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Incubation Temperature and Gonadal Sex Affect Growth and Physiology in the Leopard Gecko (*Eublepharis macularius*), a Lizard With Temperature-Dependent Sex Determination

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ABSTRACT Temperature-dependent sex determination (TSD), in which the temperature at which an egg incubates determines the sex of the individual, occurs in egg-laying reptiles of three separate orders. Previous studies have shown that the embryonic environment can have effects lasting beyond the period of sex determination. We investigated the relative roles of incubation temperature, exogenous estradiol, and gonadal sex (testis vs. ovary) in the differentiation of adult morphological and physiological traits of the leopard gecko, *Eublepharis macularius*. The results indicate that incubation temperature, steroid hormones, and gonads interact in the development of morphological and physiological characters with incubation temperature resulting in the greatest differences in adult phenotype. Incubation temperature did not affect reproductive success directly, but may influence offspring survival in natural situations through effects on adult female body size. Postnatal hormones seem to be more influential in the formation of adult phenotypes than prenatal hormones. These results demonstrate that TSD species can be used to investigate the effects of the physical environment on development in individuals without a predetermined genetic sex and thus provide further insight into the roles of gonadal sex and the embryonic environment in sexual differentiation.

The adult phenotype emerges from the interaction of genes and environment during development. Growth rate and body size are common results of natural selection, whereas sexually dimorphic traits are the most physically apparent results of sexual selection. In species having genetic sex determination (GSD), the sex of the embryo is established at fertilization. Because males and females differ genetically, they respond differently to environmental challenges. Further, in litter-bearing mammals, the embryos are subjected to maternal hormone fluctuations as well as the influence of the hormones produced by their fetal neighbors; females situated between two males are masculinized, whereas males situated between two females are feminized in their morphology, physiology, and behavior (vom Saal, '81; Houtsmuller and Slob, '90).

Many oviparous reptiles have an alternate form of sex determination known as temperature-dependent sex determination (TSD). In contrast to GSD, in TSD there are no visibly distinct sex chromosomes inherited from the parents (reviewed in Bull, '80; Janzen and Paukstis, '91). The temperature at which the egg is incubated determines the sex of the individual with little or no parental genetic contribution predisposing embryos to become one sex or the other (Bull, '83; Lang et al., '89; Ewert and Nelson, '91). There is a window of temperature-sensitivity during which the cumulative exposure to certain temperatures gradually determines the sex of embryos; prior to this period each embryo can become either sex (Bull et al., '90; Wibbels et al., '91a). Application of exogenous estradiol during the period of temperature-

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sensitivity can irreversibly override the sex-determining effect of temperature in a dose-response fashion that covaries with temperature (Gutzke and Bull, '86; Bull et al., '88; Crews et al., '89, '91, '94; Wibbels et al., '91b). Unlike most mammals and birds, which provide their young with significant amounts of nutrition and social experience postnatally, most TSD species studied to-date provide little if any additional parental influence after birth in the form of nutritional or social factors. For these reasons, the TSD system is well suited for studying the effects of the internal and external environments on the development of sex differences.

Several patterns of TSD are observed in reptilian species (Ewert and Nelson, '91). In most turtles, males are produced at low temperatures and females at high temperatures, whereas in many lizards, low temperatures produce females and high temperatures produce males. In a third pattern characteristic of all crocodilians thus far examined and the snapping turtle, females are produced across the entire range of viable incubation temperatures and males are produced only at intermediate temperatures. Viets et al. ('93) determined that the leopard gecko (*Eublepharis macularius*) has this latter pattern, rather than the previously reported pattern of females produced at low temperatures and males produced at high temperatures (Bull, '80, '87a,b; Wagner, '80).

In the adult leopard gecko, differences in behavior and physiology are correlated to the individual's incubation temperature (Gutzke and Crews, '88). Females from a male-biased incubation temperature are more masculinized in their behavior and physiology than females from female-biased incubation temperatures. These findings suggest that incubation temperature might parallel the organizational effects of hormonal (androgen) variation experienced during embryonic development in polytocous mammals owing to proximity in the uterus to male fetuses. The present study was undertaken to determine the relative contributions of incubation temperature, gonadal sex, and the pre- and postnatal hormonal environment on the growth and adult morphology and physiology of individual leopard geckos incubated at different temperatures.

MATERIALS AND METHODS

Animals

Eggs of the leopard gecko (*Eublepharis macularius*) were collected on the day of laying from our captive-breeding population during two seasons. All fertile eggs were weighed, placed singly in covered plastic cups containing moist vermiculite (1.5:1; water: vermiculite by weight), and housed in upright incubators (Precision Scientific: Chicago, IL) set at either 30°C (first season), or at 26°C or 32.5°C (second season). The incubators maintained constant temperature ±0.2°C. The leopard gecko usually lays clutches of two eggs. Successive clutches from individual females were distributed across temperatures; when possible, the clutches from the second season were split between incubation temperatures of 26°C and 32.5°C. Eggs were monitored daily for hatching.

Posthatching rearing

Upon hatching, animals were weighed and placed into individual polypropylene containers (28 × 13 × 9 cm). The containers held a 1.5 × 8-cm petri dish for water, small plastic shelter, and a paper towel substrate. Animals from all egg incubations were reared in individual boxes within controlled environmental chambers.

During weeks 1–10 posthatching, the animals were housed at a constant temperature of 30°C and a light-dark cycle of 14:10 L:D. During weeks 10–65, the chambers were maintained on a diel temperature cycle of 30°:18°C (14:10 hr) and 14:10 L:D photic cycle. Humidity was not controlled and ranged from 40–70%.

Food initially consisted of crickets (Flukers Cricket Farm) and giant mealworms (Rainbow Mealworms) with neonatal mice being added to the diet after the geckos reached 10 wk of age (1 neonatal mouse offered per week). The food was dusted with pulverized dicalcium phosphate and a vitamin/mineral powder (Petco Animal Supplies). Food was offered three times weekly, and the amount offered was held constant among individuals.

Morphological measurements

All hatchlings were weighed and with the exception of hatchlings from the 30°C incubation temperature, the snout-to-vent length (SVL) and jaw width were also measured at hatch. Animals from all incubation temperatures were then weighed weekly and had SVL and jaw measurements taken at 10, 45, and 65 wk, times that correspond to juveniles, adults at reproductive maturity, and adults in the plateau phase of body growth, respectively.

Reproductive success

Upon reaching 65 wk of age, all unmanipulated females were randomly assigned and transferred to a large plexiglass breeding cage (43 × 22 × 20 cm, L × W × H) and paired with an unrelated male proven to be a breeder. Environmental conditions were maintained as described above for the reach-in chambers. Each cage was provided with a nest box filled with moist sand and a localized heat source placed beneath the cage. Food and water were available as described above. After one full breeding season, a new proven male was introduced to each cage. All females were weighed and visually inspected for eggs weekly to determine parentage of any eggs found in the cage. All eggs were collected on the day of laying and checked for fertility. All mothers and their fertile eggs were weighed and the eggs incubated as described above. Infertile eggs and fertile eggs which had dried out prior to discovery were collected and catalogued, but were not weighed or incubated.

Radioimmunoassay of steroid hormones

A single 250-μl whole blood sample was collected from individuals at 50 wk of age by cardiocentesis. The time of day of sampling was held constant for all individuals. The blood was centrifuged in capillary tubes at 2,000 rpm for 12 min. The serum was collected and stored at −20°C until assayed. Celite chromatography was used to separate steroids for individual analysis. The individual steroid fractions were eluted with increasing concentrations of ethyl acetate in isooctane as follows: progesterone (P), 3.6 ml of 1%; dihydrotestosterone (DHT), 4.5 ml of 10%; testosterone (T), 4.5 ml of 20%; estradiol-17β (E), 4.0 ml of 40%; and corticosterone (B), 4.0 ml of 50%. The fractions were dried and resuspended in 300 μl of phosphate-buffered gelatin and finally assayed for levels of hormone using a single antibody competitive radioimmunoassay as described by Whittier et al. ('87). Intraassay (mean) and interassay coefficients of variation were as follows: P: 1.1%, 16.3%; DHT: 6.5%, 16.1%; T: 7.8%, 12.6%; E: 7.2%, 14.3%; B: 8.5%, 10.2%.

Embryonic hormonal manipulation

Some eggs incubated at 26°C and 32.5°C had either 5 μl of 95% ethanol (control, $n = 34$) or 10 μg of estradiol benzoate (EB) dissolved in 5 μl 95% ethanol (hormone treatment, $n = 36$) applied noninvasively to the eggshell during incubation (Crews et al., '91). Eggs at 26°C were manipulated on Day 11 (d11) following oviposition, whereas those incubated at 32.5°C were manipulated on Day 5 (d5) following oviposition. Histological analysis of two embryos each at both temperatures showed that, owing to the differences in developmental rate at the different temperatures, eggs incubated at 32.5°C and treated on d5 were manipulated at approximately the same stage of embryonic development as those incubated at 26°C and treated on d11.

Posthatching hormonal manipulation

A subsample of individuals hatching from untreated eggs at the same incubation temperature was randomly assigned to either a gonadectomy group ($n = 10$ for 26°C and $n = 15$ for 32.5°C) or a sham-operation group ($n = 7$ at 26°C and 32.5°C). All surgeries were conducted on the day of hatch, performed under hypothermally induced anesthesia, and lasted no more than 5 min. In all gonadectomies, the excised tissue was preserved in Bouin's fixative for later histological analysis. Animals were sutured and allowed to recover at room temperature.

Statistical methods

Comparisons of body size for different incubation temperatures within a sex were performed using a one-way analysis of variance (ANOVA) on actual body weights at hatching, and at 10, 45, and 65 wk of age. A two-way ANOVA was used to test for differences in body weight and SVL for males and females incubated at 30°C and 32.5°C with sex and incubation temperature as fixed factors. Adult weights were also compared with analysis of covariance (ANCOVA) using SVL as a covariate. Jaw-dimension comparisons were also performed using ANCOVA with SVL as a covariate. Raw data for ANCOVA were log-transformed. Tests for reproductive success were made using repeated measures analysis of variance with incubation temperature as a main effect and reproductive season as the repeated measure. Linear regressions were calculated for egg and hatchling weights of the first successful clutch of a female regressed on her body weight at the time the eggs were laid. Analysis of hormone values were made using ANOVA on log-transformed values to increase homogeneity of variance among groups. All probabilities for statistical tests are two-tailed and were considered significant at the 0.05 level. Probability values for growth data are listed in Tables 1 and 2.

RESULTS

Growth in unmanipulated individuals

Totals of 46 unmanipulated female geckos incubated at 26°C (n = 12), 30°C (n = 25), or 32.5°C (n = 9) and 15 male unmanipulated geckos incubated at 30°C (n = 5) or 32.5°C (n = 10) were successfully raised to adulthood (65 wk).

Sex differences in morphological characteristics were determined through comparison of individuals from 30°C and 32.5°C incubation temperatures, both of which produce males and females. Analysis of adult growth data showed that there was a significant sex difference for both body weight (BW) and SVL, with males being heavier and longer ($P \leq 0.002$, df = 48; 0.001, df = 45 respectively) (Fig. 1a). The analysis also showed an effect for incubation temperature on BW ($P \leq 0.003$), but not SVL ($P \geq 0.123$) within a sex. However, ANCOVA with SVL as a covariate indicated that incubation temperature accounted for a significant portion of the variation observed for BW ($P \leq 0.01$, df = 44), but sex did not. There was a significant effect of gonadal sex ($P \leq 0.0001$, df = 46), but not incubation temperature in the analysis of the data on jaw width for males and females whether or not corrected for SVL with ANCOVA. Although the mean adult BW for males from a 32.5°C incubation temperature was greater than that for males from a 30°C incubation temperature, there were no significant differences between males for BW, SVL, or jaw width.

There were significant differences correlated to incubation temperature among females for both BW and SVL at adulthood ($P \leq 0.018$, df = 45; $P \leq 0.02$, df = 41), with the individuals from the male-biased incubation temperature of 32.5°C having the highest mean (Fig. 1b). However, analysis of covariance with the SVL as a covariate to correct for the increased overall body size of females from a 32.5°C incubation temperature indicated no significant difference among females from different incubation incubation temperatures in BW. There were no significant differences in either variable for individuals as hatchlings or at reproductive maturity. There was a significant difference in BW as juveniles at 10 wk of age ($P \leq 0.006$), with females from an incubation temperature of 30°C being the heaviest. There was no significant difference for SVL at this stage

TABLE 1. Results of statistical analysis of morphometric measurements for unmanipulated male and female leopard geckos (Eublepharis macularius) *incubated at 26°C, 30°C, or 32.5°C*

Comparison group	Measure	Age group	P value	Significance
Females	Body weight	Hatchling	0.922	
		Juvenile	0.006	30°C females heavier
		Sexual maturity	0.090	
		Adult	0.017	32.5°C females heavier
	SVL (mm)	Hatchling[1]	0.838	
		Juvenile	0.183	
		Sexual maturity	0.094	
		Adult	0.019	32.5°C females larger
Females (ANCOVA) SVL as covariate	Body weight	Adult	0.418	
	Jaw width		0.562 (sex; inc. temp.)	
Male vs. female at 30°C and 32.5°C	Body weight	Hatchling	0.403; 0.807	
		Juvenile	0.745; 0.039	30°C individuals heavier
		Sexual maturity	0.182; 0.016	32.5°C individuals heavier
		Adult	0.002; 0.002	males heavier, 32.5°C individuals heavier
	SVL (mm)	Juvenile	0.174; 0.651	
		Sexual maturity	0.131; 0.068	
		Adult	0.001; 0.123	Males larger than females at given incubation temperature
Male vs. female at 30°C and 32.5°C (ANCOVA) SVL as covariate	Body weight	Adult	0.259; 0.010	32.5°C individuals are proportionally heavier
	Jaw width		0.001; 0.549	Males have proportionally wider jaws

[1]Analysis for females incubated at 26°C and 32.5°C only.

TABLE 2. Results of statistical analysis of morphometric measurements for manipulated female leopard geckos (Eublepharis macularius) incubated at 26°C or 32.5°C[1]

Comparison group	Measure	Age group	*P* value (inc. temp.; treatment)	Significance
Females from eggs treated with either ethanol or estradiol at 26°C and 32.5°C	Body weight	Hatchling	0.825; 0.843	
		Juvenile	0.389; 0.719	
		Sexual maturity	0.012; 0.293	32.5°C females heavier
		Adult	0.002; 0.613	
	SVL (mm)	Hatchling	0.178; 0.471	
		Juvenile	0.482; 0.672	
		Sexual maturity	0.011; 0.868	32.5°C females longer
		Adult	0.001; 0.737	
(ANCOVA) SVL as covariate	Body weight	Adult	0.179; 0.240	
	Jaw width		0.138; 0.081	
			*(26°C; 32.5°C)	
Females sham-operated or gonadectomized on the day of hatch	Body weight	Hatchling	0.175; 0.646	
		Juvenile	0.181; 0.183	
		Sexual maturity	0.087; 0.116	
		Adult	0.016; 0.644	Gonadectomized 26°C females heavier
	SVL (mm)	Hatchling	0.101; 0.854	
		Juvenile	0.060; 0.146	
		Sexual maturity	0.111; 0.496	
		Adult	0.046; 0.530	Gonadectomized 26°C females longer
(ANCOVA) SVL as covariate	Body weight	Adult	**; 0.586	
	Jaw width		0.008; 0.490	Gonadectomized 26°C females have proportionally wider jaws than sham-operated females

[1]Prenatal treatment consisted of application of ethanol alone (control) or estradiol in ethanol (hormone treatment) to eggs at similar embryonic stages. Postnatal treatment consisted of a sham operation (control) or gonadectomy (treatment) on the day of hatch.
*Individuals from 26°C and 32.5°C incubation temperatures were analyzed separately using one-way ANOVA after it was determined that there were significant interactions between temperature and treatment. This may either indicate a differential response or reflect the small sample size for castrates from a 32.5°C incubation temperature.
**The assumption of homogeneity of slopes was violated for females from a 26°C incubation temperature and thus no statistic is presented.

($P \geq 0.18$). Analysis of jaw width indicated that there were no significant differences between groups whether or not corrected for body size (Table 1).

Growth in manipulated individuals

A total of 26 females treated as embryos with either ethanol only ($n = 7$ for 26°C, $n = 4$ for 32.5°C) or estradiol in ethanol ($n = 6$ for 26°C, $n = 9$ for 32.5°C) were raised to adulthood. Only one male individual hatched from an egg treated with estradiol and incubated at 32.5°C. Treatment of eggs incubating at a temperature of 32.5°C with EB resulted in a significant shift in the observed hatchling sex ratio compared to that for hatchlings from eggs treated with ethanol only ($P \leq 0.0001$, data for hatchling sex ratios reported by Tousignant and Crews, '94). There was no effect of EB or ethanol treatment on the observed sex ratio of hatchlings from eggs incubated at 26°C.

The morphological results for these embryonically manipulated geckos paralleled those for unmanipulated individuals with increasing incubation temperature correlating with increased size. Regardless of hormonal egg manipulation, females from a 32.5°C incubation temperature were significantly larger than those from a 26°C incubation temperature at reproductive maturity (45 wk) in BW ($P \leq 0.012$, df = 27) and SVL ($P \leq 0.011$, df = 24). These differences in BW and SVL were also present in individuals as adults at 65 wk ($P \leq 0.015$, $P \leq 0.004$, respectively) (Fig. 1c). There were no significant differences on the day of hatch or at 10 wk of age for either variable. No significant differences in morphological characters were observed for eggs receiving control ethanol treatment or EB treatment for either incubation temperature at any age examined.

Fourteen females incubated at 26°C and 10 females incubated at 32.5°C were success-

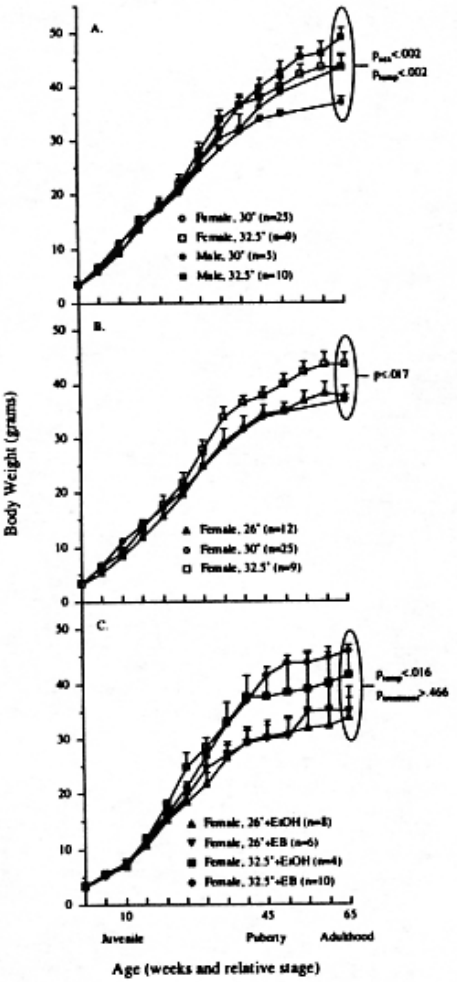

Fig. 1. *Eublepharis macularius*. The relative influence of incubation temperature, gonadal sex, and prenatal steroid hormones on the growth rate of the leopard gecko. Illustrated is the mean body weight (+1 SE) of males and females from different incubation temperatures or hormonal manipulations. The probability value(s) adjacent to each ellipse is from an ANOVA at 65 wk of age. **A.** Males and females from incubation temperatures of either 30°C (female-biased sex ratio) or 32.5°C (male-biased sex ratio). **B.** Females from incubation temperatures of 26°C (all-female sex ratio), 30°C, or 32.5°C. **C.** Females from incubation temperatures of either 26°C or 32.5°C and treated as embryos with either 5 µl of 95% ethanol or 10 µg of estradiol benzoate in 5 µl of ethanol.

fully ovariectomized (7 from 26°C and 3 from 32.5°C) or sham-operated (7 each from 26°C and 32.5°C) and reared to 65 wk. The analysis of BW at adulthood indicated that gonadectomy resulted in a significant increase in body weight for females incubated at 26°C ($P \leq 0.016$, df = 13), but not for females incubated at 32.5°C (Fig. 2). There was no effect of surgery; shams and unmanipulated individuals grew similarly at both incubation temperatures. There were no significant differences in BW at any of the other time periods analyzed (Table 2). The analysis of SVL and jaw width paralleled the results for BW. Gonadectomy on the day of hatch resulted in females incubated at 26°C growing significantly longer ($P \leq 0.046$) and having proportionally wider jaws ($P \leq 0.008$) than either sham-operated or unmanipulated females, but there was no effect for females from a 32.5°C incubation temperature for either SVL or jaw width.

Hormones

No individual sampled showed detectable levels of progesterone. The androgens DHT and T were found to be significantly higher in males than in females (DHT: $P \leq 0.01$, df = 38; T: $P \leq 0.001$, df = 44). Although mean levels of DHT and T were higher in females incubated at 32.5°C, there was no significant difference in the circulating concentration of these hormones among females from the different incubation temperatures (Fig. 3). There was a significant sex differ-

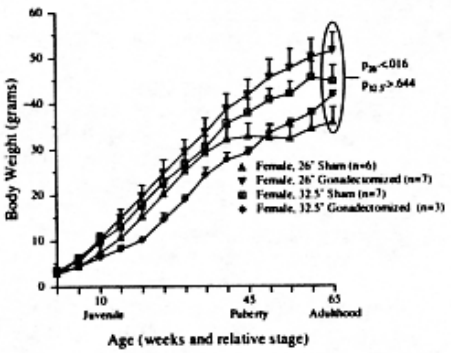

Fig. 2. *Eublepharis macularius*. The effect of gonadectomy on the growth of female leopard geckos. Illustrated is the average body weight (+1 SE) of females incubated at either 26°C or 32.5°C followed by a sham operation of ovariectomy on the day of hatch. The probability value adjacent to the ellipse is from an ANOVA at 65 wk of age.

ence in levels of B with males having lower levels ($P \leq 0.006$, df = 40), but no significant differences between incubation temperatures ($P \geq 0.52$) (Fig. 3).

Estradiol was the only hormone to show a significant difference correlated to incubation temperature. In both males ($P \leq 0.002$, df = 14) and females ($P \leq .0029$, df = 37), estradiol levels were highest in animals from a 30°C incubation temperature (Fig. 3).

The effect of embryonic treatment with exogenous hormones on hormonal profiles in reproductively mature females was investigated in females from a 32.5°C incubation temperature. There were no significant differences in circulating concentrations of DHT, T, or E between females treated with EB in ethanol vehicle and those treated with ethanol alone.

The effects of postnatal hormonal manipu lation on hormonal profiles of reproductive mature individuals was investigated in f males from a 26°C incubation temperatur Except for T, mean circulating concentr tions of all hormones measured were th same or lower in females gonadectomized o the day of hatch compared to sham-operate females; these differences were not statist cally significant (mean ± standard error, g nadectomized vs. sham-operated; T, 0.84 0.64, 0.83 ± 0.33; E, 0.46 ± 0.12, 2.78 ± 1.4 DHT, 0.39 ± 0.13, 0.88 ± 0.28; B, 7.79 1.32, 11.13 ± 3.24). Increased variation i sham-operated females may have resulte from degree of ovarian activity, as indicate by the presence or absence of vitellogeni follicles.

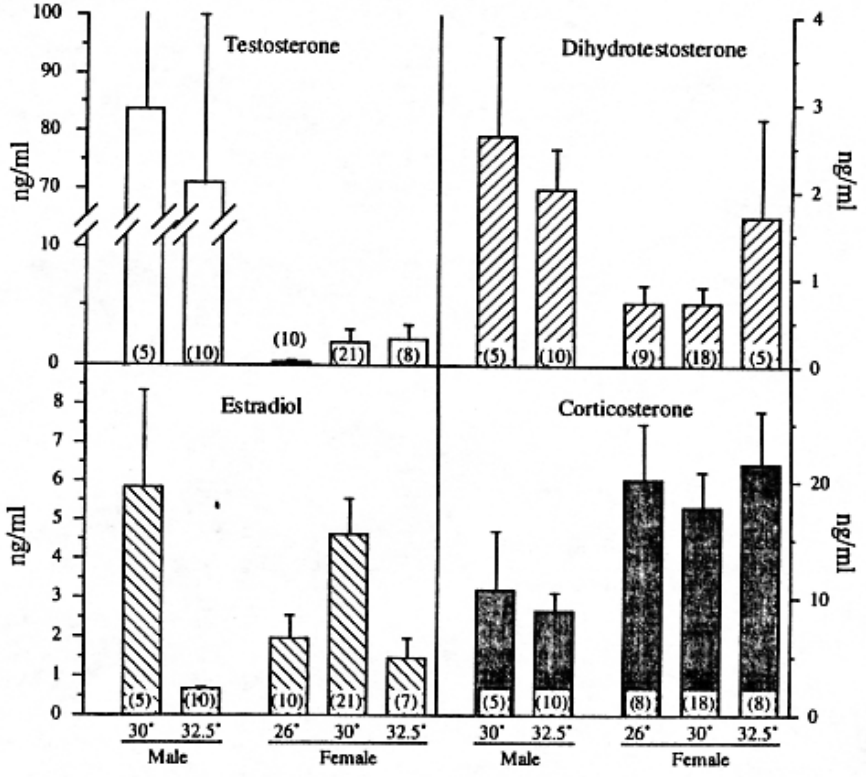

Fig. 3. *Eublepharis macularius*. Steroid hormone profiles for reproductively mature, unmanipulated leopard geckos sampled at 50 wk of age. Illustrated are mean values expressed as ng/ml (+1 SE). Males are from incubation temperatures of 30°C and 32.5°C, whereas females were incubated at 26°C, 30°C, or 32.5°C.

Reproductive success

Females from all three incubation temperatures produced hatchlings during the 2-yr study. There were no significant differences in the total number of eggs laid, the proportion of those eggs that was infertile, or the number of hatchlings produced between females from different incubation temperatures in either season (Fig. 4). Although females tended to produce more hatchlings during the second breeding season compared to the first, the effect was not significant ($P = 0.075$). There was a significant increase in the interclutch interval observed during the second season compared to the first season ($P \leq 0.001$) (Fig. 4).

Hatchling weight was significantly correlated to egg weight ($r = 0.872$, $P \leq 0.001$). Dam weight at the time of egg-laying was significantly correlated to both clutch weight ($r = 0.576$, $P \leq 0.001$) and hatchling weight ($r = 0.732$, $P \leq 0.001$) (Fig. 5).

DISCUSSION

The present data provide evidence for a significant effect of both incubation temperature and gonadal sex on the body weight of adult leopard geckos. Male and female geckos from the same incubation temperature showed a sex difference in all of the morphological characteristics measured. Although the male is the larger sex in general, incubation temperature plays a significant role in the growth of individual leopard geckos. In fact, the mean BW for females from the male-biased incubation temperature of 32.5°C is not significantly different from that of males from a 30°C incubation temperature. There was a significant effect of incubation temperature for both BW and SVL among adult females from the three incubation temperatures. Females from the 32.5°C incubation temperature are larger than females from the 26°C and 30°C incubation temperatures; however, these same three groups of females did not differ in the widths of their jaws.

It is interesting that the males from 30°C and 32.5°C incubation temperatures did not differ significantly as adults in the morphological characteristics we measured. Gutzke and Crews ('88) hypothesized that the smaller range of incubation temperatures producing males relative to that of females may have been responsible for decreased differences in behavioral measures for males as compared to females. The same hypothesis might account for the present data on morphology. Taken together, these results indicate that some characteristics are sexually dimorphic (jaw width) and others (body weight and SVL) correlate with incubation temperature. These differences in the leopard gecko do not seem to result from caloric intake; amount of food consumed does not differ significantly among groups incubated at different temperatures or between the sexes (A. Tousignant and D. Crews, pers. obs.).

In an analysis of the growth of male and female American alligators (*Alligator mississippiensis*), Joanen et al. ('87) examined body weight and total length after 18 mo of growth. When they pooled data for males and females, they observed an overall significant effect of incubation temperature on body weight and total length. However, their data were confounded by a differential "runt" effect, with individuals incubated at the lowest and highest temperatures having high incidences of poor growth. By comparing only intermediate temperatures, they were able to demonstrate a significant effect for gonadal sex on total length, but not body weight. Interestingly, the females from a female-biased incubation temperature grew larger than males from the same incubation temperature, whereas males grew larger than females if both were from a male-biased incubation temperature. It must be emphasized, however, that the alligator is a long-lived species that does not reach reproductive maturity until 7 or 8 yr of age. These findings may be analogous to our results for juvenile individuals from a 30°C incubation temperature compared to other incubation temperatures. It is possible that early growth rates may reflect more accurately the stress induced by particular incubation temperatures than the maximum body size attained by individuals. In the leopard geckos in our colony, extreme incubation temperatures near the lethal maximum are associated with a decrease in growth rate as juveniles, even though normal size is attained as adults (A. Tousignant and D. Crews, pers. obs.).

The steroid hormone profiles of individuals from TSD species are of interest because of the apparent role of steroids in the process of sex determination in these species (for review see Crews et al., '94). In turtle embryos, both steroidal hormones (White and Thomas, '92) and the production of the enzyme aromatase (Desvages and Pieau, '92) vary according to incubation temperature during the sex-determining period of develop-

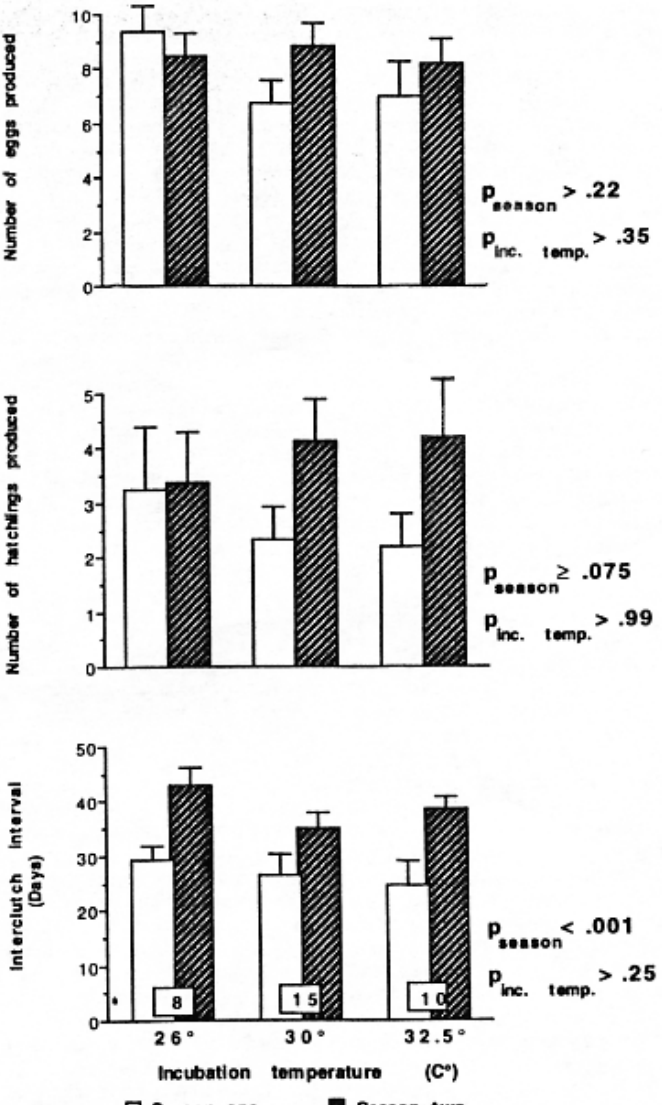

Fig. 4. *Eublepharis macularius*. Measures of reproductive success in female leopard geckos from incubation temperatures of 26°C, 30°C, and 32.5°C. Results are presented as means (+1 SE) for the first and second breeding season. All probabilities are from repeated measures ANOVA. The top panel depicts the number of eg laid. The middle panel depicts the number of hatchlin produced. The bottom panel depicts the interclutch inte val. The boxed numbers in the bottom panel represe the sample sizes for each group.

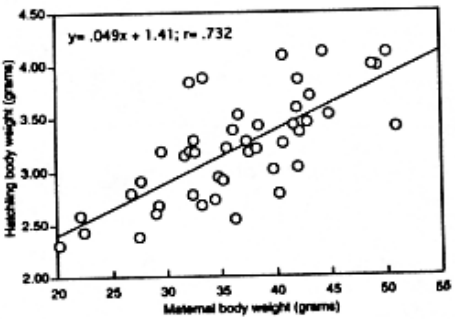

Fig. 5. *Eublepharis macularius*. The line represents the results of a linear regression of body weight (gm) of hatchling leopard geckos on the mother's body weight at the time the egg was laid.

ment. There has been little investigation of the levels of hormones present in adult individuals of TSD species in which the incubation history of the individual is known. Gutzke and Crews ('88) found that hormonal profiles varied significantly both between and within the sexes. In that study, androgens, but not estrogens, differed significantly between the sexes. Among females, individuals from relatively high (male-biased sex ratio) incubation temperatures had elevated levels of testosterone and decreased levels of estradiol compared to females incubated at lower (female-biased sex ratio) temperatures. Although the blood sampling regimen differed somewhat in that we took blood samples prior to behavior testing and at a standard age rather than a standard weight as in the previous study of Gutzke and Crews, there were several similar findings in the two studies. As in the previous study, we observed significant differences in hormonal profiles both between the sexes and within groups of same-sex individuals incubated at different temperatures. Androgen levels were significantly higher in males than females, but did not differ significantly in animals of the same sex from different incubation temperatures, even though mean levels of DHT were twice as high in females from a male-biased incubation temperature compared to females from female-biased temperatures. The loss of several samples from the DHT fractions of female geckos owing to poor recovery of hormone during extraction resulted in a decreased sample size that may have influenced this result. Estradiol varied significantly between groups of females incubated at the different temperatures. Individuals from the female-biased incubation temperature of 30°C had the highest levels of estradiol. This finding is significant in light of the fact that males from a 30°C incubation temperature also had significantly higher levels of estradiol than did males from a 32.5°C incubation temperature. Interestingly, neither study found any significant differences in androgens between males. These findings may again underscore the relative importance of estradiol in the sexual differentiation of TSD species.

Significant differences in corticosterone levels between males and females were found. It is interesting to note that the smaller sex has the higher levels of hormone. This is consistent with the finding in rats that the adrenal hormone cortisol plays a long-term role in the retardation of growth in individuals with perinatally elevated levels of hormone (Sawano et al., '69).

Reproduction is the ultimate measure of success of a phenotype. Our data indicate that the variation in morphology and physiology correlated with incubation temperature does not have a direct effect on reproduction as observed in the laboratory. We did not observe a significant difference in either egg or hatchling production by females from different incubation temperatures. However, it is possible that incubation temperature may have an effect on reproduction mediated by its effect on growth. Hatchling size was significantly correlated to maternal body size and female body size was in turn correlated to incubation temperature. Thus on average, females from higher incubation temperatures will have larger offspring. Sinervo ('90) showed that differences in hatchling sprint performance were accounted for by differences in body size; larger animals performed better. Christian and Bedford ('93) found that geckos expend more energy per offspring compared to other lizards. In a species with a high energy expenditure per offspring, slight differences in maternal investment could be more energetically costly than in species with less expenditure per offspring. In the leopard gecko, clutch weight expressed as a percentage of the female's body weight decreases as the incubation temperature that the mother experienced increases (21.1% for 26°C, 19.7% for 30°C, and 18.2% for 32.5°C) (A. Tousignant and D. Crews, pers. obs.). Thus reproduction per offspring may be less costly for

the larger females produced at higher incubation temperatures.

The effect of prenatal hormones on the growth and physiology of leopard geckos was investigated in females from 26°C and 32.5°C incubation temperatures that received exogenous EB or ethanol vehicle alone during the temperature-sensitive stage of sex determination. The results for the animals treated prenatally paralleled those for unmanipulated individuals. Incubation temperature accounted for most of the variation observed in morphology, with animals incubated at a male-biased temperature growing larger than females from lower incubation temperatures. Embryonic estrogen treatment significantly altered the observed hatchling sex ratio for eggs incubated at 32.5°C, but had no effect on the growth of the hormone-induced females. The effect on males could not be assessed from the current data because only one male was produced. Bull et al. ('88) showed that female-biased sex ratios in the leopard gecko resulting from estrogen injection of eggs are not the result of differential mortality of the sexes. The male produced in this study was small but appeared normal in all other respects.

Some individuals were gonadectomized on the day of hatch to investigate the effects of postnatal hormones. Unfortunately, the data are limited to females because gonadectomies in individuals shown to be male through histological analysis did not prove successful. Only one male gonadectomized at hatch proved not to have gonadal tissue upon adult laporoscopy. That is, although the gonads from these individuals were removed at hatch and verified histologically, remnant tissue appears to have regenerated complete testes. Although the hormone values for individuals gonadectomized on the day hatch appear to suggest that a similar phenomenon may have occurred in females, several pieces of data indicate that this is not the case. First, there was a differential growth response to gonadectomy in females from a 26°C incubation temperature compared to females from a 32.5°C incubation temperature. Gonadectomy in hatchling females from the lower temperature resulted in significantly larger adult females, whereas gonadectomy in hatchlings from a 32.5°C incubation temperature had no effect suggesting the possibility of a temperature-correlated effect. Second, the same females as presented here were later behaviorally tested in another experiment, and again there were significant diff ences between females that were gonadec mized on the day of hatch and those t were treated with a sham operation (Flo '94). Female leopard geckos gonadectomi as adults have circulating estradiol and test terone similar to those presented here neonatally gonadectomized animals (Flo '94). Thus it seems likely that other sour of hormones such as the adrenal gland responsible for the levels of steroid hormo observed in gonadectomized females in t experiment.

Several of the results from pre- and post tal hormone manipulations provide supp for a hypothesis that incubation temperatu and steroid hormones interact to produce adult phenotype and that incubation tempe ture plays the greater role: (1) exogen estradiol significantly alters the sex ratio the male-biased incubation temperature 32.5°C (Bull et al., '88; Tousignant and Cre '94), (2) incubation temperature, and prenatal application of exogenous hormo explains most of the variation observed growth of individuals, and (3) gonadecto on the day of hatch significantly affec growth in females from the all-female prod ing incubation temperature of 26°C, but those from the male-biased incubation te perature of 32.5°C. These data demonstr that several factors seem to play a role in differentiation and development of morp logical and physiological characteristics t in the leopard gecko can persist into adu hood. The recent observation that all-fem sex ratios are generated both at low (26 and high (34+°C) incubation temperatu (Viets et al., '93) presents an opportunity determine whether the same outcome is p duced by the same mechanism at differ temperatures, i.e., is a female from a 2 incubation temperature equivalent to a male from a 34°C incubation temperatu These studies are currently underway may provide insight into whether the proc of sexual differentiation in TSD species sponds differently to environmental ch lenge at temperatures that produce both se than at temperatures that produce only sex.

Finally, these data are consistent with p vious reports on other species that ha shown that nongonadal, phenotypic differ tiation in TSD species is significantly affec by early nongenetic environmental facto These findings may prove important in und

standing the role of environmental factors in species with GSD that also respond to environmental challenge, but because sex is determined at fertilization, these effects are difficult to separate from those imposed by the genetically determined sex of the individual.

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LITERATURE CITED

Bull, J.J. (1980) Sex determination in reptiles. Quart. Rev. Biol. *55*:3–21.

Bull, J.J. (1983) Evolution of Sex Determining Mechanisms. Menlo Park, CA: Benjamin/Cummings.

Bull, J.J. (1987a) Temperature-sensitive periods of sex determination in a lizard: Comparisons with turtles and crocodilians. J. Exp. Zool. *241*:143–148.

Bull, J.J. (1987b) Temperature-dependent sex determination in reptiles: Validity of sex diagnosis in hatchlings. Can. J. Zool. *65*:1421–1424.

Bull, J.J., W.H.N. Gutzke, and D. Crews (1988) Sex reversal by estradiol in three reptilian orders. Gen. Comp. Endocrinol. *70*:425–428.

Bull, J.J., T. Wibbels, and D. Crews (1990) Sex-determining potencies vary among female incubation temperatures in a turtle. J. Exp. Zool. *256*:339–341.

Christian, K., and G. Bedford (1993) High reproductive expenditure per progeny in geckos relative to other lizards. J. Herpetol. *27*:351–354.

Crews, D., T. Wibbels, and W.H.N. Gutzke (1989) Action of sex steroid hormones on temperature-induced sex determination in the snapping turtle (*Chelydra serpentina*). Gen. Comp. Endocrinol. *75*:159–166.

Crews, D., J.J. Bull, and T. Wibbels (1991) Estrogen and sex reversal in turtles: A dose-dependent phenomenon. Gen. Comp. Endocrinol. *81*:357–364.

Crews, D., J.M. Bergeron, D. Flores, J.J. Bull, J.K. Skipper, A. Tousignant, and T. Wibbels. (1994) Temperature-dependent sex determination in reptiles: Proximate mechanisms, ultimate outcomes, and practical applications. Develop. Genet. *15*:297–312.

Desvages, G., and C. Pieau (1992) Aromatase activity in gonads of turtle embryos as a function of the incubation temperature of the egg. J. Ster. Molec. Biol. *41*:851–853.

Ewert, M.A., and C.E. Nelson (1991) Sex determination in turtles: Diverse patterns and some possible adaptive values. Copeia *1991*:50–69.

Flores, D. (1994) Effects of Incubation Temperature and hormonal manipulation on the sociosexual behavior of adult leopard geckos, (*Eublepharis macularius*), a species with temperature-dependent sex determination, masters thesis, University of Texas, Austin.

Gutzke, W.H.N., and J.J. Bull (1986) Steroid hormones reverse sex in turtles. Gen. Comp. Endocrinol. *64*:368–372.

Gutzke, W.H.N., and D. Crews (1988) Embryonic temperature determines adult sexuality in a reptile. Nature *332*:832–834.

Houtsmuller, E.J., and A.K. Slob (1990) Masculinization and defeminization of female rats by males located caudally in the uterus. Physiol. Behav. *48*:555–560.

Janzen, F.J., and G.L. Paukstis (1991) Environmental sex determination in reptiles: Ecology, evolution, and experimental design. Quart. Rev. Biol. *66*:149–179.

Joanen, T., L. McNease, and M.W.J. Ferguson (1987) The effects of egg incubation temperature on post-hatching growth of American alligators. In G.J.W. Webb, S.C. Manolis, and P.J. Whitehead (eds): Wildlife Management: Crocodiles and Alligators. Chipping Norton, NSW: Surrey Beatty, pp. 533–537.

Lang, J.W., H. Andrews, and R. Whitaker (1989) Sex determination and sex ratios in *Crocodylus palustris*. Amer. Zool. *29*:935–952.

Sawano, S., A. Arimura, A. Schally, T. Redding, and S. Schapiro (1969) Neonatal corticoid administration: effects upon adult pituitary growth hormone and hypothalamic growth hormone-releasing hormone activity. Acta Endocr. *61*:57–67.

Sinervo, B. (1990) The evolution of maternal investment in lizards: An experimental and comparative analysis of egg size and its effects on offspring performance. Evolution *44*:279–294.

Tousignant, A., and D. Crews (1994) Effect of exogenous estradiol applied at different embryonic stages on sex determination, growth, and mortality in the leopard gecko (*Eublepharis macularius*). J. Exp. Zool. *268*:17–21.

Viets, B.E., A. Tousignant, M.A. Ewert, C.E. Nelson, and D. Crews (1993) Temperature-dependent sex determination in the leopard gecko, *Eublepharis macularius*. J. Exp. Zool. *265*:679–683.

vom Saal, F. (1981) Variation in phenotype due to random intrauterine positioning of male and female fetuses in rodents. J. Reprod. Fert. *62*:633–650.

Wagner, E. (1980) Temperature-dependent sex determination in a gecko lizard. Quart. Rev. Biol. *55*:21 (appendix).

White, R., and P. Thomas (1992) Whole-body and plasma concentrations of steroids in the turtle, *Trachemys scripta*, before, during, and after the temperature-sensitive period for sex determination. J. Exp. Zool. *264*:159–166.

Whittier, J.M., R.T. Mason, and D. Crews (1987) Plasma steroid hormone levels of female red-sided garter snakes, *Thamnophis sirtalis parietalis*: Relationship to mating and gestation. Gen. Comp. Endocrinol. *67*:33–43.

Wibbels, T.R., J.J. Bull, and D. Crews (1991a) Chronology of temperature-dependent sex determination. J. Exp. Zool. *260*:371–381.

Wibbels, T., J.J. Bull, and D. Crews (1991b) Synergism between temperature and estradiol: A common pathway in turtle sex determination. J. Exp. Zool. *260*:130–134.