Neuro
-endocrinology

Organization and Activation of Sexual and Agonistic Behavior in the Leopard Gecko, *Eublepharis macularius*

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Abstract

Gonadal sex is determined by the temperature experienced during incubation in the leopard gecko *(Eublepharis macularius)*. Furthermore, both factors, incubation temperature and gonadal sex, influence adult sexual and agonistic behavior in this species. Yet it is unclear whether such differences in behavior are irreversibly organized during development or are mediated by differences in hormone levels in adulthood. To address this question, we gonadectomized adult females and males generated from a female-biased (30°C) and a male-biased (32.5°C) incubation temperature and treated them with equivalent levels of various sex steroids. We found that 17β-estradiol (E_2) activated sexual receptivity in females but not males, suggesting an organized sex difference in behavioral sensitivity to E_2. There were also organized and activated sex differences in attractivity to stimulus males. Although females were more attractive than males when treated with E_2, both sexes were equally unattractive when treated with dihydrotestosterone (DHT) or testosterone (T). Likewise, sex differences in aggressive and submissive behavior were organized and activated. Attacks on stimulus males were activated by T in males but not in females. In contrast, hormones did not influence flight behavior in males but did affect female submissiveness. Overall, males also evoked more attacks by stimulus males than did females. Nevertheless, females and males treated with androgens evoked more attacks than animals of the same sex that were treated with cholesterol or E_2. Incubation temperature had some weak effects on certain behaviors and no effect on others. This suggests that temperature effects in gonadally intact geckos may be due primarily to differences in circulating levels of hormones in adulthood. We conclude that gonadal sex has both organizational and activational effects on various behaviors in the leopard gecko.

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Introduction

Expression of sex-typical reproductive and agonistic behaviors in certain vertebrates depends upon an interaction between the brain and the endocrine system during early development and/or in adulthood [reviewed in 1–6]. For example, in mice, testosterone (T) secreted from the testes during the perinatal period directs the differentiation of the neural substrates that control steroid-dependent behaviors in adults. Specifically, male mice castrated as adults do not display female-typical sex behavior even when treated with a sex steroid regimen (i.e., 17β-estradiol (E_2) followed by progesterone (P)) that activates lor-

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dosis in ovariectomized females. Female mice treated with exogenous T shortly after birth are also behaviorally insensitive to the effects of sequential E_2 and P treatment in adulthood. Conversely, male mice castrated as neonates and given the same E_2 and P treatment as adults show full lordosis like normal females. Therefore, exposure to T during early development 'defeminizes' the mouse brain and its potential for the regulation of female sex behavior. Such permanent developmental effects of sex steroids on ensuing behavior are referred to as organizational effects and have been demonstrated for various behaviors in both birds and mammals. The more transient and reversible effects of hormones on behavior in adults, called activational effects, have also been documented in these two well-studied groups.

In contrast, much less is known about sexual differentiation of the brain and behavior in other vertebrate classes. Fundamental information about sex differences in groups like reptiles, which occupy a pivotal place in the vertebrate lineage, will help us better understand the evolution of unique and shared mechanisms of neuroendocrine development and control in all vertebrates.

In the leopard gecko, *Eublepharis macularius*, we have a particularly interesting organism for studying the developmental and endocrine factors that act and interact to influence the expression of sex-typical behavior. Embryonic incubation temperature determines gonadal sex in this reptile [7], just as it does in some other lizards, many turtles, and all crocodilians [8–10]. Moreover, both embryonic temperature and gonadal sex influence adult sex steroid physiology and sociosexual behavior in the leopard gecko [11, 12, reviewed in 13]. For example, female leopard geckos from an intermediate temperature that produces a male-biased sex ratio are less attractive to males than are females from temperatures that produce female-biased sex ratios [12]. Hormone levels in adults vary in accord with embryonic temperature so that less attractive, intermediate temperature females have higher androgen levels than more attractive females from other temperatures [reviewed in 13]. Adult male leopard geckos have higher androgen levels than adult females and are unattractive to and elicit fierce aggression from other males. To date, however, there has been no systematic examination of incubation temperature or gonadal sex effects on behavior while rigorously controlling for circulating hormone levels, either during development or in adulthood.

Data in the leopard gecko and other TSD reptiles is consistent with the hypothesis that incubation temperature and gonadal sex may have organizational effects on behavior as well as effects that are mediated by differences in hormone levels in adults. Studies in TSD reptiles strongly suggest that incubation temperature determines gonadal sex by influencing sex steroid metabolism during embryonic development [14, 15]. Thus, temperature-induced variation in hormone production could also have an organizational effect on neural and behavioral differentiation [13]. Differences in gonadal sex, in turn, lead to developmental differences in sex steroid levels in the leopard gecko [16], which could result in organizational actions in the developing brain. Yet, as indicated in the preceding paragraph, there are also temperature and sex differences in hormone levels in adulthood in the leopard gecko. An alternative to this hypothesis is that incubation temperature may have more direct effects on neural and behavioral differentiation. In fact, there is evidence that sex steroids in the common snapping turtle, another TSD reptile, do not mediate incubation temperature effects on certain aspects of postnatal physiology and behavior [reviewed in 17]. Hormonal manipulations of snapping turtle embryos produce females at temperatures that normally produce males (and vice versa), but do not alter temperature effects on a number of other traits. Thus, in the leopard gecko, it is unclear whether temperature and sex differences in behavior are mediated by differences in hormone levels in adults, are organized by differences in hormone levels during development, or whether the putative organizational effects of temperature are direct or indirect (i.e., mediated by sex steroids).

We recently began to address these questions and found that embryonic temperature and gonadal sex during development have irreversible effects on the expression of male-typical behaviors elicited by female stimulus animals [18]. Differences in behavior in males and females from different temperatures are not solely dependent upon differences in levels of sex steroids in adults. In short, we found that ovariectomized females do not scent mark and display very little male-typical courtship (i.e., tail vibration) and mounting behavior even when treated with levels of hormones (primarily androgens) that activate these behaviors in castrated males. In addition, embryonic temperature has enduring effects on certain behaviors in castrated males even after circulating hormone levels were equalized. Males from a temperature that produces a male-biased sex ratio scent marked more than males from a female-biased temperature when treated with the same levels of T or dihydrotestosterone (DHT). Conversely, and across hormone treatments, males from a female-biased temperature mount stimulus females more than do males from a male-biased tempera-

ture. There were also differences in submissiveness (i.e., flight from the stimulus female) in animals from different incubation temperatures. In contrast, tail vibration (i.e., courtship) behavior is not influenced by incubation temperature. These results indicate that embryonic temperature and/or gonadal sex during development organize male-typical sociosexual behaviors and that sex steroids activate these behaviors in adulthood.

Here we test the hypotheses that female-typical sexual behaviors (i.e., receptivity and attractivity) and aggressive/submissive behaviors are similarly activated by circulating sex steroids and/or organized by embryonic temperature and gonadal sex during development. To explore these questions, we used the same set of experimental animals that were used earlier [18]. In the current report, however, stimulus animals were sexually experienced males rather than sexually receptive females. Since male and female stimulus animals provide entirely different social contexts for the display of behavior, we are reporting related but completely novel data. In short, certain behaviors are only observed in specific social contexts. Receptivity, for example, is a response to mounting attempts by a stimulus male. In contrast, stimulus females never mount experimental animals.

Methods

Animals

Animals were treated according to a research protocol approved by the Institutional Animal Care and Use Committee at the University of Texas. Leopard gecko eggs from our captive-breeding colony were collected 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°C, ±1°C). An incubation temperature of 30°C produces a female-biased sex ratio (~1 male:3 females) whereas 32.5°C produces a male-biased sex ratio (~3 males:1 female) [7]. Geckos hatched from these eggs were raised in isolation for 49–52 weeks as previously described to avoid the development of dominance/subordinance relationships that are common to group rearing [12]. Leopard geckos reach sexual maturity at roughly 45 weeks of age [19].

Surgical and Hormonal Manipulation

Approximately equal numbers of adult males and females from each incubation temperature were gonadectomized while under cold anesthesia. At the same time these animals were implanted subcutaneously with Silastic tubing containing cholesterol (C), E_2, DHT, or T for a fully factorial experimental design with embryonic temperature, gonadal sex, and adult hormone treatment as main effects. Animals were allowed 4 weeks to recover after surgery/implantation and then behavior tested. After behavior testing was completed, a blood sample was taken for radioimmunoassay to confirm hormone delivery. The procedures and data for the RIAs have been described in detail previously [18]. Briefly, 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. The steroid levels produced by these implants were also in the normal physiological ranges for intact males and/or females. More 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 adult sex steroid physiology and behavior.

Behavior Testing

Briefly, experimental geckos were tested for 5 min on each of 6 consecutive days. On days 1–3, behavior tests utilized sexually receptive females [see 18]. Here we report the results of behavior tests with gonadally intact, sexually active males on days 4–6. Stimulus males were larger and older than our experimental animals and were experienced breeders. Each experimental animal interacted with a given stimulus male only once. This set of tests allowed us to examine the factors controlling the display of female-typical sexual behaviors like attractivity and receptivity as well as agonistic interactions between the experimental animals and stimulus males.

Experimental animals were first placed into a neutral cage (43 × 22 × 20 cm) with a clean paper towel as a liner. Stimulus animals were then placed, facing the experimental subject, into the same cage. Subject and stimulus animal behavior was recorded using a keypad timer (Witt/Timer Program courtesy of Diane Witt, NIH, Bethesda, Md., USA). Tests ended after 5 min or if an attack or attempted copulation occurred. Although this behavior testing procedure is similar to that described in Flores et al. [12], 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 (versus 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- and female-typical sexual behaviors and agonistic behaviors.

Female-Typical Sexual Behavior

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 [20] 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 position the male for copulation and nearly always accompany intromission. We measured the cumulative duration (in seconds) of scent-marking and tail-vibration behavior and frequency of tests in which a body grip (i.e., mounting) occurred. Overall, these behaviors of stimulus males are a good index of female-typical sexual attractivity.

We also recorded female-typical receptivity to body grips by stimulus males. Receptivity was recorded as a composite index where experimental animals were considered receptive if they either displayed a tail lift (exposing the cloaca for intromission) or remained immobile when gripped by a male. Experimental animals were considered unreceptive if they did not tail lift and fled or attacked when gripped by the stimulus male.

Aggressive and Submissive Behavior

Intermale aggression is extreme in the leopard gecko. In an encounter with a conspecific, males will often raise their body off the ground, standing on all four limbs in a high posture display. Intact females do not exhibit this behavior as much as males do. Male-typical offensive aggression entails biting which often includes rapid body rolls. Such attacks, if not stopped at once, can lead to severe skin lacerations, loss of limbs, or the loss of a tail. In contrast, females are not aggressive toward each other or toward males and do not normally elicit offensive attacks from males. Consequently, we measured high posture duration and the frequency of tests in which an attack occurred as indices of aggressive behavior in both the experimental and stimulus animals. Conversely, submissive behavior of the experimental and stimulus animals was recorded as the duration of flight behavior.

Statistical Analyses

All data were analyzed using incubation temperature, sex, hormone treatment, and day of testing as main effects in a four-way repeated measures design. Since some behavior tests were stopped early, either due to an attack or attempted copulation, we used analysis of covariance (ANCOVA) with total test time as the covariate. In addition, stimulus male identity was a blocking factor because each male was used in multiple behavior tests. Otherwise, the analysis of continuous (i.e., scent marking, tail vibration, high posture, and flight durations) and binary (i.e., body gripped or not, receptive or unreceptive, attacked or not) dependent variables were conducted as described previously [18]. Independent variables were considered nonsignificant when $p > 0.05$. Dependent variables are pesented as least squares means ± 1 SE or as percentages. 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 = the number of individual comparisons for an experimentwise $\alpha = 0.05$ [21]. All statistics were done using Version 3.2 of JMP [22] for Apple Macintosh.

Results

Female-Typical Sexual Behavior

Receptivity. Adult hormone treatment influenced the experimental animals' receptivity to body grips by stimulus males ($LR\chi^2$ (3 d.f.) = 36.6, $p < 0.001$); only geckos treated with E_2 were receptive to mounting attempts. In addition, there was a sex difference in receptivity to body grips ($LR\chi^2$ (1 d.f.) = 8.2, $p = 0.004$). Whereas E_2-treated females were receptive in 17 out of 30 tests (57%) in which they were body gripped, males treated with E_2 were receptive in only 1 out of 12 tests (8%) in which they were body gripped. Incubation temperature, day of testing, and their interactions with other independent variables did not have any effect on receptivity ($p > 0.05$).

Attractivity. Tail-vibration (courtship) duration by stimulus males toward experimental animals was influenced by the experimental animals' sex ($F(1, 470) = 13.5$, $p < 0.001$), hormone treatment ($F(3, 470) = 14.7$, $p <$ 0.001), and a significant sex by hormone treatment interaction ($F(3, 470) = 5.2$, $p = 0.002$). There was a significant difference between experimental males and females treated with E_2, but not between males and females treated with C, DHT, or T (i.e., $\alpha' = 0.005$; see fig. 1A). We also found that DHT- and T-treated females were courted significantly less than C-treated females (i.e., $\alpha' = 0.005$; see fig. 1A). In contrast, DHT, E_2, and T treatments had no detectable effect relative to C treatment in males (i.e., $\alpha' = 0.005$; see fig. 1A). Incubation temperature, day of testing, and their interactions with other independent variables did not have any effect on tail-vibration behavior directed toward the experimental animals ($p > 0.05$). Finally, the duration of tail vibration increased with total test time, the covariate (results not shown) ($F(1, 470) = 5.0$, $p = 0.03$).

The frequency of tests in which the stimulus male body gripped (i.e., mounted) the experimental geckos was influenced by the experimental animals' sex (likelihood ratio chi-square, $LR\chi^2$ (1 d.f.) = 13.1, $p < 0.001$) and hormone treatment ($LR\chi^2$ (3 d.f.) = 51.9, $p < 0.001$). There was a significant difference between males and females treated with E_2, but not between males and females treated with C, DHT, or T (i.e., $\alpha' = 0.003$; see fig. 1B). We also found that females and males treated with DHT and T were body gripped significantly less than C-treated geckos of the same sex (i.e., $\alpha' = 0.003$; see fig. 1B). In contrast, there were no differences between E_2- and C-treated females (or males). A horomone treatment by day of testing interaction ($LR\chi^2$ (6 d.f.) = 16.3, $p = 0.01$) was caused by significant hormone effects on days 4 and 5 and no detectable hormone effect on day 6 (i.e., $\alpha' = 0.003$). Nevertheless, the general pattern of hormone effects was similar on all 3 days (results not shown). Although we also detected a marginal incubation temperature by hormone treatment interaction ($LR\chi^2$ (3 d.f.) = 8.5, $p = 0.04$), none of the individual comparisons between temperatures within hormone treatments supported this interaction (i.e., $\alpha' > 0.003$; results not shown). Other independent variables in the fully factorial model did not have any influence on the frequency of tests in which the stimulus male body gripped the experimental geckos ($p > 0.05$).
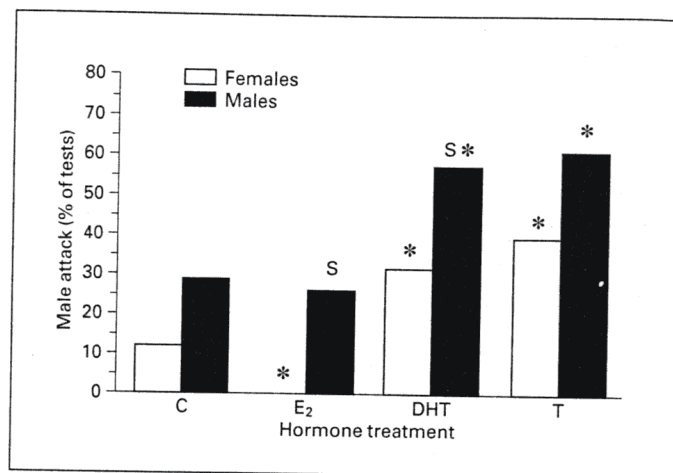
Scent marking by stimulus males in response to experimental animals was not influenced by any independent variable, i.e., sex, incubation temperature, hormone treatment, day of testing or their interactions ($p > 0.05$). However, the duration of scent marking increased with total test time, the covariate (results not shown) ($F(1, 470) = 46$, $p < 0.001$).
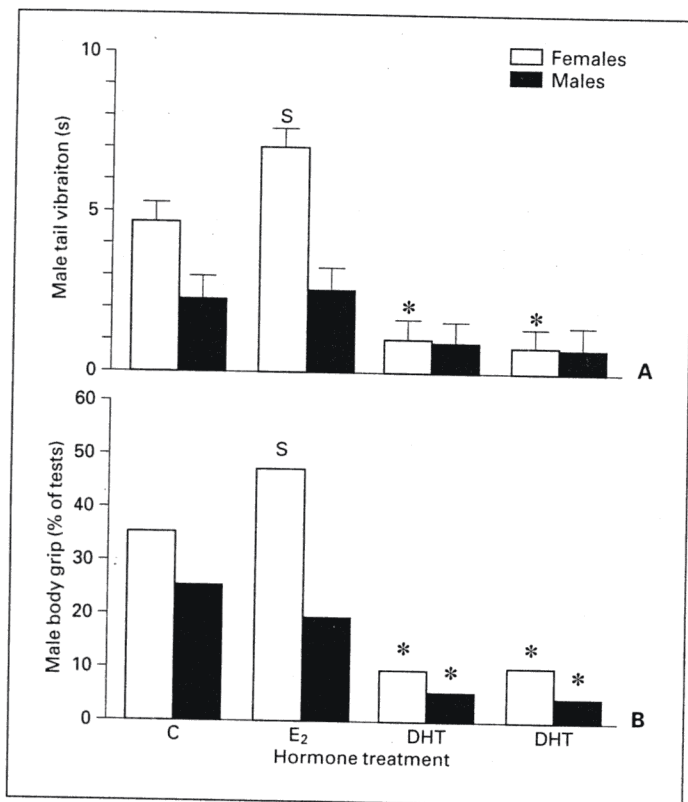
Fig. 1. Effects of adult hormone treatment on the attractivity of gonadectomized female and male leopard geckos. Attractivity was measured (**A**) as the duration of tail vibrations displayed by the stimulus male and (**B**) as the frequency of behavior tests in which the stimulus male body gripped (i.e., mounted) the experimental gecko. Individual contrasts between groups were made to determine if E_2-, DHT-, and T-treated geckos differed from C-treated geckos of the same sex (* indicates a significant difference from C-treated geckos of the same sex). Contrasts were also made between females and males within the same hormone treatment (S indicates a significant difference between the sexes).


Fig. 2. Effects of sex and adult hormone treatment on the frequency of behavior tests in which the stimulus male attacked the experimental geckos. Individual contrasts between groups were done as described in figure 1 (* indicates a significant difference between E_2-, DHT-, or T-treated geckos and C-treated geckos of the same sex; S indicates a significant difference between the sexes within a hormone treatment).

Aggressive/Submissive Behavior

Stimulus Males. The frequency of tests in which the stimulus male attacked experimental geckos was strongly influenced by the experimental animals' sex ($LR\chi^2$ (1 d.f.) = 51.1, p < 0.001) and hormone treatment ($LR\chi^2$ (3 d.f.) = 81.4, p < 0.001). Although males were attacked more than females across hormone treatments, animals of both sexes were attacked more when treated with androgens than when treated with cholesterol (see fig. 2). There was also a significant sex by hormone treatment interaction ($LR\chi^2$ (3 d.f.) = 11.3, p = 0.01). Males were attacked more than females when treated with DHT or E_2, but there was no statistically significant sex difference when geckos were treated with C or T (i.e., $\alpha' = 0.005$; see fig. 2). In addition,

females treated with DHT or T were attacked significantly more than females treated with C, yet females treated with E_2 were attacked less than females treated with C (i.e., $\alpha' = 0.005$; see fig. 2). Incubation temperature, day of testing, and their interactions with other independent variables did not influence the frequency of tests in which stimulus males attacked the experimental geckos (p > 0.05).

The stimulus males' high posture (i.e., thought to be an aggressive display) and flight (i.e., submissive) behavior did not vary according to day of testing or the experimental geckos' incubation temperature, sex, or adult hormone treatment (p > 0.05; results not shown). However, high posture duration of stimulus males increased with total test time, the covariate (results not shown) ($F(1,470)$ = 9.9, p = 0.002).
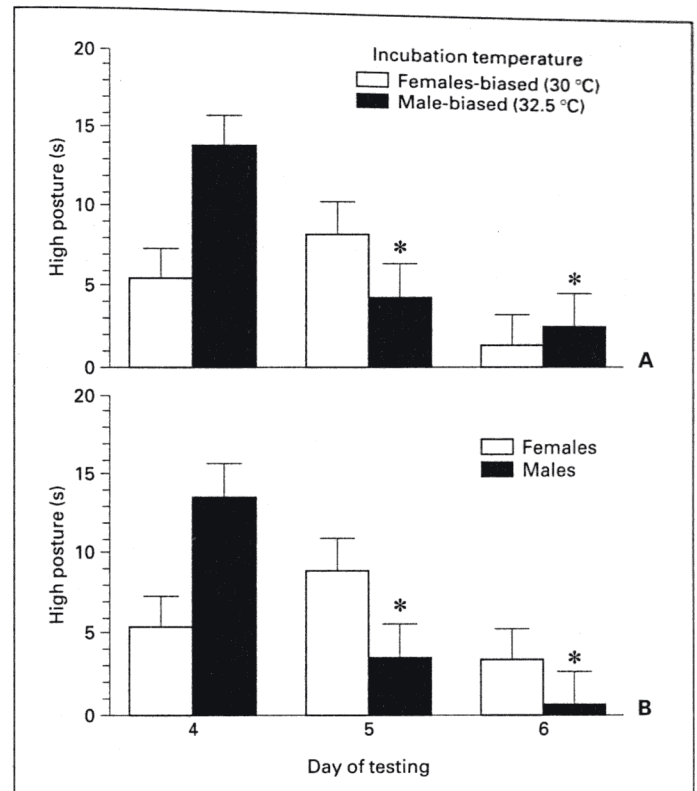
Experimental Animals

The high posture display of the experimental geckos toward stimulus males was influenced by an interaction between sex and hormone treatment ($F(3,470) = 4.2$, p = 0.006). Females displayed high postures more than males when treated with C, but there were no sex differences when geckos were treated with DHT, E_2, or T (i.e., $\alpha' = 0.002$; see fig. 3). In addition, females treated with C exhibited more high postures than females treated with T
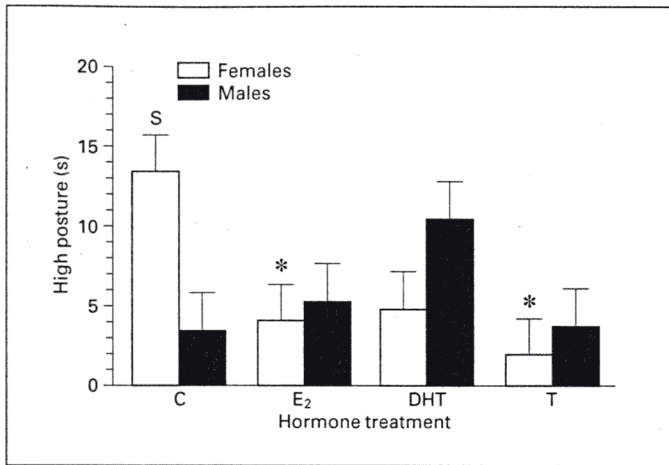
Fig. 3. The effect of adult hormone treatment on aggressive displays by gonadectomized female and male leopard geckos. Aggression was measured as the cumulative duration of high postures exhibited by the experimental geckos. Individual contrasts between groups were done as described in figure 1 (* indicates a significant difference between E_2-, DHT-, or T-treated geckos and C-treated geckos of the same sex; S indicates a significant difference between the sexes within a hormone treatment).


Fig. 4. Effects of (**A**) incubation temperature and day of testing on aggressive displays by leopard geckos (* indicates a significant difference between days 5 and 6 vs. day 4 in geckos from the same temperature). Effects of (**B**) sex and day of testing on aggressive displays by leopard geckos (* indicates a significant difference between days 5 and 6 vs. day 4 in geckos from the same sex). Aggression was measured as the cumulative duration of high postures displayed by the experimental geckos.

(i.e., $\alpha' = 0.002$; see fig. 3). Females treated with C also tended to high posture more than E_2-treated females (p = 0.006). In contrast, there were no significant differences in high posture behavior between males treated with C vs. males treated with DHT, E_2, or T. Day of testing had a significant effect on high posture behavior (F(2, 470) = 6.9, p = 0.001). Across experimental groups, geckos showed high postures more on day 4 than on day 6. However, there were also significant interactions between day of testing and incubation temperature (F(2, 470) = 4.5, p = 0.01) and day of testing and sex (F(2, 470) = 6.3, p = 0.002). Whereas geckos from 32.5 °C high postured significantly more on day 4 than on days 5 or 6, geckos from 30 °C displayed no differences in high posture behavior among the 3 days of testing (i.e., $\alpha' = 0.002$; fig. 4A). In a similar way, males displayed high postures significantly more on day 4 than on days 5 or 6 while females exhibited no differences in high posture behavior among the 3 days of testing (i.e., $\alpha' = 0.002$; fig. 4B). No other independent variables nor total test time had any effect on high posture behavior by the experimental geckos (p > 0.05).

Like high posture behavior, attacks by experimental geckos were influenced by the day of testing (LRχ^2 (2 d.f.) = 12.2, p = 0.002). The frequency of offensive attacks by experimental geckos decreased on each successive day of testing. There were 14 attacks out of 137 tests on day 4, 8

attacks out of 135 tests on day 5, and 1 attack out of 143 tests on day 6; note that the number of tests for this data is lower than the total number of tests in the experiment. We excluded data in which the experimental animal was rejecting a body grip by attacking a stimulus male (i.e., unreceptive behavior). The sex by hormone treatment interaction also affected the frequency of attacks by experimental geckos (LRχ^2 (3 d.f.) = 10.7, p = 0.01). Males treated with T attacked stimulus males more than females treated with T, but there were no differences between males and females treated with C, DHT or E_2 (i.e., $\alpha' = 0.005$; results not shown). In addition, males treated with T attacked stimulus males more often than males treated with C (i.e., $\alpha' = 0.005$; results not shown). In contrast, there were no differences between males treated with DHT or E_2 vs. males treated with C. Likewise, females treated with DHT, E_2, or T were not different than
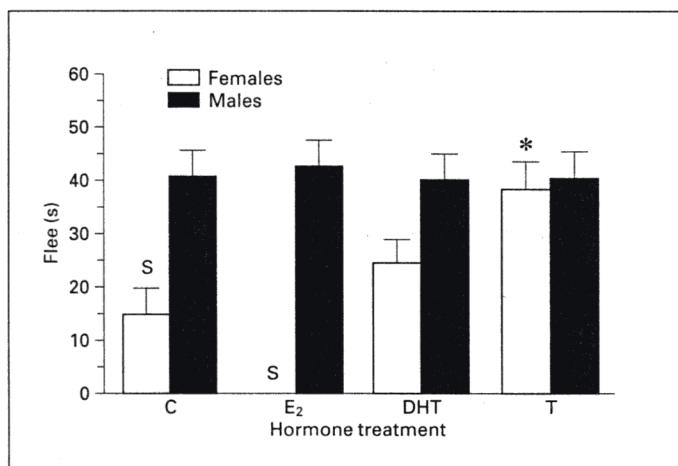
Fig. 5. Effects of adult hormone treatment on submissive (flight) behavior of gonadectomized female and male leopard geckos. Individual contrasts between groups were done as described in figure 1 (* indicates a significant difference between E_2-, DHT-, or T-treated geckos and control C-treated geckos of the same sex; S indicates a significant difference between the sexes within a hormone treatment).

females treated with C (i.e., $\alpha' = 0.005$; results not shown). Incubation temperature and its interactions with other independent variables did not have any influence on the frequency of offensive attacks by the experimental animals ($p > 0.05$).

Submissive behavior (i.e., flight from stimulus males) by experimental animals was influenced by sex ($F(1, 367) = 38.9$, $p < 0.001$). The effects of hormone treatment ($F(3, 367) = 4.4$, $p = 0.005$) and the sex by hormone treatment interaction were also significant ($F(3, 367) = 7.0$, $p < 0.001$). As in the previous analysis of offensive attacks, we excluded tests in which the experimental animal was rejecting a body grip by fleeing from a stimulus male (i.e., unreceptive behavior). Hormone treatments did not affect flight behavior in males, but did in females (i.e., $\alpha' = 0.005$; see fig. 5). Although there were no differences in flight behavior between male and female geckos treated with DHT or T, females treated with C or E_2 fled less than males treated with C or E_2, respectively (i.e., $\alpha' = 0.005$; see fig. 5). While there were no detectable differences between DHT- and E_2-treated females vs. C-treated females, females treated with T fled significantly more than C-treated females (i.e., $\alpha' = 0.005$; see fig. 5). An interaction between incubation temperature and sex also influenced flight behavior ($F(1, 367) = 4.8$, $p = 0.03$). Across hormone treatments, there was a marginal temperature effect on females: females from 30°C tended to flee less

than females from 32.5°C, while there was no difference between males from different temperatures. Flight duration increased with total test time, the covariate (results not shown) ($F(1, 367) = 43.4$, $p < 0.001$). Day of testing and its interactions with other independent variables did not have any influence on submissive behavior in the experimental geckos ($p > 0.05$).

Discussion

In this report, we examined whether incubation temperature and sex effects on behavior in the leopard gecko [11–13] were permanently organized during development or simply due to temperature and sex differences in circulating levels of sex steroids in intact adults. Since our experimental manipulations produced equivalent levels of hormones in gonadectomized female and male leopard geckos from each embryonic temperature, we effectively separated organizational vs. activational effects of temperature and sex on behavior. Our primary finding was that gonadal sex during development influenced subsequent behavior even when castrated males and ovariectomized females were treated with the same level of hormones and exposed to the same stimulus males. These results strongly suggest that the behaviors we studied are permanently organized during development by gonadal sex. Separate activational effects of adult hormone treatments on behavior further bolster this conclusion. Although incubation temperature also had persistent effects on certain agonistic behaviors, these effects were weak compared to sex differences in behavior. This suggests that temperature-induced variation in traits like attractivity of intact females [11–13] is not firmly organized by embryonic temperature but is primarily mediated by differences in circulating levels of sex steroids in adults.

Female-Typical Sexual Behavior

The organizational effect of gonadal sex on female-typical sexual behavior in the leopard gecko was strikingly similar to that described for mice (see Introduction) and certain other mammals [reviewed in 1, 23]. Ovariectomized geckos treated with E_2 displayed female-typical receptivity whereas castrated geckos were unreceptive even when treated with the same level of E_2 and mounted by the same stimulus males. Since intact males have higher levels of DHT and T than females throughout postnatal development [16], we surmise that androgens (or estrogenic metabolites) defeminize the neural substrate controlling receptivity in the leopard gecko. However, a

definitive test of this hypothesis will require the experimental manipulation of androgen levels in both males and females during ontogeny and the determination of behavioral responsiveness to adult E_2 treatment.

Attractivity of leopard geckos was also activated by adult hormone treatments and organized by sex during development. The sexes responded differently when treated with the same dose of E_2 such that females were courted and mounted more than males. An analogous sex difference in E_2-induced attractivity was observed in adult red-sided garter snakes, but not in newborns [24]. In contrast to the sexually differentiated response to E_2 treatment, androgen treatments in leopard geckos had a comparable effect on attractivity in both sexes: both males and females were courted and/or mounted less when treated with DHT or T than when treated with C. Such steroid effects on attractivity are probably mediated by altered levels of sex-specific pheromones that elicit male sex behavior [20, 25, 26]. Although it is unclear exactly how these effects are mediated, we envisage at least two ways in which estrogens and androgens could have their antagonistic effects on attractivity. In the first scenario, androgens and estrogens might have opposing effects on the expression of the same putative attractivity pheromone: androgens could decrease levels of an attractivity pheromone in both sexes while estrogens increase its expression, but only in females. A second possibility is that there are distinct androgen- and estrogen-dependent pheromones. In this case, an androgen-dependent, male-typical pheromone would inhibit sex behavior in stimulus males while an estrogen-dependent, female-typical pheromone would elicit male sex behavior. Considering that multiple skin-derived compounds are differentially expressed between male and female leopard geckos [20], these two hypotheses are not mutually exclusive. Consequently, an interesting prospect is that levels of different pheromones are regulated by sex steroids in different ways (see further discussion of pheromone effects on aggression below).

Regardless of how sex and circulating hormones influence attractivity, any temperature-induced differences in attractivity of intact females (see Introduction) are most likely activational sex steroid effects. In fact, plasma levels of DHT and T in adulthood are influenced by a female's prior incubation temperature even when strictly controlling for changes in hormone levels during the reproductive cycle [27]. Moreover, administration of exogenous T reduces female attractivity in a dose-dependent manner [28]. A precise test of the hypothesis that temperature effects on female attractivity are caused by physiological variation in steroid levels will require more subtle hormonal manipulations because levels of androgens in our DHT- and T-treated females were in the male-typical range.

Aggressive/Submissive Behavior

Treatment with male-typical levels of androgens not only reduced the attractivity of experimental geckos but also evoked offensive aggression from stimulus males. Sex and hormone effects on the frequency of attacks by stimulus males were almost a mirror image of their effects on attractivity. Females were attacked less (and courted/mounted more) than males when treated with the same dose of E_2. Androgens had similar effects on males and females: both sexes were attacked more (and courted/mounted less) when treated with DHT or T compared to when they were treated with C. Yet there is some evidence for independent effects of androgens on putative attractivity pheromones that release male sex behavior and male-typical pheromones that release aggression. In a previous study we compared the behavioral effects of long- vs. short-term T treatment [28]. Both long- and short-term treatment of females with male-typical levels of T decrease attractivity, just as long-term treatment did in the current study. However, females treated with T for a short duration (i.e., less than 8 days) were not attacked by stimulus males. This indicates that androgens have differential effects on the expression of distinct pheromones that evoke sexual vs. aggressive behavior in males. Our current findings of an organized sex difference in attractivity in response to E_2 treatment and purely activational effects of androgen treatment on attractivity further suggest that a suite of sex-specific pheromones are involved in sexual attraction and sex recognition and that these pheromones are regulated in different ways during development and in adulthood. Despite these inferences, additional experiments are required to demonstrate that sex-specific skin chemicals act as behavioral releasers and that their expression is truly under hormonal control [20, 25, 26, 29, 30].

Gonadal sex and adult hormone treatment not only affected how experimental animals were perceived by conspecific males, but also had strong effects on their own aggressive behavior. The propensity for experimental males to attack the stimulus male was increased by T treatment. Moreover, females treated with T did not attack as frequently as males did, which suggests an organized sex difference in T-dependent aggression. Interestingly, this pattern of organization and activation of offensive aggression is remarkably similar to that described for mice: male mice are generally more sensitive to the activa-

tional effects of T on aggression than are females and, like sexual behavior, this sex difference is organized by early exposure to T [reviewed in 6].

High posture behavior was also organized by sex and activated by adult hormone treatment in the leopard gecko. Females treated with C displayed more high postures than males treated with C. High postures were significantly inhibited by T in females and tended to be repressed in E_2-treated females. In contrast, hormones did not significantly alter high posture behavior in males. Although both overt attacks and high posture displays decreased on successive days of testing, the decline in aggressive displays also varied with the gonadal sex and incubation temperature of the gecko (see discussion of temperature effects below). Overall, these results imply that incubation temperature during early development modifies subsequent aggressive behavior and that gonadal sex during development also permanently alters the way hormones regulate aggressiveness in adulthood.

Submissive behavior was also organized and activated differently in males and females. Although T-treated males initiated offensive attacks significantly more than C-treated males, males from all hormone treatment groups were equally submissive to stimulus males. In contrast, submissive behavior in females was influenced by hormone treatments. Females fled as much as males when treated with DHT or T, but fled significantly less than males when treated with C or E_2. Since flight behavior was correlated with how frequently females, but not males, were attacked, it is possible that females base their submissiveness on the aggressiveness of a stimulus male, whereas males develop a relatively inflexible response to conspecific males that depends upon other cues. In support of the hypothesis that males base their submissiveness on different social cues (i.e., pheromones and relative body size), males fled more from stimulus males in the current study than they did from stimulus females in the previous study [18].

Considering the asymmetry that we observed in overt attacks, it may also be that stimulus males were perceived as more aggressive because they were more likely to initiate agonistic encounters. Neither male attacked in slightly over half of the tests, only the stimulus male attacked in approximately one third of the tests, only the experimental male attacked in <1% of the tests, and both males attacked each other in 7% of the tests. Indeed, even though behavior tests were conducted in a neutral arena, stimulus males may have been more motivated to fight because they had been continuously housed with females whereas experimental males had relatively limited sexual

experience, namely three behavior tests with stimulus females [18]. It is likely that such experience increases aggressiveness in male leopard geckos just as it does in male mammals [reviewed in 31]. Alternatively, stimulus males may have been perceived by experimental males as more apt to win a fight because they were larger. Staged encounters between gonadally intact males with extensive sexual experience indicate that larger males always win fights [J.T. Sakata, D. Crews, pers. commun.]. Thus relative body size may provide an unambiguous signal of 'resource holding potential' [sensu 32]. In any event, the neural tissues that mediate social submission also appear to be organized and activated differently in males and females.

Incubation Temperature Effects

Temperature had a significant effect on aggressive displays (i.e., high posture behavior) by the experimental animals. Aggressive displays in animals from the male-biased temperature closely paralleled the aggressive displays by males. This suggests, perhaps, that the male-biased temperature has a masculinizing effect on aggressive behavior that is independent of gonadal sex, much like intrauterine position influences aggression in both sexes in mice [33]. Incubation temperature also influenced flight behavior in females, but not males. Females from the female-biased temperature fled less than females from the male-biased temperature. These organized temperature effects, however, were not very strong when compared to the observed sex differences in behavior.

Conclusions

In sum, we have demonstrated sex differences in the display of female-typical sexual behaviors and agonistic behaviors in adult leopard geckos exposed to stimulus males. Some sex differences were due to organizational effects during development, others were due to sex differences in the circulating levels of hormones in adulthood (i.e., activational effects), and still others were both organizational and activational in nature. In conjunction with our earlier study [18], the current results indicate that sexual differentiation of behavior is, at least in part, evolutionarily conserved among amniotic vertebrates (i.e., reptiles, birds, and mammals). In other words, sexual differentiation of brain and behavior depends upon the sexually dimorphic production of steroids by the gonads during development and in adulthood. Yet our results also suggest that there are some unique mechanisms of neuroendocrine development in the leopard gecko.

Embryonic incubation temperature appears to have organizational influences on neural and behavioral differentiation as well as effects that are mediated by differences in circulating levels of sex steroids. In the current study, incubation temperature had persistent effects on aggressive displays and submissive behavior in response to stimulus males. Temperature effects on these behaviors were relatively weak, however, and other behaviors like receptivity and attractivity were not affected by the temperature experienced during incubation, suggesting that much of the temperature effect on these behaviors may be due to differences in circulating levels of sex steroids in adulthood. In a previous study with female stimulus animals, incubation temperature had much stronger organizational effects on male-typical scent-marking, mounting, and submissive behaviors [18]. We have proposed that incubation temperature may influence sex steroid metab-olism during embryogenesis and thereby have pleiotropic developmental effects on sex determination, body and brain phenotype, and behavior. Alternatively, temperature may have direct effects on neural and behavioral differentation. While there is some support for both mechanisms, how incubation temperature exerts its persistent effects on subsequent behavior in the leopard gecko is currently unknown [also see discussion in 18].

References

1 Goy R, McEwen BS: Sexual Differentiation of the Brain. Cambridge, MIT Press, 1980.
2 Meisel RL, Sachs BD: The physiology of male sexual behavior; in Knobil E, Neil JD (eds): The physiology of Reproduction, ed 2. New York, Raven Press, 1994, vol 2. pp 3–105.
3 Balthazart J, Ball GF: Sexual differentiation of brain and behavior in birds. Trends Endocrinol Metab 1995;6:21–29.
4 Adkins-Regan E: Hormonal mechanisms of mate choice (abstract). Am Zool 1996;36:88.
5 Kendrick AM, Schlinger BA: Independent differentiation of sexual and social traits. Horm Behav 1996;30:600–610.
6 Simon NG, McKenna Se, Lu SF, Cologer-Clifford A: Development and expression of hormonal systems regulating aggression. Ann NY Acad Sci 1996;794:8–17.
7 Viets BE, Tousignant A, Ewert MA, Nelson CE, Crews D: Temperature-dependent sex determination in the leopard gecko, *Eublepharis macularius*. J Exp Zool 1993;265:679–683.
8 Ewert MA, Jackson D, Nelson C: Patterns of temperature-dependent sex determination in turtles. J Exp Zool 1994;270:3–15.
9 Lang JW, Andrews HV: Temperature-dependent sex determination in crocodilians. J Exp Zool 1994;270:28–44.
10 Viets B, Ewert MA, Talent LG, Nelson CE: Sex determining mechanisms in squamate reptiles. J Exp Zool 1994;270:45–56.
11 Gutzke WHN, Crews D: Embryonic temperature determines adult sexuality in a reptile. Nature 1988;332:832–834.
12 Flores D, Tousignant A, Crews D: Incubation temperature affects the behavior of adult leopard geckos *(Eublepharis macularius)*. Physiol Behav 1994;55:1067–1072.

13 Crews D, Sakata J, Rhen T: Developmental effects on intersexual and intrasexual variation in growth and reproduction in a lizard with temperature-dependent sex determination. Comp Biochem Physiol C 1998;119:229–241.
14 Crews D: Temperature-dependent sex determination: The interplay of steroid hormones and temperature. Zool Sci 1996;13:1–13.
15 Pieau C: Temperature variation and sex determination in reptiles. Bioessays 1996;18:19–26.
16 Sakata JT, Rhen T, Crews D: Ontogeny of secondary sex structures and gonadal steroids in the leopard gecko (abstract). Am Zool 1998;38:86.
17 Rhen T, Crews D: Sex and the evolution of gender; in Fox CW, Roff DA, Fairbairn DJ (eds): Evolutionary Ecology: Perspectives and Synthesis. Oxford, Oxford University Press, 2000.
18 Rhen T, Crews D: Embryonic temperature and gonadal sex organize male-typical sexual and aggressive behavior in a lizard with temperature-dependent sex determination. Endocrinology 1999;140:4501–4508.
19 Tousignant A, Viets B, Flores D, Crews D: Ontogenetic and social factors affect the endocrinology and timing of reproduction in the female leopard gecko *(Eublepharis macularius)*. Horm Behav 1995;29:141–153.
20 Mason RT, Gutzke WHN: Sex recognition in the leopard gecko, *Eublepharis macularius* (Sauria: Gekkonidae): Possible mediation by skin-derived semiochemicals. J Chem Ecol 1990;16:27–36.
21 Sokal RR, Rohlf FJ: Biometry, ed 2. New York, Freeman, 1981.
22 SAS Institute: JMP User's Guide, Vers 3.2. Cary, SAS Institute Inc, 1997.
23 Gerrell AA, Molz H, Ward IL: Sexual differentiation; in Handbook of Behavioral Neurobiology. New York, Plenum Press, 1992, vol 11.

24 Crews D: Effects of early sex steroid hormone treatment on courtship behavior and sexual attractivity in the red-sided garter snake, *Thamnophis sirtalis parietalis*. Physiol Behav 1985;35:569–575.
25 Cooper WE Jr, Steele LJ: Pheromonal discrimination of sex by male and female leopard geckos *(Eublepharis macularius)*. J Chem Ecol 1997;23:2967–2977.
26 Steele LJ, Cooper WE Jr: Investigations of pheromonal discrimination between conspecific individuals by male and female leopard geckos *(Eublepharis macularius)*. Herpetologica 1997;53:475–484.
27 Rhen T, Sakata JT, Crews D: The reproductive cycle of female leopard geckos (abstract). Am Zool 1998;38:88.
28 Rhen T, Ross J, Crews D: Effects of testosterone on sexual behavior and morphology in adult female leopard geckos, *Eublepharis macularius*. Horm Behav 1999;36:119–128.
29 Wilson EO, Bossert WH: Chemical communication among animals. Recent Prog Horm Res 1963;19:673–716.
30 Schwenk K: Of tongues and noses: Chemoreception in lizards and snakes. Trends Ecol Evol 1995;10:7–12.
31 Albert DJ, Jonik RH, Walsh ML: Hormone-dependent aggression in male and female rats: Experiential, hormonal, and neural foundations. Neursci Biobehav Rev 1992;16:177–192.
32 Harper DGC: Communication; in Krebs JR, Davies NB (eds): Behavioral Ecology. Oxford, Blackwall Scientific, 1991, pp 374–397.
33 Palanza P, Parmigiani S, vom Saal FS: Urine marking and maternal aggression of wild female mice in relation to anogenital distance at birth. Physiol Behav 1995;58:827–835.