the latter animals was monitored to sexual maturity.

Differences in the resulting sex ratios and mortality rates were analyzed using either Fisher's exact test or chi-square analysis for comparisons of more than two groups. Analysis of body weight data was performed using single classification analysis of variance on data from females at 45 weeks of age.

RESULTS

Stage at treatment

Embryos at d5 exhibited forelimb bud development with demarcation of the digital plate. These features correspond approximately to stages 31–32 of Dufaure and Hubert ('61). Histologically these embryos had distinct, but undifferentiated gonads protruding into the body cavity covered at the lateral tip by a distinct germinal epithelium. Embryos at d11 were considerably more advanced having well developed limbs with digital formation beginning corresponding to stages 34–35 of Dufaure and Hubert ('61). The gonads of these embryos were also more advanced with some sexual differentiation apparent. Presumptive females had distinct cortical layers containing many dividing cells with a distinct basal membrane opposing the medullary region. Müllerian ducts were also present. A presumptive male had a cortical layer with less organization and neither an apparent basal membrane opposing the medullary region nor an apparent müllerian duct.

Sex ratio

Estradiol significantly altered the hatchling sex ratio in a dose-response pattern whether applied at d5 of incubation or d11 ($\chi^2 = 9.37, 8.37$, $P < .002, .016$; $df = 1, 2$ respectively) (Fig. 1A). While the lower dosages at both times of application decreased the percentage of male hatchlings, only the highest dose (10 μg) resulted in significant differences from ethanol controls (Fisher's exact test, $P < .0005$ for both groups). Sex ratios between similar treatments at different stages did not differ ($P > .8$ for comparisons of ethanol, 1.0 μg, and 10 μg EB).

Mortality

Hormone-treated eggs spotted on d11 with 10 μg of estradiol had a significantly higher mortality than all other treatments ($P < .038$) (Fig. 1B). There was some mortality in the ethanol groups, but not more than observed in untreated eggs from the same colony (18% for 44 ethanol treated eggs compared to 29% for 305 untreated eggs).

Length of incubation

Significant differences between the length of incubation were observed for the two ethanol treatment groups with individuals treated at d5 taking longer to hatch than individuals treated a d11 (mean of 39.0 ± days for d5, 34.6 ± days for d11; $P \leq .0006$). A significant reciprocal relationship between the day of spotting and increasing dosage of estradiol was observed ($P \leq .001$) with increasing dosages applied at d5 correlating to a decrease in length of incubation and the opposite effect for dosages applied at d11 (Table 1). There were no significant differences in length of incubation between male and female hatchlings for any treatment group ($P \geq .65$).

Growth pattern

There were no significant differences in growth patterns between untreated, ethanol-, or estradiol-treated female hatchlings at reproductive maturity (45 weeks of age; $P \geq .39$; Fig. 2).

DISCUSSION

The mechanism by which temperature exerts its action to determine sex in reptiles with TSD remains unclear. There are several lines of evidence to support the hypothesis that in the case of temperature-induced female development, estrogen is the physiological equivalent of a female-producing temperature. (i) Exogenous estrogen can override the effects of a male-producing temperature to result in female hatchlings (Bull et al., '88; Crews et al., '89, '91; Gutzke and Bull, '86; Pieau, '74: Raynaud and Pieau, '85; Wibbels et al., '92). (ii) The window of sensitivity to exogenous estrogen corres-

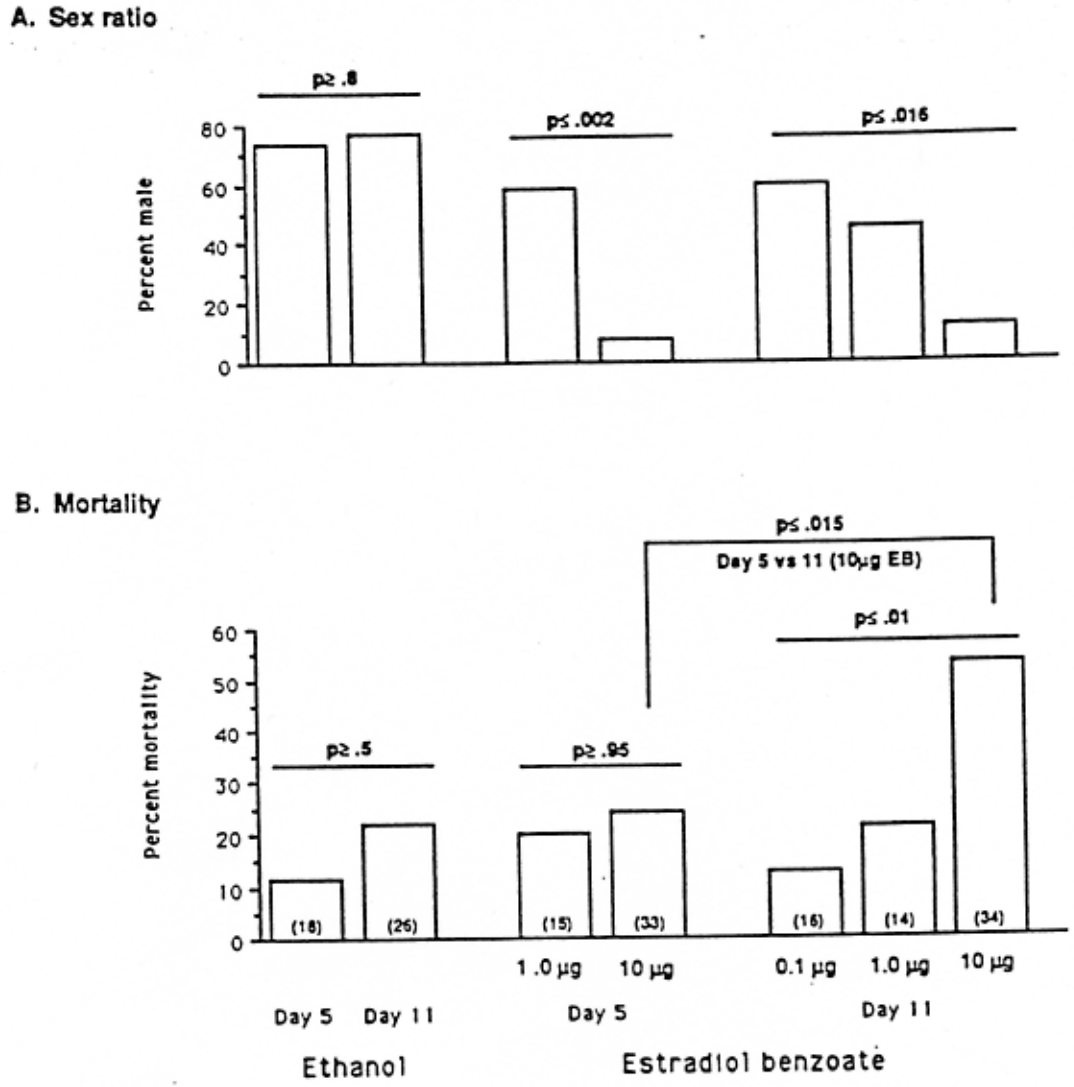

Fig. 1. A: Percentage of male leopard geckos (*Eublepharis macularius*) produced from a 32.5°C incubation temperature. Eggs received treatment of either ethanol (control) or estradiol (0.1, 1.0, 10.0 µg) at day 5 or day 11 following oviposition. B: Percentage of eggs which died during the incubation period for each treatment.

ponds to the window of temperature sensitivity (Gutzke and Chimy, '88; Wibbels et al., '91a). (iii) Temperature and estrogen interact such that the nearer the incubation temperature is to the threshold for producing females, the less estrogen is required to cause female development (Wibbels et al., '91b). (iv) The morphological changes induced by estrogen administration are similar to the changes induced by incubation temperature (Wibbels et al., '93).

Previous research with the leopard gecko indicated that injection of 50 µg/5 µl of EB was effective in causing female development when administered at an early stage of embryonic development (3–6 days post-oviposition), but not when administered at a later stage of embryonic development (10–14 days post-oviposition) (Bull et al., '88). Mortality associated with injection was high in the first year (42%) but lower in the second year (24%) and presumably resulted from infection of the egg following penetration of the shell barrier. Analysis indicated that the sex ratios observed did not result from a differential mortality of males due to the infection. The results in the present study indicated a definite dosage- and stage-specific effect on mortality compared to the year-to-year variation observed in Bull et al. ('88). It is clear that the injection of estrogen into eggs causes significantly greater

TABLE 1. Length of incubation for male and female leopard geckos (Eublepharis macularius) incubated at 32.5°C and treated with either ethanol or various dosages of estradiol benzoate on day 5 or day 11 following oviposition[1]

Treatment	Dosage (μg)	Sex	Sample size (d5, d11)	Day of treatment	
				Day 5	Day 11
Ethanol					
		Female	(6, 2)	38.8 (3.1)	34.5 (0.7)
		Male	(17, 9)	39.1 (2.9)	34.7 (0.9)
Estradiol benzoate					
	0.1	Female	(*, 4)	*	36.0 (0.8)
		Male	(*, 7)	*	35.3 (1.0)
	1.0	Female	(5, 6)	37.2 (1.3)	35.5 (0.8)
		Male	(7, 5)	38.6 (2.5)	37.4 (3.9)
	10.0	Female	(19, 14)	36.5 (1.7)	37.5 (2.6)
		Male	(2, 2)	35.5 (0.7)	36.5 (0.7)

[1]No. given are means with one standard deviation within the parentheses.
* No data for this dosage at day 5.

mortality than does spotting the hormone on the egg shell surface. In the red-eared slider (*Trachemys scripta*) mortality from injection ranged from 50 to 100%, whereas spotting averaged 3%, the mortality observed in untreated eggs (Crews et al., '91). The present results also indicate that EB applied at either d5 or d11 altered the sex ratio. It is significant that EB altered the ratio when applied at the later stage of development at a dosage five times lower than that used by Bull et al. ('88). It is likely that in that study, embryogenesis was more advanced and hence the embryos were outside of the window of estrogen sensitivity. The present dose-response experiment indicates that a threshold level of hormone is required to override the effects of a male-producing temperature. The previous result of no sex ratio change with a higher dosage at the later embryonic stage also suggests that there may be an hormonal threshold above which the cellular physiology directing male development is not affected.

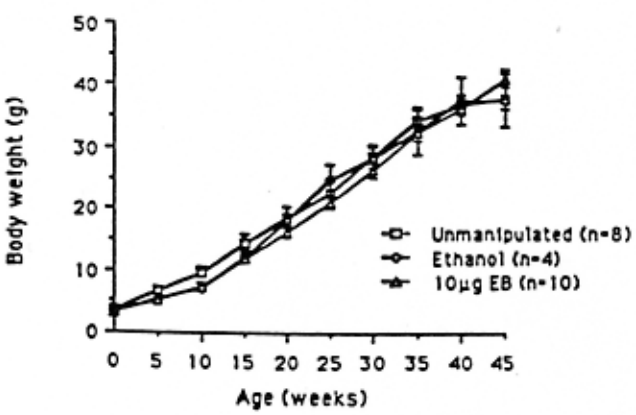

Fig. 2. Growth rate in female leopard geckos (*Eublepharis macularius*). Illustrated is the average body weight ± one standard error of unmanipulated individuals, and individuals that received ethanol or estradiol benzoate (EB) treatment 5 days after oviposition.

The significant difference in the length of incubation for eggs treated with ethanol may be a response to an arresting of development induced by the ethanol. It is perhaps significant that treatment at d5 with increasing dosages of estradiol resulted in a decrease in length of incubation while treatment at d11 resulted in an increase. In the red-eared slider, hatchlings spotted with high dosages of estradiol emerge with large amounts of unabsorbed yolk, suggesting that estradiol interferes with the absorption of yolk (T. Wibbels and D. Crews, unpublished research). This could explain the effect for treatment at d11. While our hatchlings did not have excess yolk at the time of hatching, they may have delayed hatching until the yolk was fully absorbed. Perhaps the difference in length of incubation may result from stage-specific expression of enzymes capable of degrading estradiol. An alternative explanation may be that at d11 gonadal differentiation has progressed farther along a male-biased developmental path, thus requiring a longer amount of time to revert to female development.

The growth data for unmanipulated, ethanol- and estradiol-treated individuals followed through reproductive maturation is in agreement with the observation that sex determination in TSD systems is "all or none". In other words, the females from all three groups grew similarly regardless of treatment, even though presumably approximately 75% of the individuals treated with estradiol were presumptive males at the time of treatment.

TSD has obvious and severe implications for conservation efforts of endangered species where incubation temperature either is not or cannot be

controlled. Sex ratios resulting from such efforts can be far from optimal and can even be 100% male. The use of estradiol to produce females regardless of incubation temperature has great potential for areas where controlled incubation regimes may be difficult to maintain. This work demonstrates that although application of estradiol has beneficial effects, it is important to know at what stage the application is made to minimize mortality.

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