0013-7227/99/$03.00/0
Endocrinology

Vol. 140, No. 10
Printed in U.S.A.

Embryonic Temperature and Gonadal Sex Organize Male-Typical Sexual and Aggressive Behavior in a Lizard with Temperature-Dependent Sex Determination*

TURK RHEN AND DAVID CREWS

Section of Integrative Biology, School of Biological Sciences, University of Texas, Austin, Texas 78712

ABSTRACT

Temperature during embryonic development determines gonadal sex in the leopard gecko, *Eublepharis macularius*. Moreover, both embryonic temperature and gonadal sex influence adult behavior. Yet it remains unclear whether the effects of embryonic temperature and gonadal sex on behavior are irreversibly organized during development. To address this question, we gonadectomized adult females and males generated from a temperature that produces mostly females (30 C) and a temperature that produces mostly males (32.5 C). Females and males from both temperatures were then treated with equivalent levels of various sex steroids. We found that both embryonic temperature and gonadal sex had persistent effects on the expression of male-typical sexual and aggressive behaviors. For example, adult females do not scent mark and display very little courtship and mounting behavior even when treated with levels of hormones (primarily androgens) that activate these behaviors in males. In contrast, species-typical aggressive displays were less sex specific and were activated by both dihydrotestosterone and testosterone (T) in males and by T in females. Nevertheless, the average duration of aggressive displays was significantly shorter in T-treated females than that in T-treated males. With regard to submissive behavior, androgens decreased flight behavior in males, but had no effect in females. Embryonic temperature had enduring effects on certain behaviors in males. For instance, males from a male-biased embryonic temperature scent-marked more than males from a female-biased embryonic temperature when treated with dihydrotestosterone or T. Conversely, and across hormone treatments, males from a female-biased embryonic temperature mounted more than males from a male-biased embryonic temperature. Finally, treatment with 17β-estradiol decreased submissive behavior in males from a male-biased embryonic temperature compared with that in males from a female-biased embryonic temperature. Courtship and aggressive behavior were not influenced by temperature. These results strongly suggest that male-typical behaviors in the adult leopard gecko are permanently organized by both embryonic temperature and gonadal sex during development. (*Endocrinology* **140:** 4501–4508, 1999)

WHEREAS SEX chromosomes determine gonadal sex in mammals and birds (1), embryonic temperature determines sex in some lizards, many turtles, and all crocodilians (2–4). Despite this dramatic difference in the signal that initiates testicular or ovarian development, it appears that much of the molecular machinery for gonadogenesis is evolutionarily conserved. Indeed, genes clearly involved in mammalian sex determination [*e.g.* anti-Müllerian hormone, steroidogenic factor-1, Wilms' Tumor (WT-1) gene, and Sry-like, High Mobility Group Box-like (SOX)9 gene] (5–7) have also been identified and implicated in avian sex determination (5, 8–10) and temperature-dependent sex determination (TSD) in reptiles (5, 11–13). Moreover, the gonadal anlagen is initially bipotential and consists of a cortical region that gives rise to the ovary and a medullary region that gives rise to the testis in all amniotic vertebrates (14). Considering such similarities in gonadal differentiation, a fundamental question is whether other aspects of sexual differentiation are also alike in mammals, birds, and reptiles with TSD.

In this respect, the sexual differentiation of reproductive and aggressive behavior is very well studied in mammals and birds and depends upon the sexually dimorphic production of steroids by the differentiated gonads. Our understanding of sex differences in these behaviors is based on the organization-activation paradigm formulated by Pheonix *et al.* (15). Classically, behavioral activation is the process by which circulating sex steroids affect specific neural substrates to induce sexual or aggressive behavior in adults that are exposed to the appropriate external stimuli (*i.e.* individuals of the opposite or same sex, respectively). For example, sex differences in the display of male-typical sex behavior occur because of sex differences in plasma testosterone (T) levels in intact rats. In fact, exogenous T can activate male-typical mounting behavior in gonadectomized male and female rats (reviewed in Refs. 16–19). In contrast, castrated male rats do not display female-typical sex behavior when treated with a sex steroid regimen [*i.e.* 17β-estradiol (E_2) followed by progesterone] that activates lordosis in ovariectomized female rats. A perinatal T surge in male, but not female, rats causes this dimorphic response to hormonal activation of female sex behavior in adulthood (reviewed in Refs. 16 and 19). Such permanent developmental effects of sex steroids on subsequent behavior are called organizational effects.

Although details vary, the basic paradigm of organizational *vs.* activational effects of sex steroids has been supported in a variety of mammals and birds. For instance, female quail are demasculinized (*i.e.* organized) by circulating estrogens during the perinatal period and lose the ability to display male-typical mounting behavior when

Received March 22, 1999.
Address all correspondence and requests for reprints to: Dr. Turk Rhen, Section of Integrative Biology, School of Biological Sciences, University of Texas, Austin, Texas 78712. E-mail: turkrhen.uta@mail.utexas.edu.
* This work was supported by Individual National Research Service Award MH-11369 from the NIMH (to T.R.), NSF Dissertation Improvement Grant IBN-9623546 (to T.R.), and NIMH Grant MH-57874 (to D.C.).

treated with T as adults (20, 21). Gonadectomized male and female quail, when treated with E_2 as adults, can display female-typical receptive behavior in response to male sexual overtures. Copulatory behavior in the zebra finch also fits this general pattern, even though its song system is paradoxical in that exogenous E_2 organizes the male phenotype (22). Thus, the sexual differentiation of reproductive behaviors in birds can be classified as activational and/or organizational in nature. In contrast, very little is known about the sexual differentiation of behavior in reptiles with TSD.

In the leopard gecko, *Eublepharis macularius*, an embryonic temperature of 30 C produces a female-biased sex ratio (approximately one male to three females), whereas 32.5 C produces a male-biased sex ratio (approximately three males to one female) (23). Furthermore, both embryonic temperature and gonadal sex influence reproductive and aggressive behavior in intact adult leopard geckos (24, 25; reviewed in Ref. 26). However, it is unclear whether these effects are organizational or activational, because embryonic temperature and gonadal sex also influence adult sex steroid physiology. For example, female leopard geckos have lower circulating levels of T and 5α-dihydrotestosterone (DHT) than males and normally do not exhibit male-typical sex behaviors (24, 25, 27, 28), yet females treated with male-typical levels of T can display male-typical courtship behavior (27). However, in this latter study, there was no quantitative comparison between levels of courtship behavior in males and females given identical hormone treatments and tested in exactly the same manner. Consequently, it is not clear whether sex differences in courtship behavior are purely activational in nature.

Similarly, males from the male-biased incubation temperature (*i.e.* 32.5 C) are more aggressive but less sexually active toward females than are males from the female-biased incubation temperature (*i.e.* 30 C) (25). Males from the male-biased incubation temperature also have lower E_2 levels than males from the female-biased incubation temperature, whereas their T levels are similar (28, 29). Overall, the combined data clearly show that sexual differentiation of behavior in the leopard gecko depends upon both gonadal sex and embryonic temperature (reviewed in Ref. 26). Nevertheless, it is uncertain whether such effects are activated or organized because there has been no systematic examination of temperature and sex effects on reproductive and aggressive behavior while controlling for circulating hormone levels.

A definitive answer to this question would provide fundamental information concerning sexual differentiation of behavior in a reptile with TSD. The following study of male-typical behaviors was designed to determine whether the sexes are behaviorally organized in the way that the mammalian and avian sexes are organized. Another goal was to determine whether embryonic temperature has permanent (*i.e.* organizational) effects on behavior within each sex. Overall, this experiment illuminates how embryonic temperature and gonadal sex during development and sex steroids in adulthood act and interact to influence sexual and aggressive behaviors in the leopard gecko.

Materials and Methods

Animals

Animals were treated according to a research protocol approved by the university's institutional animal care and use committee. Leopard gecko eggs from our captive breeding colony at the University of Texas were collected within 24 h of oviposition and candled for fertility. Fertile eggs were placed in individual cups filled with moist vermiculite (1 part water/1 part vermiculite) and split between two constant incubation temperatures (30 and 32.5 $\pm$ 0.1 C). An incubation temperature of 30 C produces a female-biased sex ratio, whereas 32.5 C produces a male-biased sex ratio (23). Geckos hatched from these eggs were raised in isolation for 49–52 weeks as previously described (25). Leopard geckos reach sexual maturity at roughly 45 weeks of age (28).

Surgical and hormonal manipulation

Approximately equal numbers of adult males and females from each incubation temperature were gonadectomized under cold anesthesia. At the same time these animals were implanted sc with SILASTIC brand tubing (Dow Corning Corp., Midland, MI) containing cholesterol (C), E_2, DHT, or T for a fully factorial experimental design, with embryonic temperature, gonadal sex (before gonad removal), and adult hormone treatment as main effects. Although sample sizes ranged from 8–15 for each group, all except 1 group had 10 or more individuals (see Table 1). Implant length was 10 mm for C, E_2, and DHT and 20 mm for T. Otherwise, implants were identical in dimension (id, 1.47 mm; od, 1.95 mm), were packed in the same manner, and were all soaked in reptilian Ringers solution for 24 h before surgery. Animals were allowed 4 weeks to recover after surgery/implantation, and then behavior was tested. One day after behavior testing was completed, a blood sample was taken via cardiac puncture for RIA to confirm hormone delivery. Animals were then killed, dissected, and examined for residual gonadal tissue. Gonadectomies were complete in all cases.

Behavior testing

We used a behavior testing procedure similar to that described by Flores *et al.* (25). Briefly, animals were tested three times for 5 min each time in a neutral cage with one of two types of stimulus animals to assess levels of male-typical and female-typical sexual and aggressive behavior (six tests total per animal). In this paper we report the results of behavior tests in which experimental animals were exposed to intact vitellogenic females (*i.e.* sexually receptive females) on 3 consecutive days; each experimental animal interacted with a given female only once. This set of tests allowed us to examine the factors controlling the display of male-typical behaviors toward female stimulus animals. Experimental animals were first placed in a neutral cage ($43 \times 22 \times 20$ cm) with a clean paper towel as a liner. Stimulus females were then placed, facing the experimental subject, in the same cage. Subject animal and stimulus female behavior was recorded using a keypad timer (Witt/Timer Program courtesy of Diane Witt, NIH, Bethesda, MD). Tests ended after 5 min or if an attack or attempted copulation occurred. In contrast to the testing protocol used previously (25), experimental animals in the current study were tested on 6 consecutive days (*vs.* over a 5-week period) and were first tested with female stimulus animals for 3 days and then with male stimulus animals for 3 days (*vs.* a randomized sequence). The latter change was made because aggressive behavior of stimulus males toward experimental animals could alter subsequent behavior and thus would have confounded our measures of male-typical and female-typical sexual behaviors.

We measured scent marking, courtship (*i.e.* tail vibrations), and mounting (*i.e.* body grips) as male-typical sex behaviors in our experimental animals. We also recorded aggressive (*i.e.* high posture display and attacks) and submissive (*i.e.* flight) behaviors. 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 (30) 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

TABLE 1. Circulating concentrations of DHT, E_2, and T (nanograms per ml plasma) in female and male leopard geckos from two incubation temperatures (30 or 32.5 C) after receiving SILASTIC implants filled with C, DHT, E_2, or T

Treatment groups			Circulating steroid levels			Sample size
Sex	Temperature (C)	Hormone	E_2	DHT	T	
Female	30	C	1.4 ± 0.3^a	0.3 ± 0.1^a	0.3 ± 0.05^a	13
		E_2	7.2 ± 2.4^b	0.3 ± 0.1^a	0.4 ± 0.1^a	10
		DHT	$2.9 \pm 0.4^{a,c}$	42.9 ± 5.3^b	1.6 ± 0.2^b	14
		T	1.7 ± 0.3^a	17.4 ± 1.6^b	172 ± 15^c	15
	32.5	C	0.8 ± 0.1^a	0.2 ± 0.05^a	0.3 ± 0.07^a	11
		E_2	$6.7 \pm 1.3^{b,c}$	0.2 ± 0.04^a	0.2 ± 0.05^a	10
		DHT	1.2 ± 0.2^a	39.6 ± 8.3^b	1.4 ± 0.3^b	10
		T	1.6 ± 0.2^a	16.7 ± 3.8^b	190 ± 23^c	10
Male	30	C	1.0 ± 0.5^a	0.4 ± 0.3^a	0.3 ± 0.05^a	10
		E_2	8.1 ± 1.7^b	0.3 ± 0.2^a	0.4 ± 0.1^a	10
		DHT	1.1 ± 0.3^a	35.0 ± 4.3^b	1.3 ± 0.2^b	10
		T	$2.4 \pm 0.3^{a,c}$	17.9 ± 4.4^b	227 ± 15^c	10
	32.5	C	1.4 ± 0.3^a	0.3 ± 0.2^a	0.4 ± 0.1^a	8
		E_2	7.8 ± 1.1^b	0.4 ± 0.2^a	0.3 ± 0.1^a	10
		DHT	$2.0 \pm 0.4^{a,c}$	44.2 ± 6.3^b	1.5 ± 0.3^b	10
		T	2.0 ± 0.2^a	14.7 ± 2.6^b	187 ± 25^c	10

Mean hormone levels are shown in nanograms per ml plasma ± 1 SE. Numbers of samples assayed are shown. For each hormone, groups with different superscripted letters are significantly different from each other using Tukey's *post-hoc* comparisons.

they position the male for copulation and nearly always accompany intromission. We measured the cumulative duration (in seconds) of scent marking, tail vibration, and mounting (*i.e.* body grip) behaviors. Overall, these behaviors are a fairly complete index of male-typical sex behavior. We also measured high posture duration (an aggressive display) and the frequency of tests in which an attack occurred as an index of aggressive behavior. Conversely, submissive behavior was recorded as the cumulative duration (in seconds) of flight from the stimulus female.

RIA

On the day after the last behavior test (with a male), 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, and plasma was stored in plastic microfuge tubes at −80 C until assayed for levels of DHT, E_2, and T. The antibodies used for RIA were DT3–351 for DHT, $E_2$6–47 for E_2, and T3–125 for T (Endocrine Sciences, Inc., Calabasas Hills, CA). Column chromatography and RIAs were performed as previously described (28). Recoveries averaged 57%, 56%, and 70% for DHT, E_2, and T, respectively. Assay sensitivity was 71 pg DHT/ml plasma, 92 pg E_2/ml plasma, and 86 pg T/ml plasma. For a pooled plasma sample, intraassay coefficients of variation were 16%, 18%, and 17% for DHT, E_2, and T, respectively. Interassay coefficients of variation for the same sample were 18%, 17%, and 13% for DHT, E_2, and T, respectively. We also ran quality control standards of known concentration in the low, medium, and high ranges of the standard curve for each steroid. For DHT, intraassay coefficients of variation were 12%, 6%, and 6% in the low, medium, and high parts of the curve, respectively. Interassay coefficients of variation for DHT were 18%, 9%, and 11% in the low, medium, and high parts of the curve, respectively. For E_2, intraassay coefficients of variation were 11%, 4%, and 6% in the low, medium, and high parts of the curve, respectively. Interassay coefficients of variation for E_2 were 10%, 8%, and 9% in the low, medium, and high parts of the curve, respectively. For T, intraassay coefficients of variation were 9%, 4%, and 5% in the low, medium, and high parts of the curve, respectively. Interassay coefficients of variation for T were 14%, 9%, and 10% in the low, medium, and high parts of the curve, respectively.

Statistical analyses

All data were analyzed using embryonic temperature, gonadal sex (before gonadectomy), adult hormone treatment, and day of testing as main effects in a four-way repeated measures design. All dependent variables, scent marking, tail vibration, body grip, high posture, and flight durations, were analyzed with univariate ANOVA. Independent variables were considered nonsignificant when $P > 0.05$. Dependent variables are presented as least squares mean ± one SE. *Post-hoc* comparisons were made using the Dunn-Sidák method to provide a significance level of $\alpha' = 1 - (1 - 0.05)^{1/k}$, where k is the number of individual comparisons for an experimentwise $\alpha = 0.05$ (31). Hormone concentrations were first log transformed and then compared using Tukey's honestly significant difference test. All statistics were performed using version 3.1 of JMP (32) for Macintosh (Apple Computer, Inc., Cupertino, CA).

Results

Hormone levels

As expected, treatment with SILASTIC capsules containing E_2, DHT, and T elevated plasma levels of these hormones above the levels observed in geckos treated with C (see Table 1). Importantly, treatment with a given steroid resulted in equivalent levels of hormones in gonadectomized female and male leopard geckos from each embryonic temperature. Consequently, our experimental manipulations achieved the desired goal, which was to separate the normally confounding effects of embryonic temperature and gonadal sex on sex steroid physiology and behavior. The steroid levels produced by these implants are in the normal physiological ranges for intact males and/or females of this species (24–28).

Scent marking behavior

Scent marking behavior was organized by embryonic temperature [$F(1,458) = 12.1$; $P = 0.0005$], gonadal sex [$F(1,458) = 24.0$; $P < 0.0001$], and a significant interaction between embryonic temperature and gonadal sex during development [$F(1,458) = 12.1$; $P = 0.0005$]. Specifically, females never scent marked regardless of their embryonic temperature (results not shown), whereas, overall, males from the male-biased temperature marked significantly more than did males from the female-biased temperature (see Fig. 1). Scent marking was activated by adult hormone treatment [$F(3,458) = 5.8$;

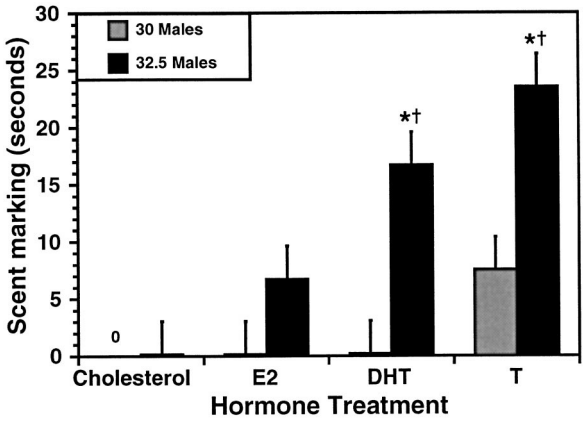

FIG. 1. Effects of embryonic temperature and adult hormone treatment on scent marking behavior of castrated male leopard geckos. Individual contrasts between groups were made to determine whether E_2-, DHT-, and T-treated males differed from C-treated males from the same incubation temperature (the *asterisk* indicates a significant difference from C-treated males from the same incubation temperature). Individual contrasts were also made between males from different embryonic temperatures within the same hormone treatment (the *cross* indicates a significant difference between temperatures). Data are presented as the least squares mean for each group ± 1 SE.

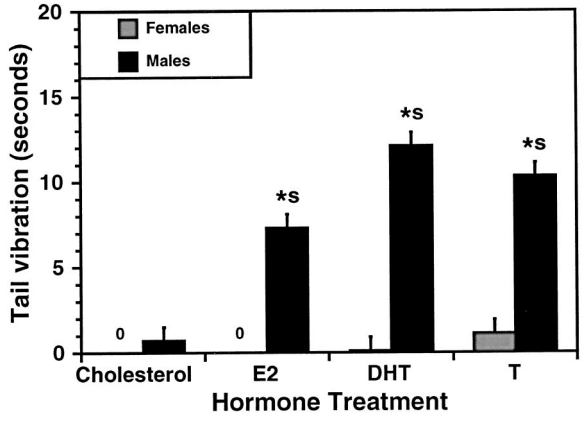

FIG. 2. Effects of gonadal sex (before gonadectomy) and adult hormone treatment on tail vibration behavior of gonadectomized female and male leopard geckos. Individual contrasts between groups were made to determine whether E_2-, DHT-, and T-treated geckos differed from C-treated geckos of the same sex (the *asterisk* indicates a significant difference from C-treated geckos of the same sex). Individual contrasts were also made between females and males within the same hormone treatment (the s indicates a significant difference between the sexes). Data are presented as the least squares mean for each group ± 1 SE.

$P = 0.0006$], but there was also a significant sex × hormone interaction [$F(3,458) = 5.8$; $P = 0.0006$]. Females never marked regardless of their hormone treatment (results not shown), whereas males marked when given sex steroids (see Fig. 1). The other independent variables (*i.e.* day of testing and its interactions, the temperature × hormone interaction, and the temperature × sex × hormone interaction) were not significant ($P > 0.05$). *Post-hoc* comparisons revealed that there were significant differences in how males from the two incubation temperatures responded to the same hormone treatments. Males from the male-biased embryonic temperature marked significantly more than males from the female-biased embryonic temperature when treated with DHT and T, but not when treated with C or E_2 (*i.e.* $\alpha' = 0.005$; see Fig. 1). Moreover, only DHT and T treatments increased marking behavior, relative to C treatment, in males from the male-biased embryonic temperature (*i.e.* $\alpha' = 0.005$; see Fig. 1).

Courtship behavior

The duration of tail vibration (male-typical courtship) behavior by experimental animals was organized by gonadal sex [$F(1,458) = 139.1$; $P < 0.0001$] and was activated by adult hormone treatment [$F(3,458) = 18.7$; $P < 0.0001$]. There was also a significant sex × hormone treatment interaction [$F(3,458) = 15.6$; $P < 0.0001$]. DHT, E_2, and T treatments all activated tail vibrations relative to C treatment in males, but did not increase this behavior in females above the levels observed in C-treated females (*i.e.* $\alpha' = 0.005$; see Fig. 2). In contrast to scent marking behavior, embryonic temperature and its interactions with other independent variables did not have any influence on courtship behavior ($P > 0.05$). Day of testing and its interactions with other independent variables did not have any influence on courtship behavior ($P > 0.05$).

Mounting behavior

The duration of body grips (a major component of male-typical mounting behavior) displayed by experimental geckos was organized by embryonic temperature [$F(1,458) = 3.9$; $P = 0.05$], gonadal sex [$F(1,458) = 25.7$; $P < 0.0001$], and a significant interaction between embryonic temperature and gonadal sex during development [$F(1,458) = 3.9$; $P = 0.05$]. Females rarely body gripped (results not shown) regardless of their embryonic temperature, whereas, overall, males from the female-biased embryonic temperature body gripped significantly more than males from the male-biased embryonic temperature (see Fig. 3). Hormone treatment [$F(3,458) = 2.5$; $P = 0.06$] and the hormone treatment × gonadal sex interaction [$F(3,458) = 2.3$; $P = 0.07$] both approached statistical significance. The other independent variables (*i.e.* day of testing and all of its interactions, the temperature × hormone interaction, and the temperature × sex × hormone interaction) were not significant ($P > 0.05$). The only significant individual *post-hoc* comparison was that between T-treated and C-treated males from 30 C (*i.e.* $\alpha' = 0.005$; see Fig. 3). In sum, males from the two embryonic temperatures responded in a similar manner to adult hormone treatment even though, across hormone treatments, males from the female-biased embryonic temperature body gripped significantly more than males from the male-biased embryonic temperature (see Fig. 3).

Aggressive behavior

Although experimental animals never attacked stimulus females (results not shown), they did display significant variation in high posture behavior (an aggressive display). The duration of high postures by experimental animals was organized by gonadal sex [$F(1,458) = 16.3$; $P < 0.0001$] and was activated by adult hormone treatment [$F(3,458) = 22.2$; $P < 0.0001$]. The other independent variables (*i.e.* day of testing

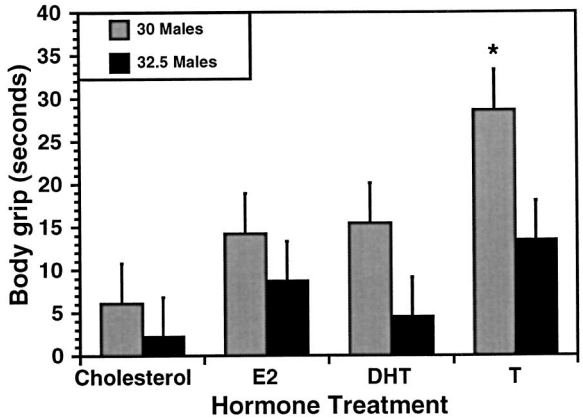

FIG. 3. Effects of embryonic temperature and adult hormone treatment on mounting (body grip) behavior of castrated male leopard geckos. Individual contrasts between groups were made to determine whether E_2-, DHT-, and T-treated males differed from C-treated males from the same incubation temperature (the *asterisk* indicates a significant difference from C-treated males from the same incubation temperature). Individual contrasts were also made between males from different embryonic temperatures within the same hormone treatment (the *cross* indicates a significant difference between temperatures). Data are presented as the least squares mean for each group ± 1 SE.

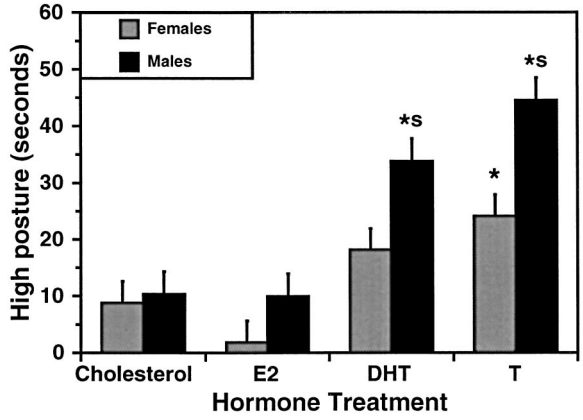

FIG. 4. Effects of gonadal sex (before gonadectomy) and adult hormone treatment on high posture behavior of gonadectomized female and male leopard geckos. Individual contrasts between groups were made to determine whether E_2-, DHT-, and T-treated geckos differed from C-treated geckos of the same sex (the *asterisk* indicates a significant difference from C-treated geckos of the same sex). Individual contrasts were also made between females and males within the same hormone treatment (the s indicates a significant difference between the sexes). Data are presented as the least squares mean for each group ± 1 SE.

and all of its interactions, embryonic temperature and all of its interactions, and the sex × hormone interaction) were not significant ($P > 0.05$). *Post-hoc* comparisons revealed that there were significant differences between males and females treated with androgens. Both DHT and T activated high posture behavior in males, whereas only T activated high posture behavior in females above the levels observed in C-treated females (*i.e.* $\alpha' = 0.005$; see Fig. 4).

Submissive behavior

Flight from female stimulus animals was organized by embryonic temperature [$F(1,458) = 11.2$; $P = 0.0009$] and gonadal sex [$F(1,458) = 6.3$; $P = 0.01$]. The temperature × sex interaction also had a marginal influence on flight behavior [$F(1,458) = 3.6$; $P = 0.06$]. Across hormone treatments, males from 32.5 C fled less than males from 30 C (see Fig. 5B), whereas there was no difference between females from different temperatures (see Fig. 5A). Hormone treatment [$F(3,458) = 5.9$; $P = 0.0006$] and the hormone treatment × gonadal sex interaction [$F(3,458) = 4.3$; $P = 0.005$] both significantly influenced flight behavior. Females did not flee very much regardless of their hormone treatment or incubation temperature; *post-hoc* comparisons revealed that there were no significant differences among different female treatment groups (*i.e.* $\alpha' = 0.005$; see Fig. 5A). In contrast, males from the male-biased temperature fled less when treated with T than when treated with C (*i.e.* $\alpha' = 0.005$; see Fig. 5B). Males from the female-biased temperature fled significantly less when treated with DHT than when treated with C (*i.e.* $\alpha' = 0.005$; see Fig. 5B). Finally, males from the male-biased embryonic temperature fled less than males from the female-biased embryonic temperature when treated with E_2 (*i.e.* $\alpha' = 0.005$; see Fig. 5B). The other independent variables (*i.e.* day of testing and all of its interactions, the temperature × hormone interaction, and the temperature × sex × hormone interaction) were not significant ($P > 0.05$).

Discussion

In this report we summarize results from an extensive study of sexual and aggressive behaviors in adult leopard geckos of both sexes from two embryonic temperatures. Our primary findings were that embryonic temperature and gonadal sex during development permanently influenced subsequent behavior. In addition, hormone treatments in adulthood had activational effects on behavior. Although these general results are robust, an important caveat concerning the following discussion is that our interpretations of the data are based on selected pairwise comparisons, and a few of these comparisons are not entirely concordant with the relevant ANOVA statistics (we point out these disparities).

Regarding sex effects, ovariectomized females displayed no scent marking behavior and very little tail vibration behavior even when treated with hormones that activated these behaviors in castrated males. In contrast to the strong hormonal activation of scent marking and tail vibrations, sex steroids did not activate mounting behavior in males. Nonetheless, there was a clear sex difference in mounting behavior (*i.e.* experimental females rarely mounted stimulus females). We also found that both gonadal sex and hormone treatment affected aggressive displays (high posture behavior). Moreover, based on our pairwise comparisons, it appeared that hormonal activation of aggressive behavior differed between the sexes. Specifically, DHT activated aggressive displays in males but not in females, whereas T significantly activated aggressive displays in both sexes. Yet the ANOVA did not indicate an interaction between gonadal sex and hormone treatment, perhaps because there was also a sex difference in aggressive displays between males and females treated with T. Finally, gonadal sex modified the hormonal control of submissive behavior. Regardless of hormone treatment, fe-

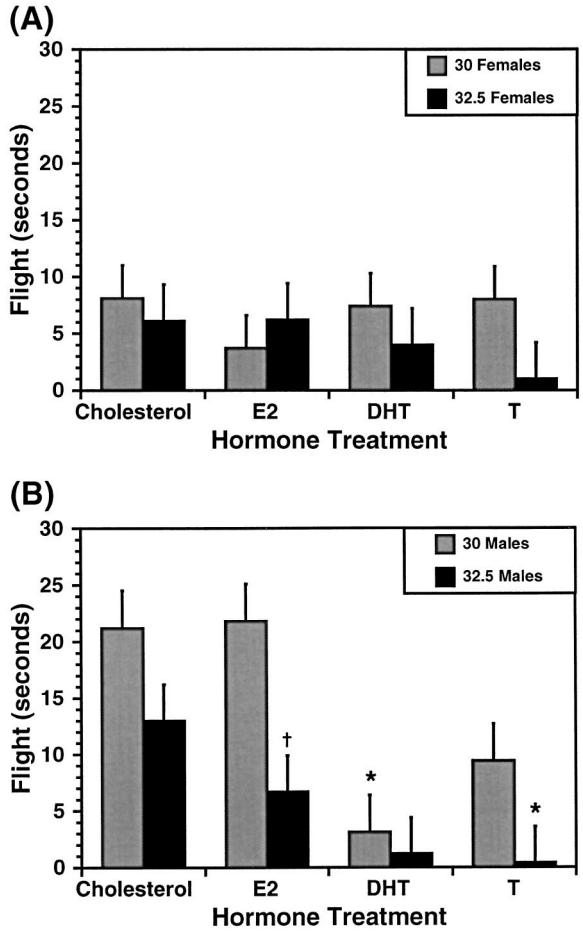

FIG. 5. Effects of embryonic temperature and adult hormone treatment on submissive behavior of ovariectomized female (A) and castrated male (B) leopard geckos. Individual contrasts between groups were made to determine whether E_2-, DHT-, and T-treated females (males) differed from C-treated females (males) from the same incubation temperature (the *asterisk* indicates a significant difference from C-treated females (males) from the same incubation temperature). Individual contrasts were also made between females (males) from different embryonic temperatures within the same hormone treatment (the *cross* indicates a significant difference between temperatures within a sex). Data are presented as the least squares mean for each group ± 1 SE.

males rarely fled, whereas males fled more when treated with C than when treated with androgens. As we made direct comparisons between the sexes under exactly the same experimental conditions (*e.g.* endocrine states and behavior testing protocol), these data strongly imply that male-typical behaviors are permanently organized by gonadal sex during development. Thus, sexual differentiation of reproductive and aggressive behavior in the leopard gecko, a reptile with TSD, occurs in a manner that is generally analogous to sexual differentiation in birds and mammals.

In accord with the hypothesis that steroid-dependent organization of behavior occurs in leopard geckos, we have found that males have significantly higher levels of DHT and T than females throughout postnatal development (33). This observation suggests that androgens may masculinize (*i.e.* organize) the neural substrates controlling sexual and aggressive behavior in the leopard gecko, much like certain behaviors are masculinized in mammals (reviewed in Refs. 16–19). However, a definitive test of this hypothesis will require the experimental manipulation of sex steroid levels in both males and females during ontogeny and the determination of behavioral responsiveness to adult hormone treatments as performed in the present experiment. Even if the developmental mechanism causing sexual organization proves to be evolutionarily conserved among TSD reptiles, birds, and mammals, a more intriguing problem will be to determine how embryonic temperature permanently influences behavioral differentiation.

In this study, we found that scent marking behavior was affected by an interaction between gonadal sex and embryonic incubation temperature. Females did not scent mark, whereas males from the male-biased embryonic temperature marked significantly more than males from the female-biased embryonic temperature. Pairwise comparisons revealed that scent marking behavior was activated by DHT and T treatments in males from the male-biased temperature, but not in males from the female-biased temperature. Despite this apparent difference in the hormonal activation of scent marking behavior between males from different temperatures, the ANOVA did not indicate such an interaction. Conversely and across hormone treatments, males from the female-biased embryonic temperature mounted (*i.e.* body gripped) significantly more than males from the male-biased embryonic temperature. Overall, embryonic temperature also strongly influenced submissive behavior, such that males from the male-biased temperature fled less than males from the female-biased temperature, whereas there was no difference between females from different temperatures. Notwithstanding the lack of a significant interaction between hormone treatment and incubation temperature for submissive behavior, the only significant pairwise comparison was that between E_2-treated males from the male-biased embryonic temperature *vs.* males from the female-biased embryonic temperature. In comparison, embryonic temperature did not affect the expression of courtship (tail vibration) or aggressive (high posture) behavior. In sum, these results clearly show that embryonic temperature has permanent developmental effects on a number of sexual and aggressive behaviors in male leopard geckos. Moreover, the pattern of these effects is the same as that reported previously in intact animals (25).

Like sex differences in behavior, temperature-induced behavioral variation may be mediated by sex steroids. Indeed, embryonic temperature effects in the leopard gecko have been compared with intrauterine position effects on behavior in rodents (25, 25, 29, 34). Although outwardly distinct, there are a number of underlying similarities between these phenomenon. First of all, TSD in reptiles and intrauterine position effects in mammals both involve exposure to sex steroids during critical periods of development. In rodents, the position of the fetus relative to that of same or opposite sex siblings *in utero* influences its exposure to androgens. Specifically, fetuses located between two males are exposed to higher androgen levels than are fetuses located between two females. Ultimately, intrauterine position, via its effect on androgen exposure, influences an entire suite of morphological, physiological, and behavioral traits, so that individ-

uals located between two males *in utero* are more masculinized as adults than individuals located between two females (reviewed in Ref. 35).

In TSD species, considerable evidence indicates that temperature determines gonadal sex by influencing sex steroid metabolism during embryonic development (36, 37). In a simplified model, the expression of aromatase enzyme is regulated in a time- and temperature-dependent manner. Aromatase then converts endogenous androgens into estrogens, which induce ovarian differentiation. As sex determination is a threshold trait, individuals with estrogen levels below a certain threshold develop as males, whereas individuals with estrogen levels above the threshold develop as females. A direct prediction of this model is that individuals of a given sex from different temperatures are exposed to different hormonal milieus during embryonic development. Consequently, temperature-induced variation in hormone levels could have pleiotropic effects on sex determination, brain phenotype, and behavior. Although we do not currently know how (or if) temperature influences prenatal hormone levels in the leopard gecko, differentiation of the diencephalon in lizards and turtles occurs at the same time as the temperature-sensitive period for gonadal differentiation (38–40).

In contrast to the hypothesis that temperature is transduced into a physiological signal (*i.e.* sex steroids) that influences sexual differentiation, there is also some support for an alternative hypothesis, namely that temperature has direct effects on neural and behavioral differentiation. In fact, female leopard geckos from E_2-treated eggs incubated at the male-biased temperature (32.5 C) do not differ in growth rates or aggressiveness from unmanipulated females incubated at the same temperature (41). Perhaps the strongest evidence that the embryonic temperature effects on postnatal physiology and behavior are not mediated by sex steroids comes from a series of experiments on the common snapping turtle, another reptile with TSD (42–46). In these experiments, eggs were incubated at three temperatures, two that produce only males and a third that produces a female-biased sex ratio. Eggs were then treated during the temperature-sensitive period with E_2, a potent aromatase inhibitor, or a vehicle control or were not treated. Whereas gonadal sex was reversed by hormonal manipulations, it was found that neither hormone treatment nor gonadal sex influenced hatchling size, residual energy stores, posthatching growth rate, or thermoregulatory behavior. Nonetheless, embryonic temperature had very strong effects on these traits. In sum, these studies suggest that temperature may directly influence neuroendocrine and behavioral differentiation in TSD reptiles. In accord with the hypothesis that temperature has direct effects on neural and behavioral differentiation, there are temperature-sensitive neurons within the anterior hypothalamus and preoptic area (AH-POA) in both mammals and reptiles (47–49).

Our finding that embryonic temperature and gonadal sex interact to influence certain hormone-dependent behaviors in adulthood (*i.e.* scent marking, mounting, and submissive behaviors) further implies that temperature and sex steroids act upon a common neural substrate during development. In fact, the AH-POA controls hormone-dependent, male-typical sex behavior in all amniotic vertebrates studied to date (50). Furthermore, this area is the most likely place for the integration of temperature and steroid effects, as there are distinct populations of temperature- and steroid-sensitive neurons within the AH-POA in the rat and presumably in the leopard gecko (51). Nonetheless, it remains to be determined exactly how, on a mechanistic level, embryonic temperature and gonadal sex act and interact to organize subsequent reproductive and aggressive behaviors in leopard geckos.

In conclusion, we have demonstrated that sex differences in the display of male-typical behaviors in the adult leopard gecko are due to both organizational effects of gonadal sex during development and sex differences in the circulating levels of sex steroids (*i.e.* activational effects of androgens) in adulthood. Moreover, embryonic temperature appears to modulate the differentiation of sexual and aggressive behavior, such that males from different temperatures are differentially sensitive to the same hormone treatments in adulthood. Although the latter conclusion is based on selected pairwise comparisons that are not entirely concordant with the relevant ANOVA statistics, the overall effects of temperature on male-typical behavior are persistent and strong. We are currently investigating neuroendocrine correlates of these behavioral differences by determining the effects of gonadal sex, embryonic temperature, and adult hormone treatment on androgen receptor, estrogen receptor, and aromatase gene expression in the brains of the geckos from the present experiment. In addition, we are characterizing gonadal sex and embryonic temperature effects on endocrine and neuroendocrine development to determine the developmental mechanisms responsible for behavioral organization. Such information will provide the basis for manipulations of sex steroid levels during ontogeny and for the interpretation of resultant effects on neuroendocrine and behavioral phenotype. Overall, the current experiment suggests that sexual differentiation of reproductive and aggressive behavior is at least in part evolutionarily conserved among amniotic vertebrates with different modes of sex determination (*i.e.* dependent upon the sexually dimorphic production of sex steroids by the differentiated gonads). However, our results also suggest that there may be unique mechanisms of sexual differentiation in TSD reptiles (*i.e.* embryonic temperature effects on neural and behavioral differentiation may be direct or may be mediated by sex steroids).

Acknowledgments

We thank Mark Zeller for assistance with RIAs, and Jon Sakata for critical reading of the manuscript. Comments from three anonymous reviewers significantly improved the manuscript.

References

1. **Bull JJ** 1983 Evolution of Sex Determining Mechanisms. Benjamin-Cummings, Menlo Park
2. **Ewert MA, Jackson D, Nelson C** 1994 Patterns of temperature-dependent sex determination in turtles. J Exp Zool 270:3–15
3. **Lang JW, Andrews HV** 1994 Temperature-dependent sex determination in crocodilians. J Exp Zool 270:28–44
4. **Viets B, Ewert MA, Talent LG, Nelson CE** 1994 Sex determining mechanisms in squamate reptiles. J Exp Zool 270:45–56
5. **Di Clemente N, Ghaffari S, Pepinsky RB, Pieau C, Josso N, Cate RL, Vigier**

B 1992 A quantitative and interspecific test for biological activity of anti-Mullerian hormone: the fetal aromatase assay. Development 114:721–727
6. **Lou X, Ikeda Y, Parker KL** 1994 A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. Cell 77:481–490
7. **Nachtigal MA, Hirokawa Y, Enyeart-VanHouten DL, Flanagan JN, Hammer GD, Ingraham HA** 1998 Wilms' tumor 1 and Dax-1 modulate the orphan nuclear receptor SF-1 in sex-specific gene expression. Cell 93:445–454
8. **Da Silva SM, Hacker A, Harley V, Goodfellow P, Swain A, Lovell Badge R** 1996 Sox9 expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. Nat Genet 14:62–68
9. **Kent J, Wheatley SC, Andrews JE, Sinclair AH, Koopman P** 1996 A male-specific role for SOX9 in vertebrate sex determination. Development 122:2813–2822
10. **Smith CA, Smith MJ, Sinclair AH** 1999 Expression of chicken steroidogenic factor-1 during gonadal sex differentiation. Gen Comp Endocrinol 113:187–196
11. **Spotila LD, Spotila JR, Hall SE** 1998 Sequence and expression analysis of WT1 and Sox9 in the red-eared slider turtle, *Trachemys scripta*. J Exp Zool 281:417–427
12. **Wibbels T, Cowan J, LeBoeuf R** 1998 Temperature-dependent sex determination in the red-eared slider turtle, *Trachemys scripta*. J Exp Zool 281:409–416
13. **Fleming A, Crews D,** Developmental expression of steroidogenic factor-1 in the red-eared slider, a species with temperature-dependent sex determination. Gen Comp Endocrinol, in press
14. **Witshi E** 1959 Age of sex determining mechanisms in vertebrates. Science 130:829–846
15. **Pheonix CH, Goy RW, Gerell AA, Young WC** 1959 Organizing action of prenatally administered testosterone proprionate on the tissues mediating behavior in the female guinea pig. Endocrinology 65:369–382.
16. **Goy R, McEwen BS** 1980 Sexual Differentiation of the Brain. MIT Press, Cambridge
17. **Sachs BD, Meisel RL** 1988 The physiology of male sexual behavior. In: Knobil E, Neill JD (eds) The Physiology of Reproduction. Raven Press, New York, vol 2:1393–1482
18. **Meisel RL, Sachs BD** 1994 The physiology of male sexual behavior. In: Knobil E, Neill JD (eds) The Physiology of Reproduction, ed 2 Raven Press, New York, vol 2:3–105
19. **Gerrell AA, Molz H, Ward IL** 1992 Handbook of Behavioral Neurobiology. Plenum Press, New York, vol 11
20. **Adkins EK** 1975 Hormonal basis of sexual differentiation in the Japanese quail. J Comp Physiol Psych 89:61–71
21. **Balthazart J, Ball GF** 1995 Sexual differentiation of brain and behavior in birds. Trends Endocrinol Metab 6:21–29
22. **Arnold AP, Schlinger BA** 1993 Sexual differentiation of brain and behavior: the zebra finch is not just a flying rat. Brain Behav Evol 42:231–241
23. **Viets BE, Tousignant A, Ewert MA, Nelson CE, Crews D** 1993 Temperature-dependent sex determination in the leopard gecko, *Eublepharis macularius*. J Exp Zool 265:679–683
24. **Gutzke WHN, Crews D** 1988 Embryonic temperature determines adult sexuality in a reptile. Nature 332:832–834
25. **Flores D, Tousignant A, Crews D** 1994 Incubation temperature affects the behavior of adult leopard geckos (*Eublepharis macularius*). Physiol Behav 55:1067–1072
26. **Crews D, Sakata J, Rhen T** 1998 Developmental effects on intersexual and intrasexual variation in growth and reproduction in a lizard with temperature-dependent sex determination. J Comp Biochem Physiol C 119:229–241
27. **Flores D, Crews D** 1995 Effect of hormonal manipulation on sociosexual behavior in adult female leopard geckos (*Eublepharis macularius*), a species with temperature-dependent sex determination. Horm Behav 29:458–473
28. **Tousignant A, Viets B, Flores D, Crews D** 1995 Ontogenetic and social factors affect the endocrinology and timing of reproduction in the female leopard gecko (*Eublepharis macularius*). Horm Behav 29:141–153
29. **Coomber P, Gonzalez-Lima F, Crews D** 1997 Effects of incubation temperature and gonadal sex on the morphology and metabolic capacity of brain nuclei in the leopard gecko (*Eublepharis macularius*), a lizard with temperature-dependent sex determination. J Comp Neurol 380:409–421
30. **Mason RT, Gutzke WHN** 1990 Sex recognition in the leopard gecko, *Eublepharis macularius* (Sauria: Gekkonidae) possible mediation by skin-derived semiochemicals. J Chem Ecol 16:27–36
31. **Sokal RR, Rohlf FJ** 1981 Biometry, ed 2. Freeman, New York
32. **SAS Institute** 1995 JMP User's Guide, version 3.1. SAS Institute, Cary, NC
33. **Sakata JT, Rhen T, Crews D,** Ontogeny of secondary sex structures and gonadal steroids in the leopard gecko. Annual Meeting of the Society for Integrative and Comparative Biology, Denver, CO, 1998, Am Zool, vol 38, no. 5, p 86A (Abstract 297)
34. **Crews D, Coomber P, Baldwin R, Azad N, Gonzalez-Lima F** 1996 Effects of gonadectomy and hormone treatment on the morphology and metabolic capacity of brain nuclei in the leopard gecko (*Eublepharis macularius*) a lizard with temperature-dependent sex determination. Horm Behav 30:474–486
35. **Clark, MM, Galef BG** 1995 Prenatal influences on reproductive life history strategies. Trends Ecol Evol 10:151–153
36. **Crews D** 1996 Temperature-dependent sex determination: the interplay of steroid hormones and temperature. Zool Sci 13:1–13
37. **Pieau C** 1996 Temperature variation and sex determination in reptiles. BioEssays 18:19–26
38. **Senn DG** 1979 Embryonic development of the central nervous system. In: Gans C, Northcutt RG, Ulinski P (eds) Biology of the Reptilia: Neurology A. Academic Press, London, pp 173–244
39. **Pearson AK, Licht P** 1974 Embryology and cytodifferentiation of the pituitary gland in the lizard *Anolis carolinensis*. J Morphol 144:85–118
40. **Pearson AK, Licht P** 1982 Morphology and immunocytochemistry of the turtle pituitary gland with special reference to the pars tuberalis. Cell Tissue Res 222:81–100
41. **Tousignant A, Crews D** 1995 Incubation temperature and gonadal sex affect growth and physiology in the leopard gecko (*Eublepharis macularius*), a lizard with temperature-dependent sex determination. J Morphol 224:159–170
42. **Rhen T, Lang JW** 1994 Temperature-dependent sex determination in the snapping turtle: manipulation of the embryonic sex steroid environment. Gen Comp Endocrinol 96:243–254
43. **Rhen T, Lang JW** 1995 Phenotypic plasticity for growth in the common snapping turtle: effects of incubation temperature, clutch, and their interaction. Am Nat 146:726–747
44. **Rhen T, Elf PK, Fivizzani AJ, Lang JW** 1996 Sex-reversed and normal turtles display similar sex steroid profiles. J Exp Zool 274:221–226
45. **Rhen T, Lang JW** 1999a Embryonic and juvenile temperature independently influence growth in hatchling snapping turtles, *Chelydra serpentina*. J Therm Biol 24:33–41
46. **Rhen T, Lang JW** 1999 Incubation temperature and sex affect mass and energy reserves of hatchling snapping turtles (*Chelydra serpentina*). Oikos, 86:311–319
47. **Cabanac M, Hammel HT, Hardy JD** 1967 *Tiliqua scincoides*. Temperature sensitive units in lizard brain. Science 158:1050–1051
48. **Rodbard S, Sampson F, Ferguson D** 1950 Thermosensitivity of the turtle brain as manifested by blood pressure changes. Am J Physiol 160:402–407
49. **Heath JE, Gasdorf E, Northcutt RG** 1968 The effect of thermal stimulation of anterior hypothalamus on blood pressure in the turtle. Comp Biochem Physiol 26:509–518
50. **Crews D, Silver R** 1985 Reproductive physiology and behavior interactions in non-mammalian vertebrates. In: Adler NT, Pfaff DW, Goy RW (eds) Handbook of Behavioral Neurobiology: Reproduction. Plenum Press, New York, vol 7:101–182
51. **Silva NL, Boulant JA** 1986 Effects of testosterone, estradiol, and temperature on neurons in preoptic tissue slices. Am J Physiol 250:R625–R632