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Brain Organization in a Reptile Lacking Sex Chromosomes: Effects of Gonadectomy and Exogenous Testosterone

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In mammals, males and females differ both genetically and hormonally, making it difficult to assess the relative contributions of genetic constitution and fetal environment in the process of sexual differentiation. Many reptiles lack sex chromosomes, relying instead on the temperature of incubation to determine sex. In the leopard gecko (*Eublepharis macularius*), an incubation temperature of 26°C produces all females, whereas 32.5°C results in mostly males. Incubation temperature is the primary determinant of differences both within and between the sexes in growth, physiology, and sociosexual behavior, as well as the volume and metabolic capacity of specific brain nuclei. To determine if incubation temperature organizes the brain directly rather than via gonadal sex hormones, the gonads of male and female leopard geckos from the two incubation temperatures were removed and, in some instances, animals were given exogenous testosterone. In vertebrates with sex chromosomes, the size of sexually dimorphic nuclei are sensitive to hormone levels in adulthood, but in all species studied to date, these changes are restricted to the male. Therefore, after behavior tests, morphometrics of certain limbic and nonlimbic brain areas were determined. Because nervous system tissue depends on oxidative metabolism for energy production and the level of cytochrome oxidase activity is coupled to the functional level of neuronal activity, cytochrome oxidase histochemistry also was performed on the same brains. Hormonal manipulation had little effect on the volume of the preoptic area or ventromedial hypothalamus in geckos from the all-female incubation temperature, but significantly influenced the volumes of these brain areas in males and females from the male-biased incubation temperature. A similar relationship was found for cytochrome oxidase activity of the anterior hypothalamus, amygdala, dorsal ventricular ridge, and septum. The only sex difference observed was found in the ventromedial hypothalamus; males showed no significant changes in cytochrome oxidase activity with hormonal manipulation, but females from both incubation temperatures were affected similarly. The results indicate that incubation temperature organizes the brain directly rather than via hormones arising from its sex-determining function. This is the first demonstration in a vertebrate that factors other than steroid hormones can modify the organization and functional activity of sexually differentiated brain areas.

In species with genetic sex determination (male or female heterogamety), the sexes differ in two fundamental ways, namely in genetic constitution and in the nature and pattern of sex steroid hormone secretion. Regarding the former, the genetic basis for male-typical sexual behavior is distinct and separate from that for female-typical sexual behavior (Goy and Jakway, 1962) and genetic mechanisms of sex determination may also influence the brain directly (Arnold, Wade, Grisham, Jacobs, and Campagnoni, 1996). Regarding the latter, gonadal steroid hormones are important in brain differentiation during development and can, in the adult, alter the structure and neurochemical physiology of specific brain regions (reviewed in Arnold and Gorski, 1984). Because males and females differ genetically, and hence hormonally, the relative contribution of the embryonic environment cannot be distinguished easily from the genetic environment. Thus, the extent to which individual and sexually dimorphic traits can be sepa-

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rated from genetic sex and its associated hormones is problematic.

Factors independent of sex chromosomes may play important roles in the differentiation of the neural mechanisms underlying individual and sex differences in aggressive and sexual behavior. One source is the physical environment experienced during development. In the leopard gecko (*Eublepharis macularius*), gonadal sex is determined by the temperature of the incubating egg, not by sex chromosomes (Viets, Tousignant, Ewert, Nelson, and Crews, 1993). Incubation of eggs at 26°C produces only female hatchlings, 30°C produces a female-biased sex ratio (75:25), and 32.5°C produces a male-biased (25:75) sex ratio. Further, the temperature an individual experiences during incubation influences the frequency of aggressive and sexual behaviors in adulthood, accounting for much of the variation among individuals within a sex (Gutzke and Crews, 1988; Flores and Crews, 1995; Flores, Tousignant, and Crews, 1994). Not only does the behavior of adults vary according to their incubation temperature, but the behavioral sensitivity to hormone manipulation also differs in individuals from different incubation temperatures (Flores and Crews, 1995). Finally, incubation temperature, rather than gonadal sex hormones, is the primary organizer of the volume and metabolic capacity of brain nuclei that, in other vertebrates, are involved in adult aggressive and sexual behavior (Coomber, Gonzalez-Lima, and Crews, 1996).

If brain organization in the leopard gecko is largely dependent upon incubation temperature, and is independent of gonadal sex and its associated hormones, then several predictions can be made regarding the effects of gonadectomy and hormone treatment during adulthood. One set of predictions is related to the response to gonadectomy between and within the sexes. Hypothesis 1: Males and females from the same incubation temperature will show the same response to gonadectomy in terms of brain nuclei volume and metabolic capacity; for example, if the volume of the ventromedial hypothalamus (VMH) increases in males, it will also increase in females. Hypothesis 2: Because nuclei volume differs significantly between individuals of the same sex from different incubation temperatures, there will be significant variation within each sex across incubation temperatures in response to gonadectomy; for example, if the volume of the VMH increases in females from the male-biased incubation temperature, it will either decrease or show no change in geckos from the all-female incubation temperature.

A second set of predictions is related to the response to hormone treatment. Hypothesis 3: Males and females from the same incubation temperature will show the same response to treatment with exogenous testosterone in terms of brain nuclei volume and metabolic capacity; for example, if the volume of the VMH decreases in males, it will also decrease in females. Hypothesis 4: Because nuclei volume differs significantly between individuals of the same sex from different incubation temperatures, there will be significant variation within each sex across incubation temperatures in response to treatment with exogenous testosterone; for example, if the volume of the VMH decreases in females from the male-biased incubation temperature, it will either increase or show no change in geckos from the all-female incubation temperature. This experiment was designed to test these hypotheses.

METHODS

Animals

Animals were sexually mature, 1-year-old animals that had been raised in isolation. See Coomber *et al.* (1996) for housing, maintenance, and animal care. The following groups were represented: geckos from the all-female incubation temperature (26°C), females from a male-biased incubation temperature (32.5°C), and males from a male-biased incubation temperature (32.5°C).

Gonadectomy, Hormone Treatment, and Radioimmunoassay

Animals were anesthetized with ice, the gonads were removed, and some geckos were given a subcutaneous Silastic implant containing either testosterone (TESTO) or cholesterol (CHOL) (Sigma) as described in Tousignant and Crews (1995); other individuals remained intact (INTACT). See Coomber *et al.* (1996) for details regarding radioimmunoassay procedure.

Aggressive and Sexual Behavior

Sociosexual behaviors in the leopard gecko are easily distinguished. Aggressive behavior is sexually dimorphic in frequency and intensity, but not in form. Aggression is characterized by the animal raising and arching its body into a high-posture stance while slowly waving its tail. The animal then approaches the stimulus animal in a sideways movement. If the stimulus animal does not flee it will be quickly and viciously attacked with bites usually to the head or tail. The at-

tacking animal will then shake and flip the intruder. Males typically take the offensive toward intruders, whereas females typically restrict their behavior to high-posture stances; attacks by females are rare, usually occurring if the intruder is a male that makes persistent mating attempts when she is nonreceptive.

Sexual behaviors include attractivity and courtship and are sexually dimorphic. Attractivity is a female-typical behavior that is measured by a stimulus male's courtship response. During courtship, the male will approach the female slowly while licking the air and substrate with his tongue. Females produce an attractivity pheromone in their skin (Mason and Gutzke, 1990) that induces the male to rapidly vibrate the tip of his tail. If the female is not receptive, she will either flee or attack the male by biting him. If the female is receptive, she remains stationary while the male licks and then gently bites and shakes her tail without wounding her. He gradually shifts his grip to the female's upper back and then to her neck or head, positioning his body parallel to hers. Receptive females will lift the tail to allow the male to intromit.

Behavior Testing

Experimental females were tested in their home cage with both stimulus females from a like incubation temperature and stimulus males from the male-biased incubation temperature. Experimental males were tested with stimulus geckos from the all-female incubation temperature and stimulus males from the male-biased incubation temperature. All behavior tests lasted for 5 min and were conducted between 1400 and 1700, the period coinciding with the onset of daily activity observed in communal breeding cages. The animals were bled by cardiac puncture after initial behavior testing, and 1 week prior to manipulation.

Each experimental animal was tested with three different stimulus animals of each sex while intact and again tested with the same six stimulus females and males 3.5 weeks after manipulation. Female stimulus animals were always presented first because male stimulus animals are often aggressive, and could attack the experimental animal, affecting subsequent behavior tests. The aggressive and sexual behaviors of both the experimental and stimulus animals (high-posture aggression, attacks, tail vibrations, and tail and neck grips) were recorded using an event recorder program for Macintosh computers.

Individuals were considered aggressive if they responded with a high posture during two or more of the behavior tests, or if they attacked the stimulus animal during one or more of the tests. Experimental females were considered attractive if male stimulus animals courted them with a tail vibration and tail or neck grip during one or more of the tests. Males were considered to be courting if they responded with a tail vibration and tail or neck grip during one or more of the tests. Experimental females were considered to exhibit heterotypical sexual behavior if they responded with a tail vibration and tail or neck grip during one or more of the tests. Group comparisons for behavioral data were done with the likelihood ratio χ^2 test for nonparametric behavioral data (JUMP, SAS Institute Inc., Cary, NC).

Brain Analysis

See Coomber *et al.* (1996) for general procedures for histology and description of brain areas. Brain morphology was analyzed in terms of the volume of brain nuclei that contain sex hormone receptors and are affected by sex steroid hormone treatment in other reptiles (Young, Lopreato, Horan, and Crews, 1994; Wade, Huang, and Crews, 1993). Quantitative histochemistry of cytochrome oxidase (C.O.) was used as a functional marker to examine the oxidative activity of brain nuclei (Coomber *et al.*, 1996; Gonzalez-Lima and Cada, 1994; Gonzalez-Lima and Jones, 1994). All statistical analyses utilized SYSTAT as described in Coomber *et al.* (1996). ANCOVA verified that there was no significant interaction between forebrain volume and incubation temperature and sex. All volume indices were found to be homogeneous using Bartlett's Test for Homogeneity, so the data were not log-transformed. Volume indices were compared using ANOVA, and if $P \leqslant 0.01$, a Tukey's post hoc test was used to determine which groups were significantly different. The mean C.O. Activity Units for all groups of brain nuclei measured were found to be heterogeneous using Bartlett's Test, so the data were log-transformed; reanalysis with Bartlett's Test showed the data for all nuclei to be homogeneous after transformation. Transformed C.O. Activity Unit means and the temperature and sex were compared using ANCOVA to verify that there was no significant interaction between covariate (nucleus C.O. activity) and treatment (temperature and sex). The transformed C.O. Activity Unit means were then compared using ANOVA. If $P \leqslant 0.01$, a Tukey's post hoc test determined which individual group comparisons were significantly different.

RESULTS

Effect of Gonadectomy on Behavior

Aggression. The level of aggression exhibited by INTACT females toward stimulus males ($\chi^2 = 10.7$, P

= 0.001) or females (χ^2 = 14.5, P = 0.0001) differed significantly as a function of incubation temperature, with females from the male-biased incubation temperature being more aggressive than geckos from the all-female incubation temperature. There was no significant change in the frequency or intensity of aggression exhibited toward male (χ^2 = 0.67, P = 0.72) or female (χ^2 = 0.56, P = 0.46) stimulus animals in CHOL-treated ovariectomized females from the male-biased incubation temperature, but similarly treated geckos from the all-female incubation temperature were more aggressive toward stimulus males (χ^2 = 8.4, P = 0.01), but not toward stimulus females (χ^2 = 0.67, P = 0.72).

INTACT males from the male-biased incubation temperature were highly aggressive toward stimulus males (80%), but rarely aggressive toward stimulus females (10%). After castration and CHOL treatment, males did not change in their aggression toward stimulus males (χ^2 = 0.83, P = 0.36), but they were more aggressive toward stimulus females (χ^2 = 5.8, P = 0.01). Stimulus males were more likely to attack experimental animals following gonadectomy (χ^2 = 31.6, $P < 0.0001$). None of the females, while intact or following ovariectomy and CHOL treatment, were attacked by stimulus males, whereas 90% of the males were attacked while they were intact, and 80% were attacked after castration and CHOL treatment.

Attractiveness. Attractiveness of females differed significantly as a function of the manipulation. Stimulus males were less likely to exhibit tail vibration or other courtship behaviors toward ovariectomized females treated with CHOL (χ^2 = 27.1, $P < 0.0001$).

Courtship behavior. While intact, most of the males (10/11) exhibited tail vibration or performed a neck or tail grip toward female stimulus animals, but none performed any courtship behavior after castration and treatment with CHOL (χ^2 = 15.9, P = 0.0001).

Heterotypical behavior. None of the females or males exhibited any heterotypical behaviors while intact or following gonadectomy and treatment with CHOL.

Effect of Testosterone Treatment on Behavior

Aggression. The likelihood of aggression exhibited toward a male or female stimulus animal differed significantly as a function of hormone treatment. Following ovariectomy and treatment with TESTO, geckos from the all-female incubation temperature were more aggressive toward stimulus males (χ^2 = 14.5, P = 0.0001); females from the male-biased incubation temperature showed no change in their aggression toward males (χ^2 = 0.67, P = 0.72), but were less aggressive to stimulus females (χ^2 = 8.4, P = 0.01). After castration and TESTO treatment, males did not change in their aggression toward stimulus males (χ^2 = 0.83, P = 0.36) or stimulus females (χ^2 = 0.83, P = 0.36). Male stimulus animals attacked every gonadectomized female and male treated with TESTO in the study (χ^2 = 50.4, $P < 0.0001$).

Attractiveness. Attractiveness of females differed significantly as a function of hormone manipulation (χ^2 = 27.1, $P < 0.0001$). None of the stimulus males courted the ovariectomized females treated with TESTO.

Courtship behavior. The likelihood of male courtship behavior by experimental males toward stimulus females did not differ significantly as a function of hormone manipulation (χ^2 = 0.67, P = 0.72). Most of the males (10/11), while intact, and all of the males after castration and TESTO treatment, performed neck or tail grips and/or tail vibrations toward female stimulus animals.

Heterotypical behavior. Ovariectomized females treated with TESTO differed according to their incubation temperature (χ^2 = 23.8, P = 0.0001), with geckos from the all-female incubation temperature being less likely to exhibit heterotypical courtship behaviors toward stimulus females as compared to females from a male-biased incubation temperature. None of the castrated males treated with TESTO exhibited any heterotypical behaviors.

Hormones

Coefficients of variation for androgen and estrogen assays were 2 and 10% (inter- and intrassay variation, respectively). INTACT males had higher androgen and lower estrogen levels compared to INTACT females (both P = 0.001). INTACT females from the all-female and male-biased incubation temperatures were not significantly different in their total androgen and estrogen levels (P = 0.57 and 0.33, respectively). After gonadectomy and treatment with CHOL, androgens did not change in females (P = 0.97 and 0.63 for geckos from the all-female and male-biased incubation temperatures, respectively), but decreased in males (P = 0.0001). Estrogen levels decreased in females (P = 0.0006 and 0.0009 for geckos from the all-female and male-biased incubation temperatures, respectively), but did not change in males (P = 0.20) after gonadectomy and CHOL treatment.

In female geckos, androgen levels were higher following TESTO treatment compared to levels when intact (P = 0.0001 for geckos from both incubation tem-

TABLE 1
Female Groups Statistics

	All-female (26°C) incubation temperature			Male-biased (32.5°C) incubation temperature		
Region	Intact	Chol	Testo	Intact	Chol	Testo
Fb volume	13.87 ± 0.25	14.83 ± 0.44	13.87 ± 0.44	12.79 ± 0.27	12.49 ± 0.44	12.75 ± 0.44
HAB	0.27 ± 0.02	0.29 ± 0.05	0.29 ± 0.04	0.44 ± 0.04	0.42 ± 0.03	0.45 ± 0.05
LFB	0.42 ± 0.03	0.41 ± 0.05	0.41 ± 0.04	0.44 ± 0.04	0.42 ± 0.04	0.45 ± 0.05
POA	0.86 ± 0.03	0.84 ± 0.05	0.89 ± 0.05	1.35 ± 0.03	1.19 ± 0.05	1.55 ± 0.05
VMH	0.91 ± 0.02	0.92 ± 0.02	0.94 ± 0.04	0.80 ± 0.02	0.92 ± 0.04	0.64 ± 0.04
C.O. metabolic activity						
AH	8.7 ± 0.2	8.7 ± 0.3	8.9 ± 0.3	10.1 ± 0.2	8.2 ± 0.3	10.9 ± 0.3
AME	5.9 ± 0.2	5.8 ± 0.3	6.8 ± 0.3	10.4 ± 0.2	9.6 ± 0.3	11.3 ± 0.3
DL	11.0 ± 0.2	10.3 ± 0.3	12.3 ± 0.3	11.3 ± 0.2	10.5 ± 0.3	12.5 ± 0.3
DLH	13.1 ± 0.1	12.0 ± 0.2	12.9 ± 0.2	15.7 ± 0.1	14.7 ± 0.2	15.1 ± 0.2
DVR	6.1 ± 0.1	5.5 ± 0.2	6.9 ± 0.2	8.3 ± 0.1	7.3 ± 0.2	9.3 ± 0.2
HAB	15.0 ± 0.1	14.8 ± 0.2	14.7 ± 0.2	14.9 ± 0.1	14.6 ± 0.2	14.7 ± 0.2
LH	8.5 ± 0.6	9.4 ± 0.5	8.6 ± 0.2	8.6 ± 0.1	8.7 ± 0.2	8.5 ± 0.2
NR	14.2 ± 0.1	14.0 ± 0.1	13.9 ± 0.1	14.2 ± 0.1	14.0 ± 0.1	14.2 ± 0.1
NS	9.7 ± 0.1	10.0 ± 0.1	9.7 ± 0.1	11.5 ± 0.1	11.6 ± 0.1	11.4 ± 0.1
PH	9.3 ± 0.1	8.7 ± 0.1	9.3 ± 0.1	9.0 ± 0.1	8.4 ± 0.1	9.1 ± 0.1
POA	6.1 ± 0.3	5.7 ± 0.5	9.4 ± 0.5	7.1 ± 0.3	4.3 ± 0.5	9.4 ± 0.5
PP	6.2 ± 0.2	4.3 ± 0.3	6.2 ± 0.3	6.3 ± 0.2	4.3 ± 0.3	6.5 ± 0.3
SEP	12.8 ± 0.1	12.0 ± 0.2	13.2 ± 0.2	14.9 ± 0.1	13.4 ± 0.2	15.4 ± 0.2
STR	11.9 ± 0.1	11.9 ± 0.1	12.0 ± 0.1	13.2 ± 0.1	13.3 ± 0.1	13.2 ± 0.1
TS	13.8 ± 0.1	13.7 ± 0.2	13.7 ± 0.2	14.4 ± 0.1	13.6 ± 0.2	14.3 ± 0.2
VMH	12.4 ± 0.1	11.2 ± 0.2	12.3 ± 0.2	12.2 ± 0.2	11.1 ± 0.2	12.2 ± 0.2

Note. The volume of a fixed portion of the forebrain in mm^3 (Fb volume) and of different brain areas (Region) of adult female leopard geckos (*Eublepharis macularius*) from all-female (26°C) or a male-biased (32.5°C) incubation temperature during incubation as an egg. Manipulations include sham-operated intact (INTACT), gonadectomy and treatment with cholesterol (CHOL), or gonadectomy and treatment with testosterone (TESTO). Volume measurements are mean ratios of nucleus volume divided by a fixed portion of forebrain volume ×100 ± standard error. Cytochrome oxidase measurements are group means ± standard error (μmol/min/g tissue wet weight). Abbreviations: AH, anterior hypothalamus; AME, external amygdala; DLH, dorsal lateral nucleus of the hypothalamus; DL, dorsal lateral nucleus of the thalamus; DVR, dorsal ventricular ridge; HAB, habenula; LFB, lateral forebrain bundle; LH, lateral hypothalamus; NR, nucleus rotundus; NS, nucleus sphericus; PH, periventricular nucleus of the hypothalamus; POA, preoptic area; PP, periventricular nucleus of the preopetic area; SEP, septum; STR, striatum; TS, torus semicircularis; VMH, ventromedial nucleus of the hypothalamus.

peratures). Androgen levels in TESTO-treated male geckos tended to be higher ($P = 0.05$), but this difference did not reach the statistical criterion. After gonadectomy and TESTO treatment, estrogen levels did not change in geckos from the all-female incubation temperature ($P = 0.74$) and males from the male-biased incubation temperature ($P = 0.26$). Although estrogen levels decreased in females from the male-biased incubation temperature ($P = 0.04$), this did not reach statistical criterion.

Brain Analysis

Group means and standard errors of the volume and transformed C.O. Activity Unit measures in the various brain areas of male and female leopard geckos from the different incubation temperatures are shown in Tables 1 and 2. Presented below are the additional *P* values of specific comparisons (Tukey's post hoc test). Volume indices and transformed C.O. Activity Unit means were compared for relative effects of gonadectomy and hormone treatment within and between male and female leopard geckos from the two incubation temperatures.

Brain morphometrics. The relative forebrain volume was not significantly different between gonadectomized CHOL-treated and TESTO-treated animals (Tables 1 and 3). The volumes of the HAB and LFB were not significantly different between gonadectomized CHOL-treated and TESTO-treated animals.

When compared to INTACT animals, the following were statistically significant: (i) POA volume decreased, and VMH volume increased, in males after castration and CHOL treatment. (ii) POA volume increased after ovariectomy and TESTO-treatment only in females from the male-biased incubation tempera-

TABLE 2
Male Groups Statistics

Region	Male-biased incubation temperature (32.5°C)		
	Intact	Chol	Testo
Fb volume	14.06 ± 0.27	15.32 ± 0.44	15.39 ± 0.44
HAB	0.28 ± 0.03	0.27 ± 0.05	0.29 ± 0.05
LFB	0.42 ± 0.04	0.41 ± 0.05	0.42 ± 0.05
POA	1.45 ± 0.04	0.93 ± 0.05	1.48 ± 0.05
VMH	0.80 ± 0.02	0.91 ± 0.03	0.60 ± 0.03
C.O. metabolic activity			
AH	10.4 ± 0.1	8.3 ± 0.1	11.0 ± 0.1
AME	10.1 ± 0.2	7.7 ± 0.2	11.0 ± 0.3
DL	12.1 ± 0.3	11.2 ± 0.4	12.8 ± 0.4
DLH	14.3 ± 0.2	13.1 ± 0.3	14.3 ± 0.3
DVR	8.6 ± 0.1	7.1 ± 0.1	9.5 ± 0.1
HAB	14.7 ± 0.1	14.7 ± 0.1	14.8 ± 0.2
LH	8.6 ± 0.1	8.6 ± 0.2	8.5 ± 0.2
NR	14.2 ± 0.1	14.0 ± 0.1	14.1 ± 0.1
NS	12.3 ± 0.1	11.7 ± 0.1	12.9 ± 0.1
PH	9.2 ± 0.1	9.1 ± 0.1	9.3 ± 0.1
POA	7.5 ± 0.2	6.4 ± 0.3	9.7 ± 0.3
PP	5.1 ± 0.3	4.4 ± 0.5	5.2 ± 0.5
SEP	14.8 ± 0.1	13.9 ± 0.2	15.4 ± 0.2
STR	13.3 ± 0.1	13.4 ± 0.1	13.3 ± 0.1
TS	14.5 ± 0.1	14.4 ± 0.3	14.5 ± 0.3
VMH	9.0 ± 0.1	9.2 ± 0.2	9.1 ± 0.2

Note. The volume of a fixed portion of the forebrain in mm^3 (Fb volume) and of different brain areas (Region) of adult male leopard geckos (*Eublepharis macularius*) that had been exposed to a male-biased temperature (32.5°C) during incubation as an egg. Manipulations include sham-operated intact (INTACT), gonadectomy and treatment with cholesterol (CHOL), or gonadectomy and treatment with testosterone (TESTO). Volume measurements are mean ratios of nucleus volume divided by fixed portion forebrain volume × 100 ± standard error. Cytochrome oxidase measurements are group means ± standard error (μmol/min/g tissue wet weight). Abbreviations are as in Table 1.

ture, but not in geckos from the all-female incubation temperature. (iii) VMH volume in all gonadectomized females and males decreased with TESTO treatment.

The volume of the POA was larger, and VMH smaller, in ovariectomized and TESTO-treated females compared to ovariectomized females with CHOL treatment ($P = 0.0002$ and 0.0004 for geckos from the all-female and male-biased incubation temperatures, respectively) (Fig. 1). The volume of the POA was larger ($P = 0.0001$), and the VMH smaller ($P = 0.0001$), in castrated males receiving TESTO compared to castrated males receiving CHOL.

Brain metabolic capacity. The optic tract (OT) measured zero or less than 1 C.O. Activity Unit for all animals. There were no significant differences in C.O. activity in the HAB, LH, NR, and STR between gonadectomized animals with CHOL-treatment and gonadectomized animals with TESTO-treatment.

When compared to INTACT animals, the following were statistically significant (Tables 1 and 2, Figs. 2 and 3): (i) Metabolic capacity in the DVR and SEP decreased in males and females after gonadectomy and CHOL treatment. (ii) Metabolic capacity in the VMH and PH decreased in females after ovariectomy and CHOL treatment. (iii) Metabolic capacity decreased in the AH, AME, POA, and NS in males after castration and CHOL treatment.

In other brain nuclei, gonadectomized animals with CHOL treatment tended to have significantly less, and gonadectomized animals with TESTO-treatment significantly greater, metabolic capacity compared to INTACT animals. Metabolic capacity was greater in the DL ($P = 0.001$), DVR ($P = 0.0002$), POA ($P = 0.0002$), and SEP ($P = 0.007$) in gonadectomized TESTO-treated geckos (from both the all-female and male-biased incubation temperatures) compared to gonadectomized CHOL-treated animals; the single exception was found in the NS, in which metabolic activity was greater in ovariectomized females with CHOL ($P = 0.0001$). Meta-

TABLE 3
Effects of Gonadectomy and Treatment with Exogenous Testosterone on Brain Metabolic Capacity in Leopard Geckos (*Eublepharis macularius*)

Nucleus	Manipulation effects
DL	All-female: TESTO > INTACT = CHOL
DL	Male-biased female: TESTO > INTACT = CHOL
DL	Male-biased male: TESTO > INTACT > CHOL
DLH	All-female: TESTO > INTACT > CHOL
DLH	Male-biased female: TESTO > INTACT > CHOL
DLH	Male-biased male: TESTO > INTACT > CHOL
LH	None
PH	None
PP	None
TS	All-female: none
TS	Male-biased female: TESTO = INTACT > CHOL
TS	Male-biased male: none

Note. This table reports changes in brain nuclei not depicted in graphs. The greater than (>) symbol denotes statistically significant differences in metabolic capacity at the 0.01 confidence level or better. The equal (=) symbol denotes that metabolic capacities between groups were not significantly different. INTACT indicates intact individuals, whereas TESTO and CHOL denote gonadectomized individuals treated with testosterone or cholesterol, respectively. All-female indicates the all-female producing incubation temperature and Male-biased indicates the male-biased incubation temperature. Abbreviations are as in Table 1.

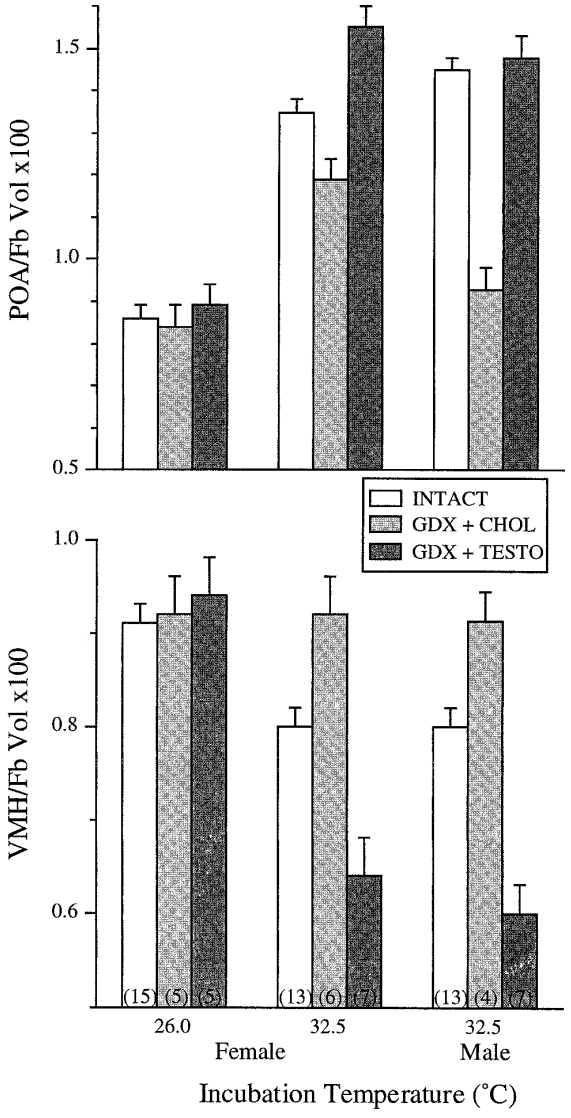

FIG. 1. Effect of gonadectomy and testosterone treatment on the volume of the preoptic area (POA) and ventromedial hypothalamus (VMH) in the leopard gecko (*Eublepharis macularius*). Mean ratio of nucleus volume divided by a fixed portion of forebrain volume × 100 is presented with vertical bars representing standard error. INTACT, intact; GDX + TESTO, castration + testosterone implant; GDX + CHOL, castration + cholesterol implant. Sample sizes are given in parentheses.

bolic capacity was greater in the AH ($P = 0.0001$) and AME ($P = 0.002$) in gonadectomized TESTO-treated geckos (females and males) from the male-biased incubation temperature compared to CHOL-treated geckos, but there was no such difference in the AH at the all-female incubation temperature. Compared to CHOL-treated animals, metabolic capacity was greater in the PH ($P = 0.0004$) and PP ($P = 0.002$) of ovariectomized females treated with TESTO from both temperatures, but not in males; a similar trend was evident in the VMH, but this did not reach the statistical criterion ($P = 0.03$). There was a significant difference in the TS ($P = 0.005$) only among females from the male-biased incubation temperature.

DISCUSSION

Hormones experienced early in life not only can modify an individual's mating behavior later in life, but also can change how the adult responds to sex steroid hormones (Goy and McEwen, 1980). In the present study we extend this concept to nonhormonal stimuli, namely the temperature of the incubating egg. Incubation temperature not only determines gonadal sex in the leopard gecko and other reptiles (Bull, 1980; Ewert and Nelson, 1991; Janzen and Paukstis, 1991), but has direct organizing effects on the growth, endocrine physiology, behavior, and brain development and activity of the individual that are independent of sex hormones (Crews, 1988; Coomber *et al.*, 1996; Flores and Crews, 1995; Flores *et al.*, 1994; Gutzke and Crews, 1988; Tousignant and Crews, 1994, 1995; Tousignant, Viets, Flores, and Crews, 1995).

As demonstrated previously (Flores and Crews, 1995; Flores *et al.*, 1994), female leopard geckos from the male-biased incubation temperature were more aggressive compared to geckos from the all-female incubation temperature. This effect was also detected in ovariectomized females treated with testosterone, suggesting that incubation temperature affects the sensitivity to hormones in adulthood. The size of capsule used results in circulating TESTO concentrations within the physiological range observed in intact, sexually active male leopard geckos [TESTO levels are approximately 100 ng/ml in intact males and 1 ng/ml in intact females (Gutzke and Crews, 1988; Tousignant and Crews, 1994, 1995; Coomber *et al.*, 1996)].

Many of the brain areas studied contain steroid hormone receptors and are involved in the control of aggressive and sexual behavior in other lizard species as well as other vertebrates (Crews and Silver, 1985; Morrell, Crews, Ballin, Morgentaler, and Pfaff, 1978; Young *et al.*, 1994). These areas (e.g., POA, VMH, DVR, SEP, and AH) showed changes in volume and metabolic capacity following hormonal manipulation. Further, the dramatic changes in behavior seen in ovariectomized females treated with TESTO were correlated with changes in brain morphology and metabolic capacity.

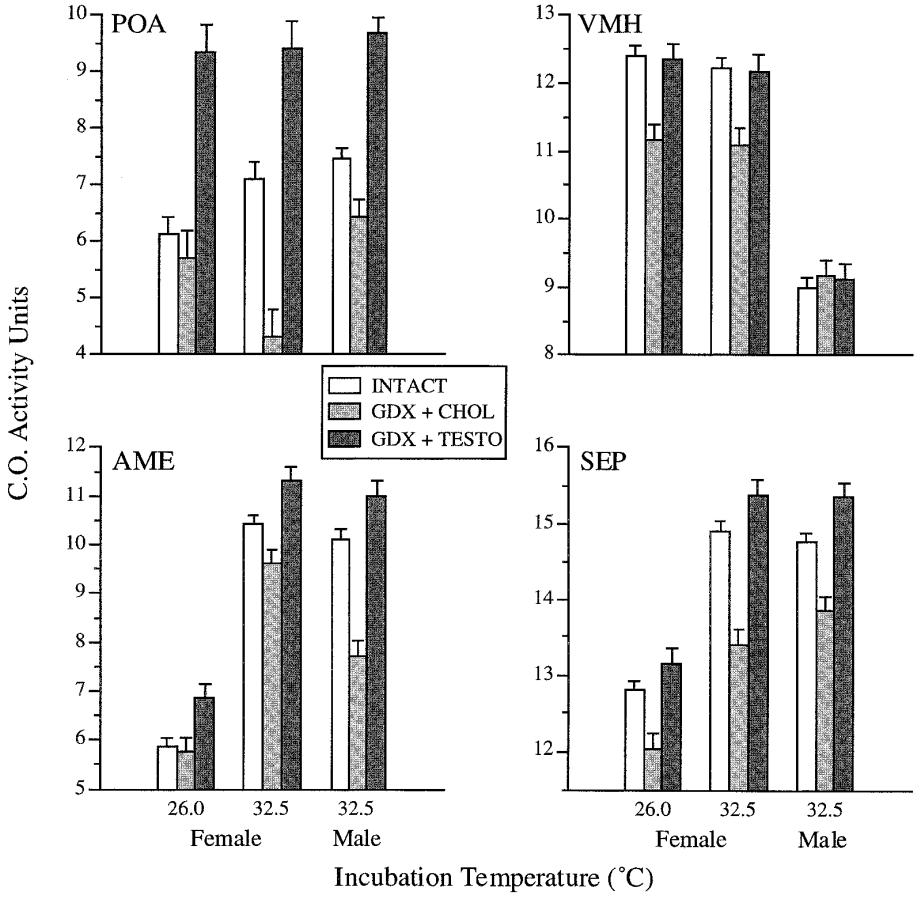

FIG. 2. Effect of gonadectomy and testosterone treatment on C.O. metabolic activity (μmol/min/g tissue wet weight) in the leopard gecko (*Eublepharis macularius*). Significant differences between treatment groups are indicated above bars. Mean C.O. Activity Units are depicted with vertical bars as standard error. POA, preoptic area; VMH, ventromedial hypothalamus; SEP, septum; AME, medial amygdala. Other abbreviations are as in the legend to Fig. 1.

Sex steroid concentrating neurons or hormone receptor mRNA have not been identified in the HAB, LFB, NR, or STR of lizards and, as expected, there were no changes in the volume or metabolic capacity of these brain regions following gonadectomy or hormone treatment.

When taken together with the results of a previous study on the effects of incubation temperature and gonadal sex (Coomber *et al.*, 1996), these data are consistent with the concept that incubation temperature has a direct organizing action on the volume of specific brain nuclei in the leopard gecko. Gonadal sex, and presumably sex hormones (i.e., TESTO, at least at the dosage used), did not appear to be responsible for the variation observed in nuclei size. After gonadectomy POA volume decreased, and VMH volume increased, in both males and females from the same incubation temperature, thereby supporting Hypothesis 1. Following gonadectomy, the low-temperature females showed no change in POA or VMH volumes, whereas POA volume decreased, and VMH volume increased, in females from the male-biased incubation temperature, thereby supporting Hypothesis 2. Further, after TESTO treatment, POA volume increased, and VMH volume decreased, in both males and females from the same incubation temperature, thereby supporting Hypothesis 3. TESTO treatment of ovariectomized females from different incubation temperatures yielded different results; the low-temperature females showed no change in POA or VMH volumes, POA volume increased, and VMH volume decreased, in females from the male-biased incubation temperature, thereby supporting Hypothesis 4.

These volume changes in the POA and VMH in the leopard gecko are different from the results of similar treatment in *Cnemidophorus inornatus,* a species with sex

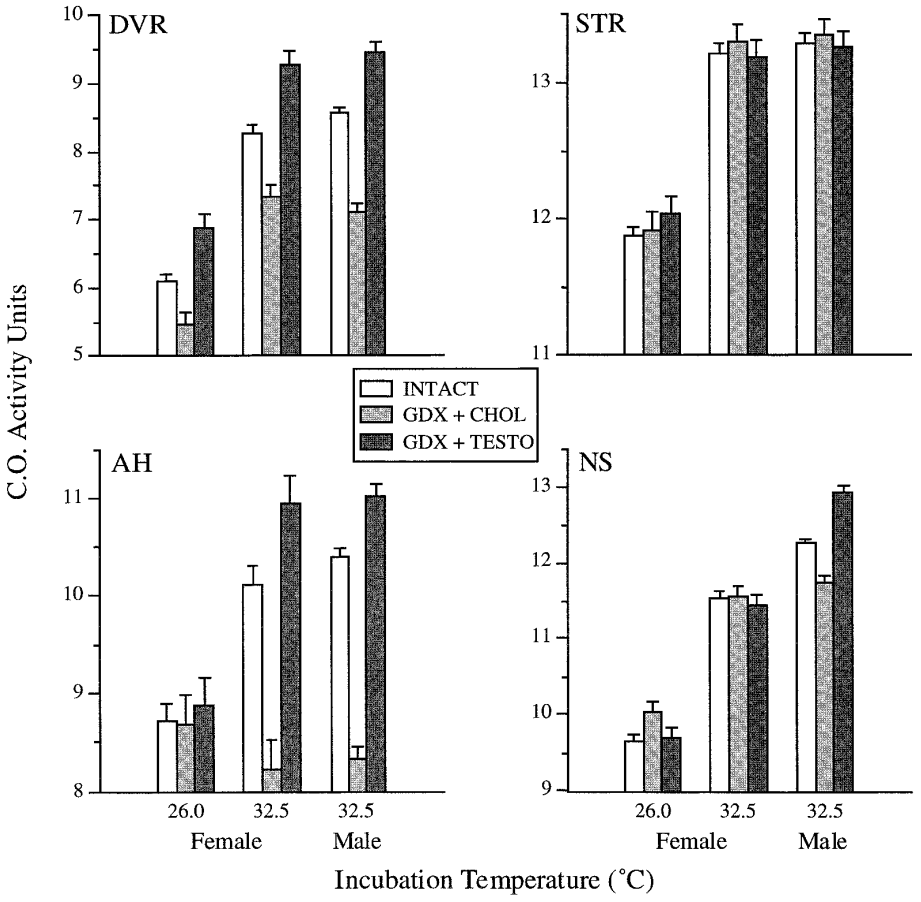

FIG. 3. Effect of gonadectomy and testosterone treatment on C.O. metabolic activity (μmol/min/g tissue wet weight) in the leopard gecko (*Eublepharis macularius*). Mean C.O. Activity Units depicted with vertical bars as standard error. DVR, dorsal ventricular ridge; STR, striatum; AH, anterior hypothalamus; NS, nucleus sphericus. Other abbreviations are as in the legend to Fig. 1.

chromosomes and male heterogamety. In the little striped whiptail, TESTO treatment following castration in males results in an increase in AH-POA volume, and a decrease in VMH volume, yet following ovariectomy and TESTO-treatment, female whiptails show no changes in the volume of these brain areas (Wade *et al.*, 1993). This sex-specific effect of exogenous testosterone in *C. inornatus* suggests that, as in mammals, testosterone is the organizing hormone of the neural circuits controlling sociosexual behaviors. In contrast, in the leopard gecko, which lacks sex chromosomes, exogenous testosterone had similar effects in both males and females from the same incubation temperature, but was without effect in geckos from an all-female incubation temperature, thereby demonstrating that temperature during development, not gonadal sex, is the principal organizer of the brain and consequently behavior.

Incubation temperature also affected the metabolic capacity in various nuclei. After ovariectomy, the AH and POA increased in metabolic capacity in females from the male-biased incubation temperature, but did not change in geckos from the all-female incubation temperature. After ovariectomy and TESTO treatment, the metabolic capacity in the POA increased by 119% in females from the male-biased incubation temperature, but only increased by 65% in geckos from the all-female incubation temperature. On the other hand, metabolic capacity in the AME increased in ovariectomized TESTO-treated geckos from the all-female incubation temperature, but did not change in females from the male-biased incubation temperature. Together, the data support previous behavioral findings that individuals from different incubation temperatures have different sensitivities to hormones (Flores and Crews, 1995). Thus, the temperature experienced during development may modify response to hormones later in life.

The change in C.O. activity with gonadectomy and hormone replacement therapy in the NS of male, but not female, leopard geckos deserves note. The NS is associated with the chemical senses, receiving projections from the accessory olfactory bulb (vomeronasal organ) and sending efferents via the bed nucleus of the stria terminalis and POA to the VMH (Halpern, 1992). It is present in garter snakes, which rely almost exclusively on pheromones for sex recognition and courtship (Crews, 1990), but is absent in anolis lizards, which are primarily visual animals with a poorly developed chemical sense (Mason, 1992; Halpern, 1992). Whiptail lizards also utilize pheromones and have a distinct NS (Crews, Wade, and Wilczynski, 1990). In leopard geckos, the role of pheromones in mating is similar to that in snakes (Mason and Gutzke, 1990), and the NS is a large, well-defined nucleus (Coomber *et al.*, 1996).

Usually there is a correlation between the presence and distribution of steroid hormone concentrating neurons in brain areas (Crews and Silver, 1985). This is not always the case, however. For example, steroid autoradiography has established that testosterone and estradiol are concentrated in the surrounding capsule (mural layer), but not in the body (hilar layer) of the NS of garter snakes (Halpern, Morrell, and Pfaff, 1982). Similarly, there is no evidence of androgen or estrogen concentrating neurons in the region of the brain where the NS would be found in anolis lizards (Morrell *et al.*, 1979); degeneration studies indicate that the accessory olfactory tract projects to the appropriate area in the anolis brain, but there is no discrete nucleus (N. Greenberg, unpublished). Homologous *in situ* hybridization of androgen and estrogen mRNA (Young *et al.*, 1994) found no evidence of steroid hormone receptors in the NS of whiptail lizards.

Another correlation concerns the size of certain brain nuclei, their role in the control of reproductive behaviors, and the dependence of courtship and copulatory behavior on sex steroid hormones (Crews and Silver, 1985). Again, this generalization does not stand in the face of the evidence. Although lesioning the NS facilitates sexual behavior in the garter snake (Krohmer and Crews, 1989), suggesting a central inhibitory control, other studies indicate that castration and hormone replacement therapy have no apparent effect on the size of the NS in the garter snake (Crews, Robker, and Mendonça, 1993). This latter finding is consistent with other work showing that mating behavior in the garter snake is not activated by steroid hormones (Crews, 1990). In the present study, the opposite pattern was apparent. That is, the leopared gecko has a well-developed chemical sense, its sexual behavior is activated by steroid hormones (Flores and Crews, 1995; Flores *et al.*, 1994), and, in males, the prominent NS fluctuates in size depending upon hormonal condition. Yet the NS does not contain steroid hormone receptors. The absence of any evidence of specific mRNA does not necessarily imply the absence of hormone receptor protein (Bern, 1990), but this is unlikely given other research in molecular neuroendocrinology. Thus, the conclusion that ''As many of the hormone-concentrating regions are involved in chemosensitive pathways, as well as in reproductive behaviors, one may assume that these represent the morphological basis of hormonal control of reproduction in the reptiles studied'' (Halpern, 1992, pp. 446–447) must be viewed with caution.

In mammals and other vertebrates with sex chromosomes, brain areas are sexually dimorphic as a result of the early hormone environment arising indirectly from the individual's genetic constitution. In some species hormone manipulation in adulthood alters the size of sexually dimorphic nuclei. For example, in adult male gerbils, the sexually dimorphic area of the medial preoptic area reduces in size by 50% after castration (Commins and Yahr, 1984; see also Panzica, Viglietti-Panzica, Sanchez, Sante, and Balthazart, 1991; Adkins-Regan and Watson, 1990). Indeed, recent studies indicate that the hormone environment during brain differentiation alters cytochrome oxidase capacity in the sexually dimorphic area of the preoptic area (Jones, Gonzalez-Lima, Crews, Galef, and Clark, 1996). However, in other species, areas of the brain organized by the early hormone environment remain fixed throughout adulthood. For example, in the guinea pig, the volume of the POA does not change when adult steroid hormones are manipulated by castration with or without hormone replacement therapy (Hines, Davis, Coquelin, Gay, and Gorski, 1985; see also Gorski, Gordon, Shryne, and Southam, 1978).

Differences between the intact and ovariectomized female geckos indicate that the presence of ovaries influenced behavior as well as brain morphology and metabolic activity. The loss of receptivity exhibited by females from both incubation temperatures following ovariectomy is similar to the effects of ovariectomy on the day of hatch (Flores *et al.*, 1994). This suggests that postnatal ovarian hormones can play a role in the development of adult sociosexual behaviors. These behavioral changes after ovariectomy may be linked to the decrease in metabolic activity in the VMH, an important integrative area for sexual receptivity (Crews and Silver, 1985; Pfaff, Schwartz-Giblin, McCarthy, and Low, 1994; Sachs and Meisel, 1994).

The increase in aggression after ovariectomy and

TESTO treatment in geckos from the all-female incubation temperature may be due to a reduction in fear. In heifers (Bouissou and Gaudioso, 1982; Boissy and Bouissou, 1994), ewes (Vandenheede and Bouissou, 1993), and chickens (Archer, 1973), TESTO treatment reduces fear reactions in both social and nonsocial situations. Androgens can also influence social rank and dominance; for example, TESTO-treated steers or cows raise their social rank within a group and dominate unfamiliar untreated animals (Bouissou, 1978; Bouissou, Demurger, and Lavenet, 1986). In leopard geckos from the all-female incubation temperature, the increase in aggression after TESTO treatment may relate to the increase in metabolic capacity in the AME. The amygdala's role in fear and aggression is well-documented (Greenberg, Scott, and Crews, 1984; Kling and Brothers, 1992; Davis, Rainnie, and Cassell, 1994; Treit, Pesold, and Rotzinger, 1993; Campeau and Davis, 1995). In addition, studies have also shown that lesions in the medial amygdala result in a decrease in social rank when confronted with conspecifics in lizards, dogs, and monkeys (Greenberg *et al.*, 1984; Kling and Brothers, 1992; Kling and Cornell, 1971).

The increase in the volume and metabolic capacity of the POA in males and females from the male-biased incubation temperature, as well as the increase in metabolic capacity in the POA in geckos from the all-female incubation temperature after gonadectomy and TESTO treatment, may relate to the role of TESTO in the stimulation of dendritic outgrowth in this brain nucleus. Exposure to testosterone increases process length and branching in neurons in the POA of rats (Kawashima and Takagi, 1994). Such an increase in neuronal growth would result in both increased volume and increased metabolic requirements, as seen in this study.

Manipulating the hormonal state of adult male and female leopard geckos from the same or different incubation temperatures reveals the relative contribution of incubation temperature during embryogenesis and gonadal steroid hormones during adulthood in the sexual differentiation of the brain. This study indicates that sociosexual behavior is reflected in differences in brain morphology and brain metabolic capacity. Although gonadal steroids can alter the structure and neurochemical physiology of specific brain regions, incubation temperature is still the main determinant of much of the individual and sexual variation in the behavior, endocrinology, and brain morphology in the leopard gecko. Additionally, gonadal sex hormones had distinct effects on the metabolic capacity of specific nuclei in the brain. Incubation temperature and gonadal sex had different effects on the metabolic capacity of specific brain areas. For example, in the AME, incubation temperature was the main determinant of differences within females and hence, between females from all-female vs male-biased incubation temperatures, whereas in the VMH, males had much lower C.O. capacity compared to females from either incubation temperature. These results indicate that in a reptile lacking sex chromosomes, differences within and between the sexes in sociosexual behavior, hormone sensitivity, and the morphology and metabolic capacity of specific brain regions are organized by incubation temperature and modulated by the hormone environment of the adult.

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