Sex Steroid Levels across the Reproductive Cycle of Female Leopard Geckos, *Eublepharis macularius,* from Different Incubation Temperatures

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Incubation temperature during embryonic development determines gonadal sex in many reptiles, including the leopard gecko (*Eublepharis macularius*). In this study, we examined the hormonal and behavioral changes that occur during the reproductive cycle of female leopard geckos from four (i.e., 26, 30, 32.5, and 34°C) incubation temperatures. Controlling for reproductive status, plasma levels of dihydrotestosterone (DHT), testosterone (T), and progesterone (P) varied with incubation temperature but estradiol 17-β (E2) levels did not. Controlling for the effects of incubation temperature, DHT and T levels were low when females were previtellogenic, increased slightly during early vitellogenesis, increased dramatically during late vitellogenesis (i.e., prior to ovulation), and then decreased to previtellogenic levels after ovulation. In contrast, E2 levels increased gradually from the previtellogenic stage to the early vitellogenic stage, peaked during late vitellogenesis, and decreased to previtellogenic levels after ovulation. Levels of P increased from the previtellogenic stage to the early vitellogenic stage, remained elevated during late vitellogenesis, and then decreased after ovulation. Moreover, we determined that females were not sexually receptive when previtellogenic, were somewhat receptive during early vitellogenesis (~20% receptive), were most receptive during late vitellogenesis (~80% receptive), and were again unreceptive after ovulation. Incubation temperature did not influence receptivity. Overall, these data show that hormone levels and behavior change coordinately during the reproductive cycle. Although incubation temperature has persistent effects on endocrine physiology in adult female leopard geckos, these effects are modest compared to hormonal changes across the reproductive cycle. © 2000 Academic Press

Temperature during embryogenesis determines gonadal sex in some lizards, many turtles, and all crocodilians (reviewed in Ewert *et al.,* 1994; Lang and Andrews, 1994; Viets *et al.,* 1994). Incubation temperature also influences the development of many other characteristics in reptiles with temperature-dependent sex determination (or TSD). For example, in various turtle species and in the American alligator, *Alligator mississippiensis,* embryonic temperature has effects on hatchling and juvenile traits such as body size, energy reserves, metabolism and growth, pigmentation, sex steroid physiology, secondary sex structures, and behavioral thermoregulation (Etchburger *et al.,* 1993; Allsteadt and Lang, 1995a,b; Rhen and Lang, 1995, 1999a,b; Rhen *et al.,* 1999a; Roosenburg and Kelley, 1996). Yet, it is unclear whether incubation temperature has effects that endure until later developmental stages in these long-lived, slowly maturing reptiles.

Research with the leopard gecko, *Eublepharis macularius,* indicates that such temperature-induced phenotypic variation persists into adulthood (reviewed in Crews *et al.,* 1998). Incubation temperature determines gonadal sex in this species: 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 (~65% males), and 34°C again produces a female-biased sex ratio (~5% males) (Viets *et al.,* 1993). Since both sexes are sexually mature at approximately 40 to 50 weeks of age (Tousignant *et al.,* 1995; Sakata and Crews, unpublished data), it is feasible to directly determine whether incubation temperature has any phenotypic effects in adult males and females. Previous studies have found that both incubation temperature and gonadal sex influence adult sex steroid physiology and reproductive behavior (reviewed in Crews *et al.,* 1998).

Gutzke and Crews (1988) found that females from an incubation temperature of 32°C had significantly higher levels of total androgens and lower levels of estrogens than females from 26°C, whereas females from 29°C had intermediate levels of androgens and estrogens. Whereas some of these results have been replicated in subsequent studies that measured sex steroid levels in females from different incubation temperatures, other findings have not been confirmed (Tousignant and Crews, 1995; Tousignant *et al.,* 1995; Flores and Crews, 1995; Crews *et al.,* 1996; Coomber *et al.,* 1997).

A number of potentially confounding factors may have contributed to these variable results. Variation in the age of females, for example, may account for some of the inconsistent findings (see discussion in Tousignant *et al.,* 1995). Recent work has shown ontogenetic changes in hormone levels prior to sexual maturity (Sakata *et al.,* 1998). The most likely explanation for many of the results, however, is undescribed variation in female reproductive status. In fact, in the only study to date that has examined the effect of reproductive status, females with vitellogenic follicles had higher levels of testosterone (T) than previtellogenic females or females after oviposition (Tousignant *et al.,* 1995). In contrast, levels of dihydrotestosterone (DHT), estradiol 17-β (E2), and corticosterone did not vary with reproductive status. Nevertheless, that study did not make a complete set of comparisons among incubation temperatures and across reproductive stages. Thus, it remains unclear whether incubation temperature influences sex steroid levels in adult female leopard geckos.

Here, we use a repeated-measures experimental design to precisely characterize the hormonal changes that occur during the reproductive cycle of female leopard geckos from different incubation temperatures. This design is ideal both for detecting temperature effects on circulating sex steroid levels and for documenting changes in hormone levels during the reproductive cycle. We also examined the effects of incubation temperature and reproductive status on the expression of female receptive behavior and the relationship of circulating hormone levels to this behavior.

METHODS

Animals

Leopard gecko eggs from our captive breeding colony at the University of Texas were collected and candled for fertility. Fertile eggs were placed in individual cups filled with moist vermiculite (1 part water:1 part vermiculite) and divided among four constant incubation temperatures: we used females from 26°C ($n = 6$), 30°C ($n = 11$), 32.5°C ($n = 7$), and 34°C ($n = 9$). Geckos hatched from these eggs were raised in isolation as previously described (Flores *et al.,* 1994) and placed in breeding cages upon reaching sexual maturity at roughly 40 to 50 weeks of age (Tousignant *et al.,* 1995; Sakata and Crews, unpublished data). The average ages (and distribution of ages) of females from each incubation temperature were similar: females from 26°C were 3–8 years old, females from 30°C were 1–6 years old, females from 32.5°C were 1–8 years old, and females from 34°C were 1–6 years old. Thus, the potentially confounding effect of reproductive aging was minimized. Moreover, age did not influence hormone levels in sexually mature females in another study (Crews *et al.,* 1996).

Reproductive Cycle and Behavior

The reproductive status of females was monitored every 2 or 3 days during a single reproductive cycle. Behavior tests were conducted and blood samples were taken during the same cycle. In the female leopard gecko, reproductive status can be determined easily because follicles and eggs are visible through the abdominal wall. We therefore collected blood samples

when females were previtellogenic (i.e., had no visible follicles or eggs), when their follicles were less than 9 mm (mean $\pm$ SE = 7 $\pm$ 0.2 mm) in diameter (i.e., during early vitellogenesis), when their follicles were 12–14 mm (mean $\pm$ SE = 13 $\pm$ 0.2 mm) in diameter (i.e., during late vitellogenesis), and again 1–3 days after ovulation when females were gravid. Samples during the previtellogenic stage were taken weeks before females entered the reproductive cycle. After females became reproductively active (i.e., reached the early vitellogenic stage), it took approximately 15 days (95% confidence limits = 11–21 days) to move from the early to the late vitellogenic stage. Females ovulated roughly 9 days (95% confidence limits = 7–12 days) after reaching the late vitellogenic stage and layed their eggs an additional 11 days later (95% confidence limits = 10–13 days). Blood samples were collected via cardiocentesis within 1 to 2 min and the average sample volume of whole blood was 200 μl. On average, adult females weigh 35–45 g (Tousignant and Crews, 1995).

Although the effect of repeated blood removal on hormone levels is uncertain (females were bled four times during a single cycle), multiple bleedings did not appear to influence circulating levels of sex steroids because the levels of hormones that we measured were comparable to those found in earlier studies in which females were bled only once. For example, Tousignant *et al.* (1995) reported levels of T for vitellogenic females of roughly 5 ng/ml of plasma, whereas we found an average of 6.5 ng/ml of plasma in the current study. Likewise, previtellogenic females in both studies had T levels less than 1 ng/ml of plasma. The levels of the other steroids that we measured were also similar to results reported in earlier experiments. If the stress of repeated sampling had influenced our results, we would have expected reduced hormone levels during the reproductive cycle rather than results like those from earlier studies in which females were only sampled once. Consequently, we believe that our findings more accurately characterize the hormonal changes that occur during the reproductive cycle of female leopard geckos from different incubation temperatures. All females in this study had two developing follicles and subsequently laid two eggs; female leopard geckos occasionally lay clutches with just one egg but normally lay two eggs. Moreover, the fertility of eggs produced by experimental females was similar to the rate of fertility in our colony as a whole.

Females were removed from their cages and tested with a sexually active male in a neutral cage during early vitellogenesis and again during late vitellogenesis. Females were first placed into a neutral cage (43 $\times$ 22 $\times$ 20 cm) with a clean paper towel as a liner. Stimulus males were then placed, facing the female, into the same cage. In a sexual encounter, a male slowly approaches a female, first licking the substrate or the air with his tongue and then licking the female. An attractivity pheromone in the skin of females (Mason and Gutzke, 1990) elicits a male-typical tail vibration that creates an audible buzz and a tactile vibration of the substrate. During these encounters, males may also drag their preanal pores on the substrate, presumably to deposit pheromones in a scent-marking behavior. Males then body grip the female's skin with their jaws during courtship and mounting. Body grips are a major component of mounting behavior, as they are used to position the male for copulation and nearly always accompany intromission.

In this study, we noted a female's receptivity to body grips by stimulus males. Females were considered receptive if they either displayed a tail lift (exposing the cloaca for intromission) or remained immobile when gripped by a male. Females were considered unreceptive if they fled or attacked when body gripped. Behavior tests were stopped after 15 min if a male did not attempt to court the female. Females that were not body gripped in the first test were retested with another male on the following day. We repeated this procedure until receptivity was determined for all females. Every female was body gripped by the third behavior test. Thus, no female changed reproductive status before we could ascertain their receptivity. However, handling and caging changes associated with behavior testing and variable exposure to males could have affected hormone levels.

Radioimmunoassay (RIA)

Immediately following the last behavior test, a blood sample was drawn from each experimental animal by cardiocentesis using a heparinized 1-cc syringe with a 25-gauge needle. Blood was centrifuged at 3000 rpm for 10 min at 4°C. Plasma was stored in plastic microfuge tubes at −80°C until assayed for

levels of T, DHT, E2, and progesterone (P) using hormone-specific RIAs. The antibodies used for the RIAs were T3-125 for T, DT3-351 for DHT, E26-47 for E2, and P11-192 for P (Endocrine Sciences, Calabasas Hills, CA).

The procedures used for column chromatography and the RIAs have been described in detail previously (Rhen and Crews, 1999; Rhen *et al.,* 1999a). Recoveries averaged 70, 57, 56, and 40% for T, DHT, E2, and P, respectively. Assay sensitivity was 86 pg T/ml plasma, 71 pg DHT/ml plasma, 92 pg E2/ml plasma, and 238 pg P/ml plasma. For a pooled plasma sample from intact male and female leopard geckos, intraassay coefficients of variation were 17% for T, 16% for DHT, 18% for E2, and 12% for P. Interassay coefficients of variation for the same sample were 13% for T, 18% for DHT, 17% for E2, and 20% for P. We also ran quality control standards of known concentration in the low, medium, and high ranges of the standard curve for each steroid. This data was reported previously and all intra- and interassay coefficients of variation were less than or equal to the values for the pooled plasma samples given above (Rhen *et al.,* 1999a).

Statistical Analyses

All hormone and behavior data were analyzed using incubation temperature and reproductive stage as main effects in a two-way univariate analysis (Crowder and Hand, 1990). Our data met the assumptions for the application of univariate methods to repeated-measures data. In addition, results of the univariate analysis were very similar to results from a multivariate repeated-measures analysis. Hormone concentrations were log-transformed before statistical analysis to meet the assumptions of analysis of variance (ANOVA). Of the 132 hormone measurements for each steroid, we excluded 3 outliers from the analysis of T levels and 2 outliers from the analyses of DHT, E2, and P levels. These extreme variates exceeded critical values for an outlier test and all were more than 4 standard deviations from their respective sample means (Sokal and Rohlf, 1981).

Female receptivity to male body grips was analyzed using a nominal logistic (log-linear) model fitted with a maximum-likelihood procedure (SAS Institute, 1997). The logistic model is most appropriate for binary dependent variables (i.e., receptive or unreceptive) and is analagous to the linear model for continuous variables in ANOVA (Chatterjee and Price, 1978; Sokal and Rohlf, 1981).

To further illuminate the association between hormones and behavior, we compared levels of sex steroids between receptive and unreceptive females using *t* tests. Moreover, we made comparisons only within each reproductive stage (i.e., during early or late vitellogenesis) to control for the effect of reproductive status on hormone levels. Independent variables were considered significant when $P \pm 0.05$. Dependent variables are presented as least squares means $\pm$ one standard error. The Dunn–Sidák method was used for post hoc contrasts to provide a significance level of $\alpha' = 1 - (1 - 0.05)^{1/k}$, where $k =$ the number of individual comparisons for an experiment-wise $\alpha = 0.05$ (Sokal and Rohlf, 1981). All statistics were done with Version 3.2 of JMP (SAS Institute, 1997) for Apple Macintosh.

RESULTS

Hormones

Levels of T were influenced by incubation temperature ($F = 4.3$; $df = 3, 111$; $P = 0.007$) and female reproductive status ($F = 249.3$; $df = 3, 111$; $P < 0.001$) but not by their interaction ($F = 1.35$; $df = 9, 111$; $P = 0.22$). Controlling for the effect of reproductive status, levels of T generally increased with incubation temperature (Figs. 1a and 2a). Females from 26 and 30°C had significantly lower levels of T than females from 34°C ($\alpha' = 0.009$). Controlling for the effect of incubation temperature, levels of T were lowest when females were previtellogenic, increased significantly during early vitellogenesis, increased dramatically during late vitellogenesis, and then decreased to previtellogenic levels after ovulation ($\alpha' = 0.017$; Fig. 2a).

Levels of DHT were influenced by incubation temperature ($F = 3.8$; $df = 3, 112$; $P = 0.01$) and female reproductive status ($F = 39.1$; $df = 3, 112$; $P < 0.001$) but not by their interaction ($F = 1.42$; $df = 9, 112$; $P = 0.19$). Controlling for the effect of reproductive status, levels of DHT generally increased with incuba-
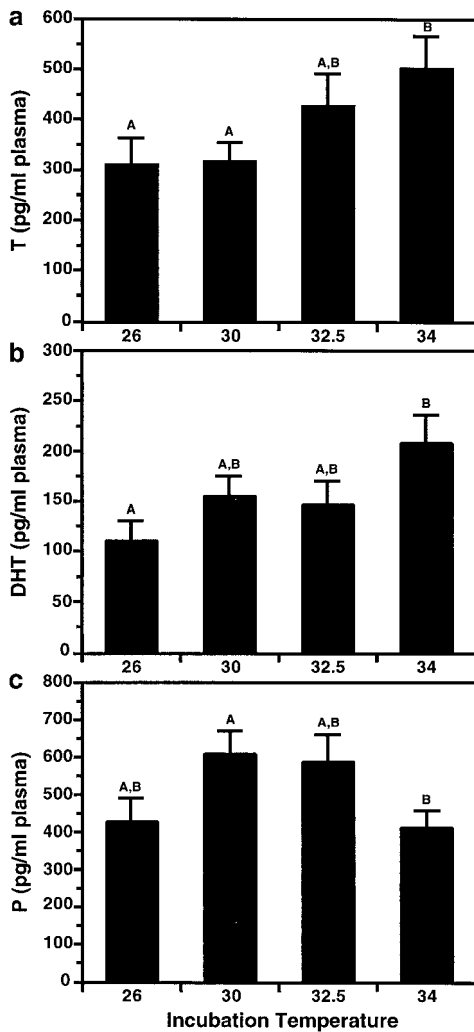
FIG. 1. Effect of incubation temperature on circulating levels of (a) testosterone, (b) dihydrotestosterone, and (c) progesterone in adult female leopard geckos. Hormone levels are least squares means (±1 SE) from the ANOVA described in the text. These means represent the means for each incubation temperature while controlling for the effects of reproductive status. Incubation temperatures having different superscripts are significantly different from each other.

tion temperature (Figs. 1b and 2b). However, the only significant pairwise difference was between females from 26°C and females from 34°C ($\alpha' = 0.009$). Controlling for the effect of incubation temperature, plasma levels of DHT were lowest when females were previtellogenic, increased slightly (but not significantly) during early vitellogenesis, increased significantly during late vitellogenesis, and then decreased after ovulation but still remained significantly above previtellogenic levels ($\alpha' = 0.017$; Fig. 2b).

Levels of E2 were influenced by female reproductive status ($F = 16.4$; $df = 3, 114$; $P < 0.001$) but not by incubation temperature ($F = 1.7$; $df = 3, 114$; $P = 0.17$) nor the interaction between reproductive status and incubation temperature ($F = 0.54$; $df = 9, 114$; $P = 0.84$). Plasma levels of E2 increased from the previtellogenic stage to the early vitellogenic stage, peaked in the late vitellogenic stage, and decreased to previtellogenic levels after ovulation ($\alpha' = 0.017$; Fig. 2c).

Levels of P were influenced by incubation temperature ($F = 4.2$; $df = 3, 114$; $P = 0.007$) and female reproductive status ($F = 5.2$; $df = 3, 114$; $P = 0.002$) but not by their interaction ($F = 0.84$; $df = 9, 114$; $P = 0.58$). Controlling for the effect of reproductive status, levels of P were generally higher at intermediate temperatures and lower at the extreme incubation temperatures (Figs. 1c and 2d). However, the only significant pairwise difference was between females from 30 and 34°C ($\alpha' = 0.009$). Controlling for the effect of incubation temperature, levels of P increased from the previtellogenic stage to the early vitellogenic stage, decreased slightly during the late vitellogenic stage (P levels were not significantly higher than previtellogenic levels), and then decreased to previtellogenic levels after ovulation ($\alpha' = 0.017$; Fig. 2d).

Receptive Behavior

Female receptivity was strongly affected by reproductive status (Likelihood Ratio $\chi^2 = 29.1$; $df = 1$; $P < 0.001$). Whereas 18% (6/33) of females were receptive during early vitellogenesis, 82% (27/33) of females were receptive during late vitellogenesis. Receptivity was not influenced by incubation temperature (Likelihood Ratio $\chi^2 = 3.9$; $df = 3$; $P = 0.27$) nor the interaction between reproductive status and incubation temperature (Likelihood Ratio $\chi^2 = 2.4$; $df = 3$; $P = 0.49$).

When we compared hormone levels, we found that there were no differences between receptive and unreceptive females for any sex steroid during early vitellogenesis (P's > 0.05). In contrast, during late vitellogenesis, receptive females had significantly higher levels of T ($t = 2.11$; $df = 29$; $P = 0.04$), DHT ($t = 2.33$; $df = 31$; $P = 0.03$), and E2 ($t = 2.32$; $df = 31$; $P = 0.03$) than unreceptive females (see Fig. 3). There were no differences between receptive and un-
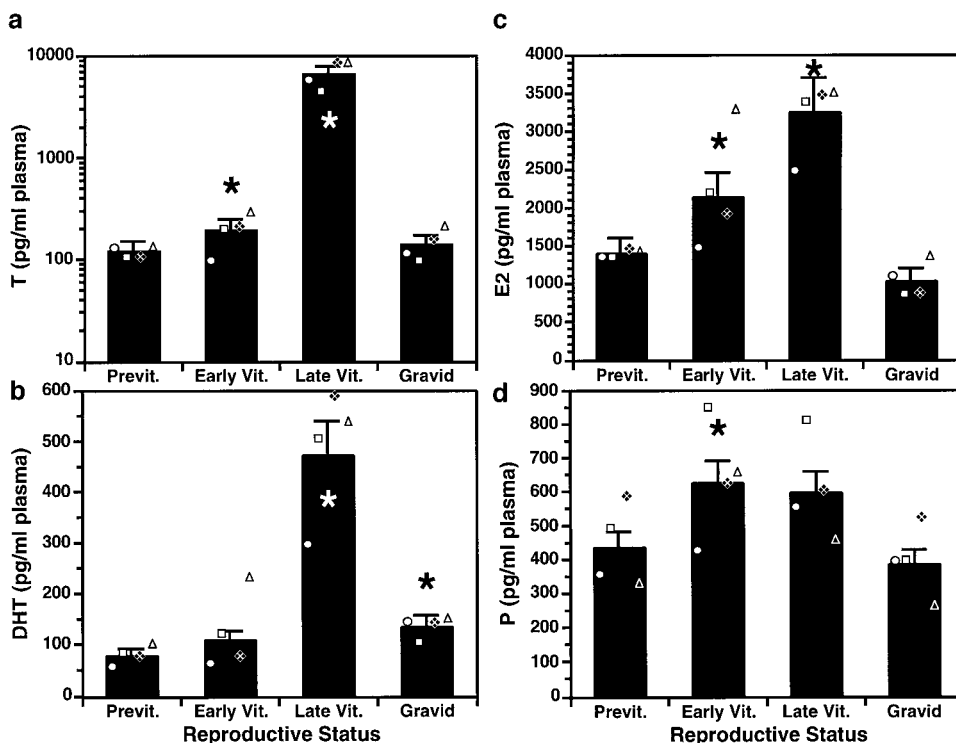
FIG. 2. Effect of reproductive status on circulating levels of (a) testosterone, (b) dihydrotestosterone, (c) estradiol 17-β, and (d) progesterone in adult female leopard geckos. Hormone levels are least squares means (± 1 SE) from the ANOVA described in the text. These means represent the means for each stage of the reproductive cycle while controlling for the effects of incubation temperature. Hormone levels during early vitellogenesis, late vitellogenesis, and the gravid stage were compared to the levels observed during previtellogenesis (significant differences from the previtellogenic stage are indicated by asterisks). Additional symbols show the means for each incubation temperature at each stage of the reproductive cycle. Open circle, 26°C; open square, 30°C; diamonds, 32.5°C; open triangle, 34°C.

receive females in levels of P during late vitellogenesis ($t = 0.02$; $df = 30$; $P = 0.93$).

DISCUSSION

Incubation Temperature Effects on Hormone Levels

In this study, we found that both embryonic incubation temperature and adult reproductive status influenced levels of sex steroids in female leopard geckos. Controlling for changes in hormone levels across the reproductive cycle, levels of T and DHT generally increased with incubation temperature. The largest temperature-induced difference in T levels was approximately 200 pg/ml of plasma. Similarly, the largest temperature-induced difference in DHT levels was approximately 100 pg/ml of plasma. These differences in androgen levels, on average, add up to 300 pg/ml of plasma. In contrast, Gutzke and Crews (1988) found that levels of total androgens differed by roughly 5000 pg/ml of plasma for females from different incubation temperatures. Since we found a difference of this magnitude only when comparing average levels of T between the late vitellogenic stage and the other reproductive stages, our results suggest that changes in androgen levels during the female reproductive cycle were confounded with incubation temperature effects in that particular study. Specifically, in Gutzke and Crews (1988), the stage of the reproductive cycle in females from different temperatures was not recorded and individuals may have been sampled at different stages in their cycle. In support of this inference and our finding of a relatively subtle temperature effect on androgen levels,
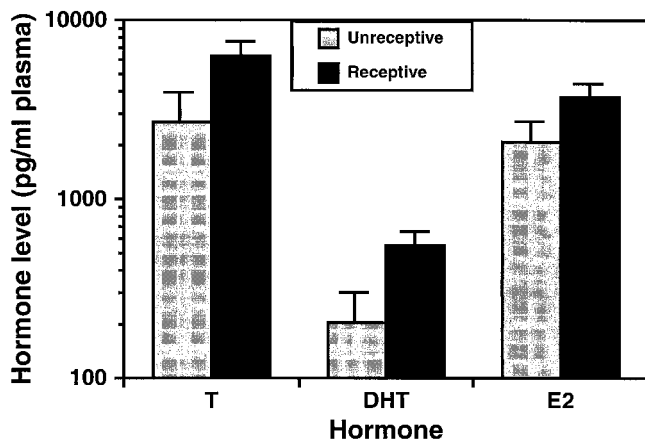
FIG. 3. Hormone levels in unreceptive vs receptive leopard gecko females during late vitellogenesis. Levels of testosterone, dihydrotestosterone, and estradiol 17-β were significantly higher in receptive than in unreceptive females.

other experiments report no detectable incubation temperature effect on total androgens or T and DHT levels (Flores and Crews, 1995; Tousignant and Crews, 1995; Tousignant *et al.,* 1995; Crews *et al.,* 1996; Coomber *et al.,* 1997). At best, however, these other studies only loosely controlled for changes in hormone levels across the female reproductive cycle. Thus, although we believe that results from the current study using a repeated-measures design better reflect the real extent of temperature-induced variation in androgen levels in female leopard geckos, it is possible that the small temperature effect that we observed is entirely due to undetected differences in reproductive condition.

We also found that P levels varied with incubation temperature. As levels of P were at the limit of detectability in previous studies (Tousignant and Crews, 1995; Tousignant *et al.,* 1995), our study is the first to indicate that temperature influences circulating levels of this steroid in the leopard gecko. In contrast to our results for plasma T and DHT levels, the magnitude of temperature effects was similar to the magnitude of reproductive status effects on P levels. The general pattern of incubation temperature effects on P levels (i.e., higher at intermediate temperatures and lower at the extreme temperatures) was also different from temperature effects on levels of T and DHT. In conjunction with our finding that levels of E2 did not vary significantly with incubation temperature, these results imply that embryonic temperature has distinct

effects on the circulating levels of different sex steroids in adult female leopard geckos. Interestingly, hormone-specific temperature effects are also seen in juvenile red-eared slider turtles, *Trachemys scripta elegans* (Rhen *et al.,* 1999a).

Reproductive Status Effects on Hormone Levels

Controlling for incubation temperature effects, sex steroid levels changed across the female reproductive cycle. Levels of T were low when females were previtellogenic, increased significantly during early vitellogenesis, increased dramatically during late vitellogenesis (i.e., shortly before ovulation), and then decreased to previtellogenic levels after ovulation. These results are in agreement with those of Tousignant *et al.* (1995), who reported that female leopard geckos with vitellogenic follicles have higher T levels than previtellogenic females or females after oviposition. Considering the large increase in T levels that we found during late vitellogenesis, the concordance between these studies is as expected.

We also found more subtle, but significant, changes in circulating levels of DHT, E2, and P through the reproductive cycle. Whereas the pattern of change in DHT levels was similar to that observed for T levels, the increase in E2 levels was more gradual from the previtellogenic stage to the late vitellogenic stage. Levels of P increased from the previtellogenic stage to the early vitellogenic stage, decreased slightly during the late vitellogenic stage, and then returned to previtellogenic levels after ovulation. In contrast, Tousignant *et al.* (1995) found that levels of DHT and E2 did not vary with reproductive state. They were also unable to test for differences in P levels because of limited assay sensitivity. In our study, power to detect changes in these hormones during the reproductive cycle was presumably maximized by using a repeated-measures design. Thus, we have more precisely characterized variation in sex steroid levels during the reproductive cycle in female leopard geckos.

Incubation Temperature and Reproductive Status Effects on Behavior

Another aim of the current study was to examine whether incubation temperature and reproductive status influence the display of female receptive behavior.

We found that incubation temperature did not affect receptivity in intact, cycling females. In support of this finding and even though temperature influences other behaviors, incubation temperature does not affect receptivity in gonadectomized females treated with various sex steroids (Rhen and Crews, 2000). Reproductive status, however, had a strong effect on receptivity. Specifically, receptive behavior increased from the early to the late vitellogenic stage. We focused on these stages of the cycle because a pilot study indicated that previtellogenic and gravid females were unreceptive to males: 1 of 28 previtellogenic females was receptive and 1 of 26 gravid females was receptive. Vitellogenic females displayed a moderate level of receptivity in this preliminary study: just 13 of 33 vitellogenic females were receptive. Our current findings may explain the latter result: pooling the data from the early and late vitellogenic stages produces an overall level of receptivity of 50%, which is not significantly different from the 39% that we found when we did not distinguish different vitellogenic stages in the preliminary study.

Hormones and Behavior

When we combine these results, variation in behavior parallels variation in levels of all the steroids that we measured. Hormone levels and receptivity are generally higher during vitellogenesis: the vitellogenic stage of ovarian development in oviparous reptiles is generally homologous to the follicular phase in mammals. Nevertheless, closer examination of hormonal and behavioral changes shows a stronger association between T levels, E2 levels, and receptive behavior. Females are not sexually receptive when previtellogenic ($<5\%$ receptive), become somewhat receptive during early vitellogenesis ($\sim20\%$ receptive), are most receptive during late vitellogenesis ($\sim80\%$ receptive), and are again unreceptive after ovulation ($<5\%$ receptive). In accord with these behavioral changes, levels of T and E2 increase significantly during early vitellogenesis, peak during late vitellogenesis, and then drop to previtellogenic levels after ovulation. Moreover, we found that receptive females had higher levels of T and E2 than unreceptive females during late vitellogenesis. Despite the relationship among reproductive status, hormone levels, and behavior, temperature effects on hormones and receptive behavior were not correlated. This disparity could be due to our finding that incubation temperature-induced differences in hormone levels are small relative to the changes in hormone levels that occur during the reproductive cycle.

Other, more direct, lines of evidence indicate that these hormones regulate receptivity in female leopard geckos. Receptive behavior can be activated by either T or E2 treatment (Rhen *et al.,* 1999b; Rhen and Crews, 2000). The finding that T treatment increases receptivity without affecting circulating levels of E2 suggests that T could play a critical role in controlling female sex behavior, perhaps via aromatization to E2 within the brain. In fact, treatment with E2 also increases receptivity. However, levels of receptivity in T- or E2-treated females are lower ($\sim60\%$; Rhen *et al.,* 1999b; Rhen and Crews, 2000) than levels of receptivity in intact females during late vitellogenesis ($\sim80\%$; this study), even though circulating levels of T or E2 in hormone treated females were above the physiological levels observed during late vitellogenesis. Moreover, there were no differences between receptive and unreceptive females in T or E2 levels during early vitellogenesis, when levels of both hormones were relatively low. These results suggest that other unmeasured factors may be required for inducing receptivity in this species. Overall, these data indicate that T and E2 have some role in controlling receptive behavior in female leopard geckos. Yet, it is still unclear whether T, E2, or both hormones normally control receptivity in the female leopard gecko because levels of both T and E2 increase in a correlated manner during the reproductive cycle.

Androgen and estrogen levels also change during the reproductive cycle in other TSD reptiles. For example, T and E2 levels both increase above basal levels during vitellogenesis in the Kemp's ridley sea turtle, *Lepidochelys kempi* (Rostal *et al.,* 1998). In this species, T levels are approximately 10-fold higher than E2 levels throughout the year but increase to 20-fold above E2 levels at the time of mating. In the Galápagos tortoise, *Geochelone nigra,* there is a similar association between hormone levels and reproductive behavior: levels of T and E2 both increase during the mating season (Schramm *et al.,* 1999). In addition, the circulating levels of T are roughly 2- to 10-fold higher than E2 levels throughout the year, with the largest difference between T and E2 levels occurring in the middle of the

mating season. A substantial increase in T levels also occurs in female American alligators at the time of mating (Guillette *et al.,* 1997). In this species, however, T and E2 levels are equivalent during late vitellogenesis (i.e., when mating occurs). Levels of E2 are 2- to 4-fold higher than T levels during the remainder of the reproductive cycle in female alligators. Although different species vary in the relative concentration of T and E2 and the exact relationship of these hormones to follicular development and mating activity, these findings suggest that T and E2 may have conserved physiological roles in vitellogenesis and/or female sexual behavior in reptiles (also see discussions in Guillette *et al.,* 1997; Rostal *et al.,* 1998).

Evidence suggests that androgens may also play an important part in normal female development and reproduction in other classes of vertebrates (reviewed by Staub and De Beer, 1997). For example, it has been clearly demonstrated that T has an essential reproductive function in the female musk shrew, *Sucus murinus.* In this mammal, levels of T greatly surpass E2 levels in intact females and physiological doses of T, but not E2, induce receptivity in gonadectomized females (Rissman and Crews, 1988; Rissman and Bronson, 1987; Rissman *et al.,* 1990). However, the normally high circulating levels of T must be aromatized within the brain to trigger female sexual behavior in the musk shrew (reviewed in Freeman and Rissman, 1996).

Circulating levels of DHT and P also change with reproductive status in female leopard geckos but their temporal pattern of change does not closely match that of receptive behavior. For instance, whereas levels of DHT differ significantly between the previtellogenic and the gravid stages, there is no difference in receptivity during these reproductive stages. Conversely, DHT levels do not increase significantly from the previtellogenic to the early vitellogenic stage, but there are significant differences in receptivity during these reproductive stages. In addition, DHT treatment does not activate receptive behavior in gonadectomized females (Rhen and Crews, 2000). Although these results suggest that DHT is not involved in regulating receptive behavior, it is important to note that DHT levels peaked during late vitellogenesis and that receptive females had significantly higher levels of DHT than unreceptive females during late vitellogenesis. In contrast, P levels did not differ between receptive and unreceptive females during the early or the late vitellogenic stages. There was also disparity between P levels and behavior during the reproductive cycle which further suggests that P may not be involved in regulating receptivity in the female leopard gecko.

In summary, our results indicate that embryonic incubation temperature has persistent effects on endocrine physiology in female leopard geckos. Our results also demonstrate that hormone levels and receptive behavior change coordinately during the reproductive cycle. We found the strongest association between T levels, E2 levels, and receptivity. Consequently, these data lay the groundwork for future experiments in which sex steroid levels and sex steroid metabolism can be manipulated in a physiologically relevant manner to examine their role in regulating female receptivity.

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