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Effects of gonadal sex and incubation temperature on the ontogeny of gonadal steroid concentrations and secondary sex structures in leopard geckos, *Eublepharis macularius*

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Abstract

Incubation temperature during embryonic development determines gonadal sex in the leopard gecko (*Eublepharis macularius*). Incubation temperature and gonadal sex jointly influence the display of sexual and agonistic behavior in adult leopard geckos. These differences in adult behavior are organized prior to sexual maturity, and it is plausible that post-natal hormones influence neural and behavioral differentiation. Here we assessed incubation temperature and sex effects on sex steroid levels in leopard geckos at 2, 10, and 25 weeks of age and monitored the development of male secondary sex structures. Males had significantly higher androgen concentrations at all time points, whereas females had significantly higher 17β-estradiol (E2) concentrations only at 10 and 25 weeks. Within males, age but not incubation temperature affected steroid levels and morphological development. Male androgen levels increased modestly by 10 and dramatically by 25 weeks of age, whereas E2 levels remained unchanged over this period. Most males had signs of hemipenes at 10 weeks of age, and all males had hemipenes and open preanal pores by 25 weeks of age. In females, age and incubation temperature affected E2 and dihydrotestosterone (DHT) but not T concentrations. Controlling for age, females from 34 °C have higher DHT and lower E2 levels than females from 30 °C. Further, E2 concentrations increased significantly from 2 to 10 weeks, after which E2 levels remained steady. Together, these results indicate that sexually dimorphic levels of steroids play a major role in the development of leopard gecko behavior and morphology. Furthermore, these data suggest that the organizational effects of incubation temperature on adult female phenotype could be, in part, mediated by incubation temperature effects on steroid hormone levels during juvenile development.
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1. Introduction

Embryonic temperature determines gonadal sex in certain reptiles (reviewed in Ewert et al., 1994; Lang and Andrews, 1994; Viets et al., 1994). Although incubation temperature also causes phenotypic variation in post-hatching growth, morphology, physiology, and behavior, incubation temperature and sex are confounded variables in many species with temperature-dependent sex determination (TSD) (reviewed in Rhen and Lang, 2004). The leopard gecko, *Eublepharis macularius*, is an appealing model in this regard because both sexes are produced over a broad range of incubation temperatures. This pattern of TSD allows evaluation of sex differences in animals produced at the same incubation temperature as well as temperature effects within each sex. Moreover, male and female leopard geckos are sexually mature at

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~45 weeks of age (Crews et al., 1998; Sakata and Crews, 2004a; Tousignant et al., 1995), which makes it feasible to study the effects of incubation temperature on adult phenotype. For example, adult males hatched from eggs incubated at 30 °C, a temperature that results in a female-biased sex ratio, are more sexually active and less aggressive than adult males hatched from eggs incubated at 32.5 °C, a temperature that results in a male-biased sex ratio (Flores et al., 1994). Temperature-induced differences in behavior between adult males persist even after castration and after hormone replacement produces equivalent levels of testosterone (T), dihydrotestosterone (DHT), and 17β-estradiol (E2) (Rhen and Crews, 1999; Sakata and Crews, 2004b). These observations indicate that temperature during development has organizational effects on the brain and behavior.

Sex differences in social and aggressive behavior are also evident in the leopard gecko (Flores et al., 1994). For instance, ovariectomized females rarely display male-typical mounting behavior even when treated with androgens that robustly activate this behavior in adult males (Rhen and Crews, 1999). Similarly, castrated males do not display female-typical receptive behavior when treated with E2, which induces receptivity in females (Rhen and Crews, 2000). These results suggest that neural and behavioral differentiation in the leopard gecko could depend upon sexually dimorphic production of steroids by the gonads both early in development and later in adulthood.

To summarize, incubation temperature and gonadal sex influence behavior and morphology in accord with the organization–activation theory of sexual differentiation (Nelson, 2000; Rhen and Crews, 1999, 2000). While several studies have examined incubation temperature and sex effects on circulating levels of sex steroids in adult leopard geckos (reviewed in Crews et al., 1998; Rhen et al., 2000), there is currently no information on developmental changes in sex steroids prior to sexual maturity in this species. Here we report that incubation temperature and gonadal sex influence circulating levels of T, DHT, and E2 in the leopard gecko during juvenile development (i.e., shortly after hatching through puberty). We also relate ontogenetic changes in the concentration of androgens to the development of hemipenes and preanal pores in males. Such data are important for understanding how and when temperature and sex have their developmental effects on the brain, behavior, and morphology.

2. Methods

2.1. Animals

Animals were cared for in accordance with a research protocol approved by the Institutional Animal Care and Use Committee at the University of Texas. Eggs from our leopard gecko colony were collected and candled for fertility. Fertile eggs were incubated in cups filled with moist vermiculite (1 part water:1 part vermiculite) at one of four constant incubation temperatures. A temperature of 26 °C produces all females, 30 °C produces a female-biased sex ratio (~30% males), 32.5 °C produces a male-biased sex ratio (~70% males), and 34 °C produces a female-biased sex ratio (<5% males) (Viets et al., 1993). After hatching, geckos were raised in isolation as previously described (Flores et al., 1994). Modal clutch size in leopard geckos is two, and eggs from the same clutch were always incubated at different temperatures. The hatching dates of the geckos used in this study spanned from 9/25/96 to 3/10/99, so husbandry conditions were nearly identical for individuals from different temperatures.

2.2. Sample collection and radioimmunoassay

We collected blood samples from 2-, 10-, and 25-week-old females that had been incubated at 26, 30, 32.5, or 34 °C and from 2-, 10-, and 25-week-old males that had been incubated at 30 or 32.5 °C. A sample was drawn from each animal by cardiocentesis using a heparinized thirty gauge needle on a 1-cc syringe. Blood samples were stored on ice and spun at 3000 rpm for 10 min at 4 °C within 1 h of collection. Plasma was collected and frozen in microcentrifuge tubes at −80 °C until assayed for T, DHT, and E2. All plasma samples from 2-week-old individuals and some plasma samples from 10-week-old individuals were pooled within sex and within incubation temperature to have enough plasma for radioimmunoassay (RIA). The antibodies used for RIA were T3-125 for T, DT3-351 for DHT, and E26-47 for E2 (Endocrine Sciences, Calabasas Hills, CA).

Methods for column chromatography and hormone specific RIA were carried out as previously described (Rhen and Crews, 1999; Rhen et al., 1999). In brief, plasma was spiked with approximately 500 cpm of each tritiated steroid (i.e., T, DHT, and E2) and incubated for 1 h at 25 °C. Steroids were then extracted with 3 ml of ether and dried under a nitrogen stream in a dry bath at 37 °C. Steroids were resuspended in 500 μl of isooctane saturated with ethylene glycol and separated using increasing concentrations of ethyl acetate in isooctane on celite columns. Steroids were then dried under a nitrogen stream in a dry bath at 37 °C and dissolved overnight in 330 μl of phosphate-buffered saline at 4 °C. A 100 μl aliquot of each sample was used to determine individual recoveries. Duplicate 100 μl aliquots were used for RIA using approximately 2000 cpm of the appropriate labeled steroid and the corresponding antibody.

Recoveries averaged 70, 57, and 56% for T, DHT, and E2, respectively. Assay sensitivity was 43 pg T/ml plasma, 41 pg DHT/ml plasma, and 80 pg E2/ml plasma. A pooled

sample of plasma from intact male and female leopard geckos was used as a quality control. Intra-assay coefficients of variation were 17% for testosterone, 16% for dihydrotestosterone, and 18% for 17β-estradiol. Inter-assay coefficients of variation for the same sample were 13% for testosterone, 18% for dihydrotestosterone, and 17% for 17β-estradiol. We also ran quality controls in the low, medium, and high ranges of the standard curve for each steroid. These data have been reported previously and all intra- and inter-assay coefficients of variation were less than or equal to the values for the pooled plasma samples given above (Rhen and Crews, 1999).

Levels of T, DHT, and E2 were detectable in virtually all males. Levels of E2 were detectable in all females. In contrast, a higher proportion of females than males had undetectable levels of T and DHT. Nevertheless, there were no temperature induced or developmental differences in detectability of T or DHT in females. When samples did not have sufficient steroid to be detected, they were excluded from further analysis.

2.3. Development of secondary sexual traits

Beginning 5 weeks following hatching, male leopard geckos (30 °C: $n = 57$; 32.5 °C: $n = 83$) were checked every 5 weeks until 25 weeks of age for the presence of secondary sex characteristics, namely hemipenes and preanal pores. To maximize reliability, examinations were done by one investigator (JTS), and the observations were made blind to the incubation temperature of the individual. We checked for prominent bulges caudal to the cloaca, which are indicative of hemipenes, and whether preanal pores were open or closed. There was a high degree of reliability in measurements as on the vast majority of occasions in which a trait was considered to be present during one observation, the trait was considered to be present during subsequent observations. Blood was not collected from some of these males, and the rate of development of these traits was not affected by blood sampling.

2.4. Statistical analyses

Concentrations of DHT and T were $\log_{10}$-transformed before statistical analysis to meet the assumptions of analysis of variance (ANOVA). In contrast, E2 concentrations were transformed using the natural log (ln). Due to the lack of males at 26 °C and the paucity of males at 34 °C, we were unable to analyze incubation temperature, gonadal sex, and age effects simultaneously in a three-way ANOVA. Consequently, all hormone data were first analyzed using sex and age as main effects in a two-way ANOVA (Sokal and Rohlf, 1981). We then split the data for males ($n = 59$) and females ($n = 93$) and analyzed incubation temperature and age as main effects in separate two-way ANOVAs for each sex.

Given significant main effects or interactions between independent variables in ANOVA, we used Tukey's HSD test for multiple comparisons among groups. Sample sizes for experimental groups are indicated in each figure. Data on secondary sex characteristics were analyzed in two ways, and given that the traits were sex-limited, the analyses were confined to males. In the first, likelihood ratio tests were done on the data at each time point for each trait (presence of prominent hemipenile bulges, open vs. closed preanal pores) with incubation temperature as the sole independent variable. Second, we used survival analysis to see whether incubation temperature affected the rate of development of these traits. Analyses were done only on males with full datasets, and the results for these analyses were in accordance with each other.

All statistics were done using JMP 5.0.1.2 software (SAS Institute).

3. Results

3.1. Sex differences

There were significant sex differences [$F(1,122) = 291$; $P < 0.0001$] and sex by age interactions [$F(2,122) = 7.5$; $P = 0.0008$] for circulating T concentrations (Fig. 1A). The overall effect of age, however, was not significant [$F(2,122) = 1.7$; $P = 0.19$]. Levels of T increased with age in males, but not females, and therefore the magnitude of sex differences increased with age. At 2 weeks, males had concentrations of T that were roughly 10 times those of females. At 10 weeks, males had T levels that were approximately 25-fold greater than T levels in females. The sex difference increased further by 25 weeks such that males had plasma T levels 90-fold higher than females.

There were significant sex differences [$F(1,97) = 85.5$; $P < 0.0001$], age effects [$F(2,97) = 12.0$; $P < 0.0001$], and sex by age interactions [$F(2,97) = 5.0$; $P = 0.0086$] for plasma DHT concentrations (Fig. 1B). Males had concentrations of DHT that were roughly four times those found in females at both 2 and 10 weeks of age. However, the sex difference increased at 25 weeks of age when males had plasma DHT levels 14-fold higher than those in females. Another reason for the sex by age interaction was a significant increase in DHT concentrations between 2 and 10 weeks for females with a slight decrease between 10 and 25 weeks for females (Fig. 1B).

There were also significant sex differences [$F(1,144) = 17.6$; $P < 0.0001$], age effects [$F(2,144) = 4.7$; $P = 0.01$], and sex by age interactions [$F(2,144) = 10.4$; $P < 0.0001$] for plasma E2 concentrations (Fig. 1C). Levels of E2 increased with age in females but not in males. Females had elevated E2 concentrations at 10 and 25 but not 2 weeks of age. The concentration of E2 increased in

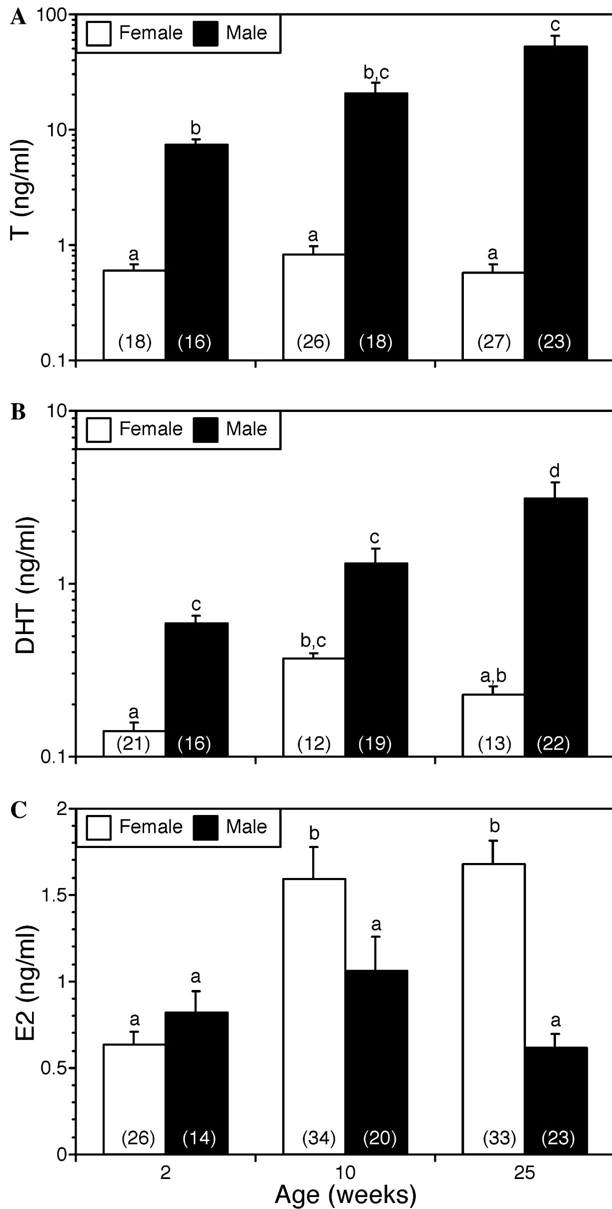

Fig. 1. Sex differences in circulating levels of (A) testosterone, (B) dihydrotestosterone, and (C) 17β-estradiol in leopard geckos at 2, 10, and 25 weeks of age. Hormone levels for females and males are means (±1 SE) from the study described in the text. Groups with significantly different hormone concentrations are shown with different letters.

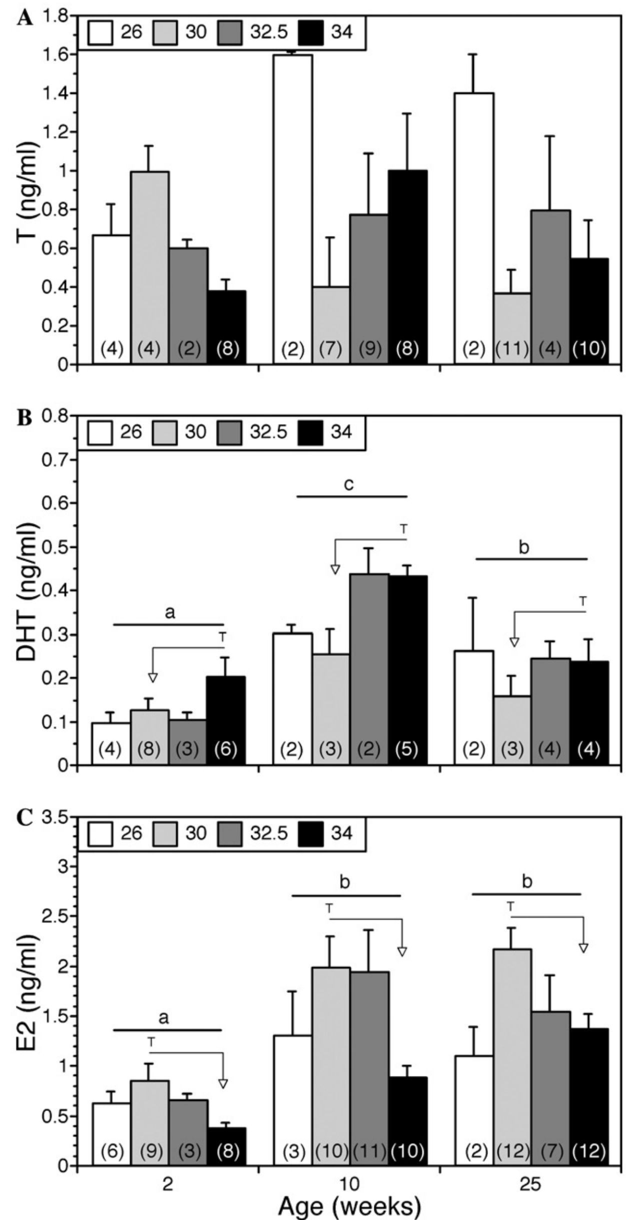

Fig. 2. Incubation temperature effects on circulating levels of (A) testosterone, (B) dihydrotestosterone, and (C) 17β-estradiol in female leopard geckos at 2, 10, and 25 weeks of age. Plasma samples were collected from females that had been incubated at 26, 30, 32.5, or 34 °C during embryogenesis. Hormone levels for females are means (±1 SE) from the study described in the text. Different letters and a thick bar over age groups show developmental changes in hormone levels. The letter T and a thin line and arrow immediately above the relevant groups designate a significant difference in hormone levels between incubation temperatures. Age and incubation temperature affected DHT and E2 but not T concentrations.

females by 10 weeks of age, and at 10 and 25 weeks females had plasma E2 concentrations that were significantly higher than in males.

3.2. Age and temperature effects in females

There were no detectable age effects [$F(2,59) = 0.29$; $P = 0.74$], incubation temperature effects [$F(3,59) = 2.1$; $P = 0.11$], or temperature by age interactions [$F(6,59) = 1.4$; $P = 0.24$] for circulating T concentration in female leopard geckos (Fig. 2A).

However, there were significant age effects [$F(2, 34) = 18.5$; $P < 0.0001$] for plasma DHT levels in females (Fig. 2B). Levels of DHT were lowest at 2 weeks of age, highest at 10 weeks of age, and intermediate at 25 weeks of age. Incubation temperature effects also approached significance [$F(3,34) = 2.6$; $P = 0.069$]. Statistically controlling for age effects, females from 34 °C had higher

levels of DHT than females from 30 °C. The incubation temperature by age interaction was not significant [$F(6,34) = 0.71$; $P = 0.64$].

Age effects [$F(2,81) = 19.7$; $P < 0.0001$] and incubation temperature effects [$F(3,81) = 8.8$; $P < 0.0001$] were significant for plasma E2 levels (Fig. 2C). Plasma E2 levels were lowest in females at 2 weeks of age but increased significantly by 10 weeks of age. There was no difference in E2 levels between 10- and 25-week-old females. Controlling for age effects, females from 30 °C had significantly higher concentrations of E2 than females from 34 °C. The temperature by age interaction was not significant for E2 concentration [$F(6,81) = 0.76$, $P = 0.60$].

3.3. Age and temperature effects in males

There were age-dependent changes in T levels in male leopard geckos [$F(2,51) = 7.2$; $P = 0.0017$] (Fig. 3A). The concentration of T roughly tripled between 2 and 10 weeks of age, but this difference did not reach statistical significance. The level of T in males at 25 weeks of age was significantly higher than the level of T in males at 2 and 10 weeks of age. However, there were no detectable effects of incubation temperature [$F(1,51) = 0.47$; $P = 0.50$] or temperature by age interactions [$F(2,51) = 0.68$; $P = 0.51$] on circulating T concentrations (Fig. 3A).

Similar effects were observed for circulating DHT levels. The concentration of DHT increased with age [$F(2,51) = 6.7$; $P = 0.0026$] (Fig. 3B). The difference in DHT levels between 2- and 10-week-old males was not statistically significant, but the concentration of DHT increased in 25-week-old males and was approximately 2.5-fold greater than in 10-week-old males. There was no detectable incubation temperature effect [$F(1,51) = 0.53$; $P = 0.47$] or temperature by age interaction [$F(2,51) = 0.44$; $P = 0.64$] for DHT levels (Fig. 3B).

There was a significant temperature by age interaction for circulating E2 concentrations in male leopard geckos [$F(2,51) = 4.1$; $P = 0.041$] (Fig. 3C). The concentration of E2 was significantly higher in males from 32.5 °C than males from 30 °C at 10 weeks of age. In addition, E2 levels decreased in males from 32.5 °C at 25 weeks of age. There were no detectable differences between males from different incubation temperatures at 2 or 25 weeks of age, but there was a trend for males from 32.5 °C to have lower E2 levels than 30 °C males at 25 weeks of age.

3.4. Development of secondary sex structures

Most males showed prominent hemipenile bulges by 10 weeks post-hatching and open preanal pores by 15 weeks post-hatching (Table 1). Virtually all males had hemipenes and open preanal pores by 25 weeks of age. Males developed hemipenes and preanal pores in parallel with increasing levels of T and DHT. At no time point were there group differences in the proportion of males with secondary sex characteristics, and the survival analysis revealed that incubation temperature did not influence the rate of development of hemipenes or preanal pores (Table 1).

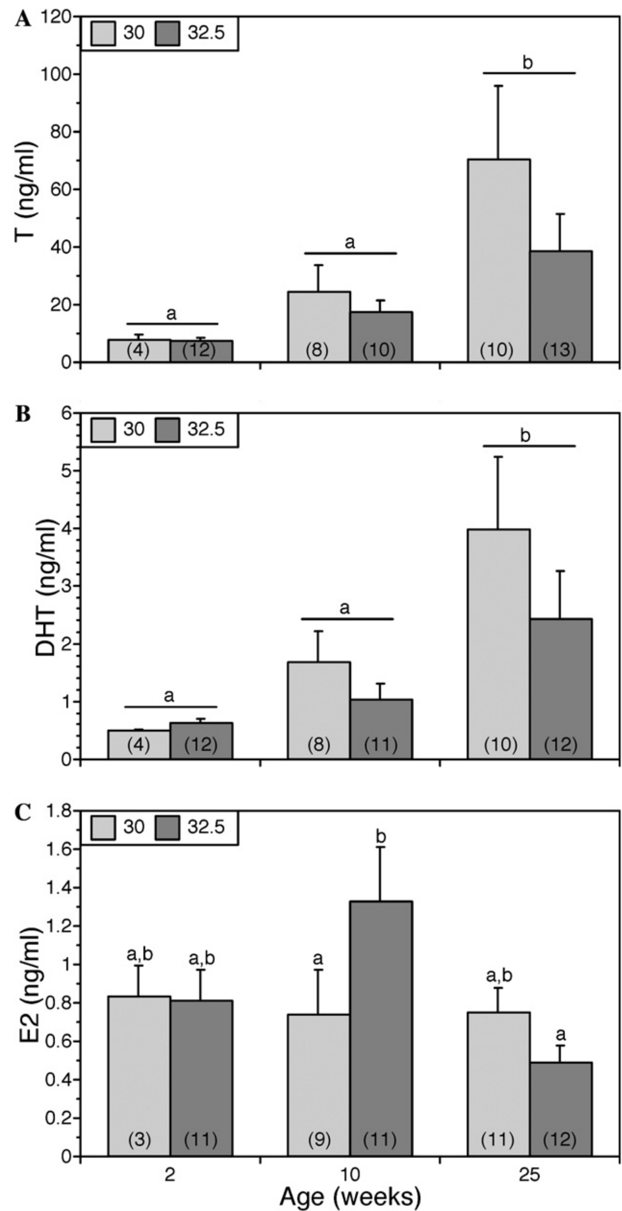

Fig. 3. Incubation temperature effects on circulating levels of (A) testosterone, (B) dihydrotestosterone, and (C) 17β-estradiol in male leopard geckos at 2, 10, and 25 weeks of age. Plasma samples were collected from males that had been incubated at 30 or 32.5 °C during embryogenesis. Hormone levels for males are means (±1 SE) from the study described in the text. Different letters and a thick bar over age groups show developmental changes in T and DHT levels in the first two panels. Groups with significantly different E2 concentrations are shown with different letters in the bottom panel.

Table 1
Percentage of male leopard geckos with secondary sex characteristics at different ages

Weeks	Incubation temperature		
	30 ($n = 57$)	32.5 ($n = 83$)	Total ($n = 140$)
Hemipenes prominent			
5	12	11	11
10	74	63	67
15	93	92	92
20	100	99	99
25	100	99	99
Preanal ridge forming/formed			
5	83	75	78
10	95	94	94
15	98	99	99
20	100	99	99
25	100	100	100
Prenanal pores open			
5	2	0	1
10	37	29	32
15	65	68	66
20	98	94	96
25	100	100	100

4. Discussion

Sexual differentiation in numerous vertebrates results from sexually dimorphic secretion of androgens by the testes and estrogens by the ovaries. These steroids can have developmental or organizational effects on phenotype that are irreversible. Steroids may also have effects that are transient or activational in nature, only persisting as long as steroid levels are elevated. Whereas organizational effects of steroids arise during embryonic or juvenile development, activational effects of steroids usually occur in animals that have reached sexual maturity. For instance, seasonal variation in reproductive traits and behaviors in many species is a consequence of changing steroid levels. Studies demonstrate that sex steroids activate sexual and aggressive behavior in adult leopard geckos and, moreover, that incubation temperature and gonadal sex have an organizational influence on the display of social behavior (Rhen and Crews, 1999, 2000). It is plausible that these organized differences are influenced by differences in circulating sex steroid hormone concentrations during development. We therefore examined whether embryonic temperature and gonadal sex influence the concentration of plasma androgens and estrogens in leopard geckos during post-hatching development.

4.1. Sex differences in hormone levels and the development of male sex structures

A primary finding was that males and females had different developmental profiles for T, DHT, and E2. Sex differences were like those observed in other vertebrates: whereas males had higher concentrations of T and DHT than females throughout juvenile development, females generally had higher concentrations of E2 than males. Levels of E2 were low in 2-week-old females, but increased by 10 weeks of age to levels found in adult previtellogenic females. Consequently, the sex difference for E2 concentration was only significant at 10 and 25 weeks of age. Preliminary data from hatchling geckos is consistent with those from our 2-week-old individuals: sex differences in T but not E2 levels were found in a small sample of hatchlings (D. Crews, unpublished data). Although plasma T concentrations differ by an order of magnitude in hatchling and 2-week-old males and females, the sexes are virtually indistinguishable based on external morphology (males rarely have any sign of hemipenes or preanal pore development at these ages). Finally, the finding that young males do not yet have male secondary sex structures is not surprising because circulating levels of T are similar to those found in intact adult females (Rhen et al., 2000). Androgen levels in this range are not sufficient to stimulate growth and differentiation of male sex structures (Rhen et al., 1999).

Many males had hemipenes and indications of preanal pore development by 10 weeks of age, when levels of T increased to 18.2 ng/ml and DHT to 1.2 ng/ml, suggesting that the threshold concentration for androgen induction of male sex structures was reached between 2 and 10 weeks. Another study indicates androgen levels must be above this theoretical threshold for at least 2 weeks to stimulate development of hemipenes (Rhen et al., 1999). Adult females grow hemipenes when treated for 2 weeks with T implants that produce androgen levels typical of adult males, but do not when treated for only 8 days (Holmes et al., 2005; Rhen et al., 1999). These studies, together with the finding that hemipenes regress following androgen removal in males and T-treated females (T. Rhen and D. Crews, unpublished data), show that hemipenes differentiate under the activational influence of androgens in this species.

Preanal pores develop more slowly than hemipenes and are only fully functional once males reach sexual maturity. As adults, males drag these pores on the substrate and swipe their tail back and forth, depositing a waxy secretion in a scent-marking behavior that may be territorial in nature. Initial differentiation of preanal pores involves formation of a ridge of epidermal tissue in front of the cloaca. The first hint of the opening of preanal pores in this ridge occurs around 10 weeks of age, when a few males had open pores. Pores open in most males in parallel with a large increase in the concentration of T and DHT between 10 and 25 weeks of age. Virtually all males secrete a waxy substance from their preanal pores between 40 and 45 weeks of age, as androgens reach levels typically found in sexually mature males (J.T. Sakata and D. Crews, unpublished data).

4.2. Incubation temperature effects on hormone levels

Generally speaking, incubation temperature had greater effects on sex steroid hormone physiology during post-hatching development in females than in males. Controlling for age-dependent changes, females from 34 °C had higher DHT concentrations than females from 30 °C, and females from 30 °C had higher E2 concentrations than females from 34 °C. The difference in DHT concentrations during development is consistent with a previous study of adult females throughout the follicular cycle (Rhen et al., 2000). On the other hand, whereas adult females from 34 °C have higher T levels than females from 26 to 30 °C (Rhen et al., 2000), we did not find differences in T concentration among juvenile females from different incubation temperatures. Finally, whereas adult females from different incubation temperatures do not show significant differences in E2 levels across the cycle, juvenile females display temperature-induced differences.

In contrast to the robust effects in females, there was a weak temperature by age interaction for plasma E2 concentration in males. Levels of E2 increased slightly from 2 to 10 weeks of age and decreased significantly from 10 to 25 weeks of age in males from 32.5 °C but not in males from 30 °C. Thus, levels of E2 were higher in males from 32.5 °C than in males from 30 °C at 10 weeks but not 2 or 25 weeks of age. It seems unlikely that this small developmental difference in E2 levels would be responsible for the organizational effect of incubation temperature on male behavior (reviewed in Crews et al., 1998; Sakata and Crews, 2004a), particularly in light of the fact that E2 levels rise in female leopard geckos at the same age. Nevertheless, definitive proof will require manipulations of E2 at the appropriate developmental stages.

4.3. Hormones and sexual differentiation

In summary, our study shows that male leopard geckos have much higher circulating levels of T and DHT than female leopard geckos from hatching through adulthood and that females have elevated E2 levels from approximately 10 weeks of age through adulthood. This study, together with previous experiments, demonstrates that androgens have an activational effect on hemipenes differentiation. Higher androgen levels in male leopard geckos during postnatal development may also masculinize regions of the brain controlling sexual and aggressive behavior. This basic mode of neural differentiation would be analogous to the mechanism underlying organization of the brain and behavior in mammals (Baum, 2003; Hughes, 2001; Nelson, 2000). Elevated levels of estrogens during early development organize the female brain in birds (Adkins, 1975; Balthazart and Ball, 1995) and may also do so in mammals (Bakker et al., 2003), and it is possible that some sex differences in adult phenotype are caused by this developmental endocrine difference. We also found that incubation temperature during embryonic development has significant effects on endocrine physiology in juveniles, particularly females, but these effects are relatively small compared to the sex differences. Nevertheless, such temperature-induced differences in sex steroid levels during development could organize the brain to produce behavioral differences among females from different incubation temperatures (Flores et al., 1994; Gutzke and Crews, 1988; Rhen and Crews, 1999, 2000).

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