

Independent Effects of Incubation Temperature and Gonadal Sex on the Volume and Metabolic Capacity of Brain Nuclei in the Leopard Gecko (*Eublepharis macularius*), a Lizard With Temperature-Dependent Sex Determination

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ABSTRACT

The extent to which variation within and between the sexes can be assigned to genes vs. environment is problematic, because, in most vertebrates, males and females differ genetically. However, factors other than sex chromosomes and the consequent sex-typical gonadal hormone secretions may play important roles in the differentiation of the neural mechanisms underlying individual and sex differences in aggressive and sexual behavior. The leopard gecko, like many oviparous reptiles, lacks sex chromosomes. Instead, gonadal sex is determined by temperature during embryogenesis, with low and high incubation temperatures producing females and intermediate temperatures producing mixed sex ratios. In essence, this allows for the study of individual and sex differences without the confounding variable of genetically determined gender. Experiments have shown that the temperature experienced during incubation plays a critical role in establishing the adult morphological, endocrinological, and behavioral phenotype. In this experiment, the independent effects of incubation temperature and gonadal sex on the morphology and metabolic capacity of specific brain nuclei were determined. Both individual and sex differences in the volume of the preoptic area and ventromedial nucleus of the hypothalamus are determined primarily by incubation temperature, not by gonadal sex. However, incubation temperature and gonadal sex are both important in determining the metabolic capacity in the anterior hypothalamus, external amygdala, dorsal lateral nucleus of the hypothalamus, dorsal lateral nucleus of the thalamus, dorsal ventricular ridge, habenula, lateral hypothalamus, nucleus rotundus, nucleus sphericus, periventricular nucleus of the hypothalamus, preoptic area, periventricular nucleus of the preoptic area, septum, striatum, torus semicircularis, and ventromedial nucleus of the hypothalamus. This is the first demonstration in a vertebrate that factors other than gonadal sex hormones, which arise from the individual's genetic constitution, can affect the sexual differentiation of the brain. *J. Comp. Neurol.* 380:409–421, 1997. © 1997 Wiley-Liss, Inc.

Indexing terms: reptile; differentiation; steroid hormones; oxidative metabolism; sexual dimorphism

Gonadal sex is a discrete characteristic that categorizes the individual, whereas sexuality is a continuous variable that is unique to each individual. The mechanisms leading to individual differences in sexual behavior, including the most fundamental, the male-female difference, are com-

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plex. For example, in mammals, a gene on the Y chromosome that is conveyed by sperm and designated *SRY* switches on the molecular cascade that leads to testicular development (Sinclair et al., 1990). Testicular hormones, in turn, cause individuals to develop a male-typical phenotype. Female development is generally believed to result from the absence of this gene. Despite this genetic difference, each individual is bipotential in both brain and behavior, and it is the hormones secreted by the gonads that sculpt the differences between the sexes. Thus, it is difficult to address experimentally what determines "masculinity" and "femininity," because, in all of the vertebrates that have been studied to date, sex chromosomes determine gonadal sex. Sexual differentiation is complicated further by the considerable individual variation that exists in sex-related traits.

Although it is generally recognized that the environment of the embryo as well as the adult is important for complete development and organization, its contribution cannot be distinguished easily from the genetic constitution. That is, the extent to which individual and sexually dimorphic features can be separated from an individual's genetic sex is problematic. Unlike the genotypic sex-determining mechanisms characteristic of mammals and birds, many reptiles lack sex chromosomes, and the embryo has the potential to develop into either a male or a female, depending on the incubation temperature of the egg, a process known as *temperature-dependent sex determination* (TSD). The temperature effect is all or none. That is, an individual is either a gonadal male or a gonadal female; at incubation temperatures intermediate to all-male- and all-female-producing temperatures, intersexes are not formed, but the sex ratio varies. Thus, in TSD, each individual has an equal ability to become a male or a female, and incubation temperature serves as the trigger activating and suppressing the cascades that lead to the development of testes or ovaries (Crews et al., 1994). Such an animal model system provides a means by which to disentangle the effects of gonadal sex and its associated hormones from that of environmental influences in the development of sex differences (Fig. 1). Furthermore, because the same sex is produced at various incubation temperatures (albeit in different proportions), it allows a

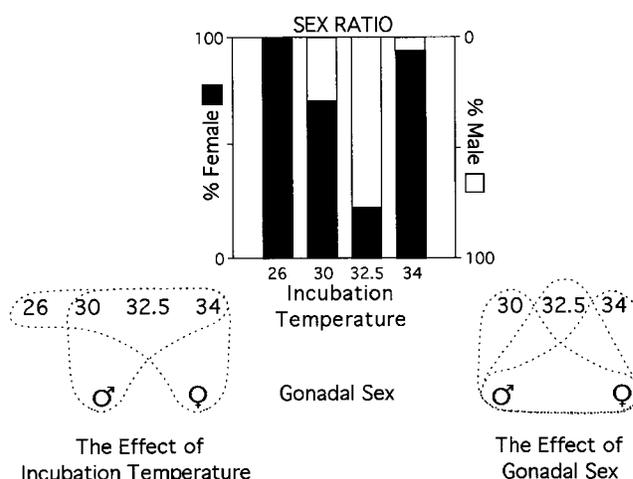


Fig. 1. Schematic diagram of the experimental design using the leopard gecko (*Eublepharis macularius*), a lizard with temperature-dependent sex determination. The bar graph (top) portrays the effect of incubation temperature ($^{\circ}\text{C}$) on sex ratio: Extreme temperatures produce females, whereas intermediate temperatures produce different ratios of males and females. Because the effects of incubation temperature and gonadal sex covary, any difference between individuals could be due to the incubation temperature of the egg, the gonadal sex of the individual, or both factors combined. If the contribution of each is to be assessed, then they must be dissociated. Studying animals of the same sex that differ only in the incubation temperature experienced reveals the effects of temperature (bottom left), whereas comparing males and females from the same incubation temperature reveals the effects of gonadal sex (bottom right). Dotted lines group comparisons made in each condition. This and other line drawings were generated by using Jandel Sigma Plot 4.11 and Deneba Canvas 3.0.3 on a PowerMac 8100/80AV.

new means of investigation of individual differences within a sex.

In the leopard gecko (*Eublepharis macularius*), the sex ratio varies with incubation temperature: 26°C produces only female hatchlings, 30°C produces a female-biased sex ratio, and 32.5°C produces a male-biased sex ratio. An incubation temperature of 34°C (at or near the lethal maximum) again produces virtually all females (Viets et al., 1993; see Fig. 1). Experiments indicate that the incubation temperature of the egg has profound effects on the morphology (Crews, 1988; Tousignant and Crews, 1994), physiology (Gutzke and Crews, 1988; Coomber et al., 1994; Tousignant and Crews, 1994, 1995; Tousignant et al., 1995), growth (Tousignant and Crews, 1994, 1995), and behavior (Gutzke and Crews, 1988; Flores et al., 1994; Flores and Crews, 1995) of the adult. For example, intact males typically average 75 ng/ml testosterone (range 15–400 ng/ml) or 10- to 20-fold higher circulating levels of androgens compared with reproductively active females with vitellogenic follicles and 50- to 200-fold higher levels than reproductively inactive females with nonvitellogenic follicles (Tousignant and Crews, 1995; Tousignant et al., 1995). Although males have substantially higher circulating concentrations of androgen than females, the androgen titer varies according to incubation temperature within each sex, being highest in animals from a male-biased incubation temperature (Gutzke and Crews, 1988; Tousignant et al., 1995). The differences in behavior and physiology among individuals of the same sex but from different

Abbreviations

AH	anterior hypothalamus
AME	external amygdala
CO	cytochrome oxidase
DLH	dorsal lateral nucleus of the hypothalamus
DVR	dorsal ventricular ridge
HAB	habenula
III	third ventricle
IV	fourth ventricle
LFB	lateral forebrain bundle
LH	lateral hypothalamus
MCX	medial cortex
NR	nucleus rotundus
NS	nucleus sphericus
OC	optic chiasm
OT	optic tract
PH	periventricular nucleus of the hypothalamus
POA	preoptic area
PP	periventricular nucleus of the preoptic area
SEP	septum
STR	striatum
TS	torus semicircularis
VE	ventricular ependymal organ
VMH	ventromedial nucleus of the hypothalamus

incubation temperatures suggest that incubation temperature has direct organizing effects on the brain.

This study investigated the relative effects of gonadal sex and incubation temperature on the sexual differentiation of the volume and metabolic capacity of specific brain nuclei in the adult leopard gecko. Two methods were employed: a well-established method, volumetric analysis of specific brain nuclei, and a promising new method of investigation into the sexual differentiation of brain function, cytochrome oxidase (CO) histochemistry. The target nuclei for volume measurements were the preoptic area (POA) and the ventromedial hypothalamus (VMH), because 1) they have been found to be sexually dimorphic and under the control of gonadal sex steroid hormones in a variety of vertebrates (Arnold and Gorski, 1984; Crews, 1993), and 2) they are critical as integrative areas for mounting and intromission behavior in gonadal males and for sexual receptivity in gonadal females (Pfaff et al., 1994; Sachs and Meisel, 1994). Two areas unlikely to contain sex steroid-concentrating neurons, the lateral forebrain bundle (LFB) and the habenula (HAB), were measured to evaluate whether gonadal sex and incubation temperature influences are specific to putative steroid binding areas.

In addition to changes in regional brain volumes, CO histochemistry was used as a metabolic marker of the functional neural effects of temperature and gonadal sex. Changes in CO activity revealed by quantitative histochemistry reflect changes in the endogenous oxidative metabolic capacity of nervous tissue (Gonzalez-Lima and Cada, 1994). Tissues with greater CO activity have a greater metabolic capacity for oxidative energy production than tissues with lower CO activity (Wong-Riley, 1989). One analogy to explain the energy metabolic process mapped by CO activity is to distinguish between *kinetic* and *potential* energy. Kinetic energy, as in the uptake of 2-deoxyglucose, visualizes short-term changes in brain metabolic activity (Gonzalez-Lima, 1992), whereas potential energy, as in CO histochemistry, reveals long-term changes in the endogenous metabolic capacity of the brain (Gonzalez-Lima, 1992). The energy metabolic processes mapped by these techniques are different: 2-Deoxyglucose uptake determines acute changes in tissue metabolic activity due to energy use (kinetic energy) over a period of minutes, whereas CO activity assesses the chronic alteration in tissue metabolic capacity (potential energy) resulting from sustained metabolic demands over a period of weeks or months.

CO is the oxygen-activating enzyme in the mitochondria that enables plant and animal cells to use oxygen to oxidize food materials, and its activity provides an elegant way to display the oxidative metabolic capacity of various tissues (Seligman et al., 1968; Wong-Riley, 1989; Gonzalez-Lima, 1992). Gonzalez-Lima and Garrosa (1991) developed a method to quantify CO activity from histochemically stained brain sections by the use of calibrated standards of known CO activity together with computerized image analysis. This offered the advantage of combining the anatomical resolution achieved with histochemistry with the biochemical quantification of CO activity (Gonzalez-Lima and Jones, 1994). This approach was used here to quantify relative differences in the metabolic capacity of brain nuclei in the leopard gecko. CO activity was analyzed in 13 putative steroid-binding areas (Morrell et al., 1979; see Materials and Methods). In addition to the POA and VMH, CO activity was measured in the anterior

hypothalamus (AH), external amygdala (AME), dorsal lateral nucleus of the hypothalamus (DLH), dorsal ventricular ridge (DVR), lateral hypothalamus (LH), nucleus sphericus (NS), periventricular nucleus of the hypothalamus (PH), periventricular nucleus of the preoptic area (PP), septum (SEP), striatum (STR), and torus semicircularis (TS). Other nuclei were also measured, because they lack sex hormone receptors: the HAB and the nucleus rotundus (NR). Other comparisons involved nuclei that do not appear to be involved in aggressive and sexual behavior in other vertebrates and/or have not been found to be sexually dimorphic (e.g., POA, VMH, AME, and NS vs. TS, NR, and DVR). Two brain regions were chosen to serve as neuron-dense controls (HAB) and neuron-sparse controls [LFB; optic tract (OT)].

MATERIALS AND METHODS

Animal Care and Maintenance

Animals. All subjects in this study were sexually mature geckos raised in isolation; hence, they were sexually inexperienced (virgin). They were killed at the age of 1 year. Sexual maturity in the leopard gecko occurs at 45 weeks of age (Tousignant et al., 1995). The following groups were used in this study: females from the low incubation temperature (N = 15), females (N = 13), and males (N = 13) from the female-biased incubation temperature; females (N = 13) and males (N = 9) from the male-biased incubation temperature; and females (N = 12) and males (N = 4) from the high incubation temperature. The sample size for males from the high incubation temperature was low, because males from this incubation temperature are extremely rare. These four males are the only ones our laboratory has ever produced at this incubation temperature.

Egg incubation. Eggs were collected on the day of laying from animals in our captive-breeding population and incubated singly in plastic-wrap-covered plastic cups containing sterilized moist vermiculite (1.5:1 water: vermiculite, by weight). Eggs were incubated in constant temperature ($\pm 0.1^\circ\text{C}$) incubators (Precision Scientific, Chicago, IL) at four different temperatures: 26.0, 30.0, 32.5, and 34°C. Leopard geckos usually lay two eggs per clutch, and eggs from each clutch were put in different incubation temperatures: Eggs from each female during a breeding season were distributed across the four temperatures.

Posthatching environmental conditions. After hatching, each individual was numbered and marked by toe clips and housed in microprocessor-controlled environmental chambers regulating photoperiod, relative humidity, and ambient temperature. This controlled environment simulated natural daily variations experienced in the geckos' native range in Pakistan and western India. During the first 10 weeks of age, hatchlings were exposed to a 14:10 light dark (L:D) photic cycle and 30°C constant temperature. After 10 weeks, all individuals were exposed to a 14:10 L:D cycle and a corresponding 30:18°C daily thermal cycle. Relative humidity ranged from 40% to 70%.

Housing and nutrition. Animals were housed individually in shoebox-sized polypropylene containers (30 cm \times 12 cm \times 6 cm) with a water dish and plastic shelter. Hatchlings were fed three live crickets daily (Flukers Cricket Farm, Baton Rouge, LA.), whereas juveniles and adults were fed mealworms three times per week supplemented with neonatal mice once a week. Crickets and

mealworms were dusted with pulverized dicalcium phosphate and vitamin/mineral powder (Petco Animal Supplies, Atlanta, GA).

Steroid radioimmunoassay

The circulating concentration of sex steroid hormones was determined by using radioimmunoassay (RIA). Blood was collected in microhematocrit tubes and centrifuged at 2,000 RPM for 12 minutes at 10°C. The plasma was then stored in plastic microcentrifuge tubes at -20°C until analysis. Total androgen and total estrogen levels in small plasma samples (50–100 µl) were determined by using RIA following the method of Tousignant and Crews (1995), using specific androgen (6DN no. 337 11-BSA; Wein Labs, Succasunna, NJ) and estrogen antibodies (no. E17-94; Endocrine Sciences, Tarzana, CA). Interassay coefficients of variation were 14% for androgen assays and 19% for estrogen assays. Intraassay coefficients of variation were 8% for androgen assays and 13% for estrogen assays. Using the Bartlett's test for homogeneity (SYSTAT, Evanston, IL), hormone values were found to be nonhomogeneous. Hormone values were homogeneous after log transformation and were compared by analysis of variance (ANOVA) and subsequent Tukey's post hoc tests.

Histology

Brain processing. Each animal was weighed, measured for snout-to-vent length (SVL), head width, and head length, and then killed by rapid decapitation in the late afternoon between 1400 and 1700. Animal protocols were approved by the Institutional Animal Care and Use Committee Review of the University of Texas at Austin and the Office of Protection From Research Risks of the National Institute of Health (assurance no. A-1496). The brain was removed within 3 minutes, while an assistant collected the trunk blood. The brains were frozen in isopentane at approximately -50°C and then stored at -80°C.

Brain sectioning and staining. Brains were sectioned in the coronal plane at 25 µm on a cryostat (Reichert-Jung, Nussioch, Germany). Sequential sections were devoted to 1) volume determinations, 2) CO determinations, and 3) future in situ hybridization. Thus, every third section was used for volume measurements. Sections were thaw mounted on cooled slides (to prevent enzyme degradation) and were kept frozen until staining. Staining for volumetrics followed the method of Crews et al. (1990). Histochemical CO staining followed the methods of Gonzalez-Lima and Cada (1994) and Gonzalez-Lima and Jones (1994).

Volumetric image analysis. Slides were analyzed with an image-processing system consisting of a Javelin camera (model JE2362A; Meyers Instruments, Houston, TX) mounted on a Zeiss light microscope, a Data Translation DT2255 QuickCapture image processor in a Macintosh Quadra computer, and a light box (TrueLite; Meyers Instruments, Houston, TX). To determine volumetrics of specific brain areas, each brain image from the slide was projected onto the computer monitor through the microscope and camera (Fig. 2). The software program BRAIN (Drexel University, NJ) was used to calculate the area of the nucleus encircled. All measurements were done blind, without knowledge of sex or incubation temperature.

The outline of the POA, VMH, HAB, and LFB were traced in one hemisphere, the side being consistent for all

nuclei in an animal. Volumes of the forebrain were measured at a magnification of ×80, and the volumes of the POA, VMH, HAB, and LFB were measured at a magnification of ×320.

The POA measurements began in its most anterior portion: a small, darkly stained, oval-shaped cluster of cells about 150 µm rostral to the first appearance of the third ventricle in coronal sections. This oval cluster enlarges to a triangle-shaped group of cells. The posterior end of the POA usually coincides with the posterior end of the anterior commissure. The boundary between the POA and AH is distinct, because the triangular POA is followed caudally by a rounded columnar AH. The whole POA was measured rather than individual nuclei within the POA, because it was easily distinguished, whereas specific sub-nuclei were not always distinct in every brain section at this level or in every animal. This type of measurement allowed for an overall assessment of differences in POA sizes.

The VMH is located in the caudal diencephalon. The most anterior portion of the VMH is a leaf-shaped group of cells parallel to the midline. Caudally, the VMH widens to a tear-shaped cell group just ventral to the ventricular ependymal organ and then reduces to a small oval shape. The VMH is separated from the ventricle by the PH.

Cross-sectional areas for the brain regions measured were determined in every third section on one side of the brain three times, as in Crews et al. (1990). The volume of each nucleus was then calculated unilaterally with the following formula, where a is the average area of the brain region in a coronal section, a_n is the first area measured for a particular brain nucleus or region, a_{n+1} is the next area measured, and t represents the thickness of three sections of tissue (75 µm). For purposes of calculation, a_{n+1} is equal to a_n for the n th section.

$$\sum_{i=1}^n t \left(\frac{a_n + a_{n+1}}{2} \right)$$

For purposes of analysis, areas of sequential sections were averaged, multiplied by the distance between them (75 µm), and then summed. A fixed portion of the forebrain volume was determined by measuring the right hemisphere in every third section from the beginning of the POA to the end of the VMH. This takes into account brains of different lengths. Measurements were only made on one side of the brain to minimize error due to plane of section. The volumes of specific brain nuclei were divided by forebrain volume to compensate for any individual differences in brain size and to avoid confounding any sex or incubation temperature differences in brain size with sex or incubation temperature differences in brain structure. Other studies have used body size measurements, such as body weight (Grober and Bass, 1991; Grober et al., 1991), standard length (Grober et al., 1991, 1994), brain weight (Gorski et al., 1978; Swaab and Fliers, 1985), and SVL (Crews et al., 1990), to control for allometric differences in brain size. In leopard geckos, SVL correlates with incubation temperature and sex (Tousignant and Crews, 1995), but ANCOVA showed no significant interaction between the covariates SVL ($P = 0.73$) or forebrain volume ($P = 0.46$) and incubation temperature and sex. However, because same-size animals could have different-size brains, forebrain volume represents a more accurate control.

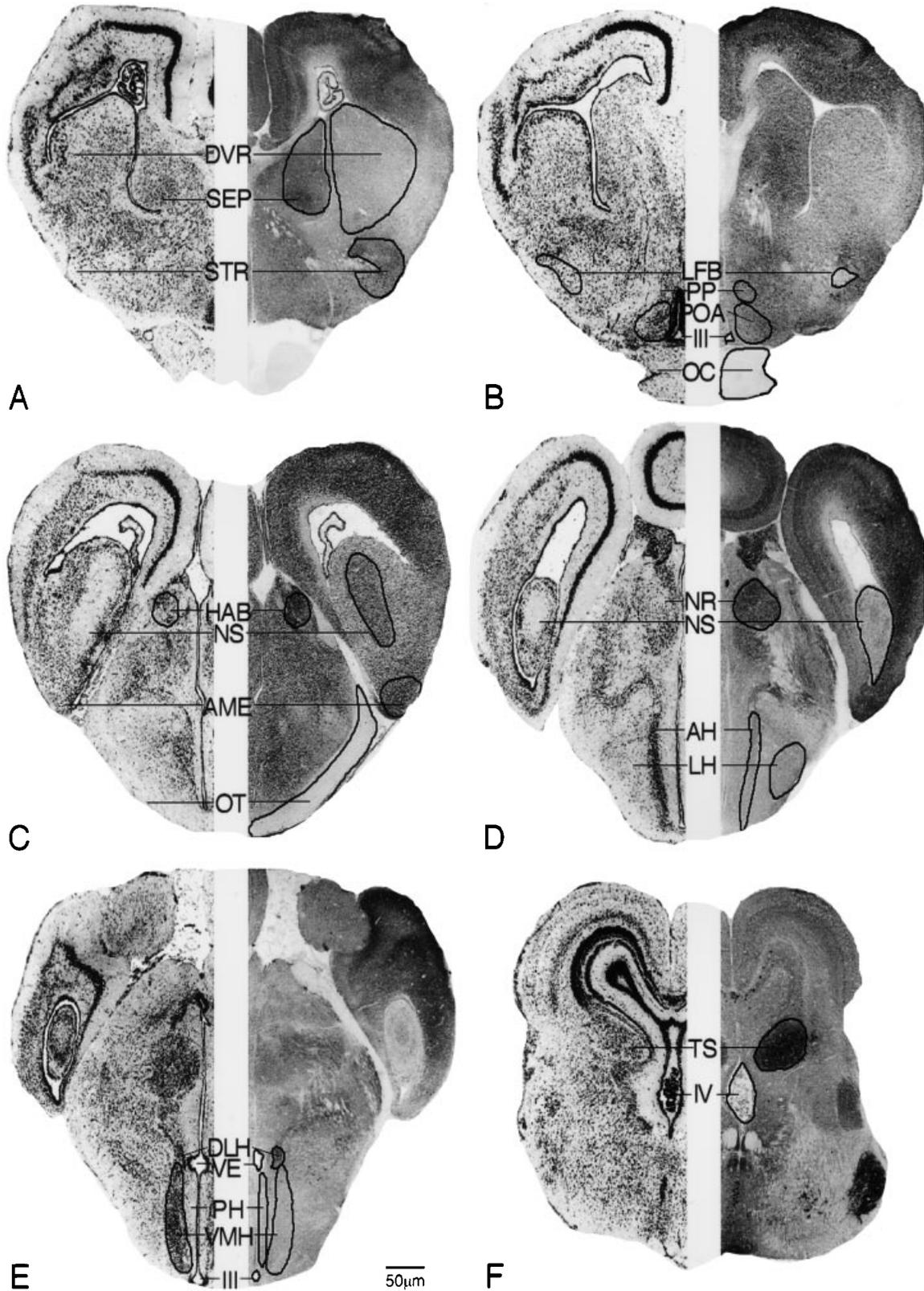


Fig. 2. Adjacent sections from the leopard gecko (*Eublepharis macularius*) stained with cresyl violet (left) or histochemically stained for cytochrome oxidase (CO) activity (right); coronal sections with dorsal to the top and medial to the center of each picture. The brain depicted is from a 1-year-old male from an egg incubated at a male-biased temperature. A-F represent a rostral-caudal series of

sections. Boundaries of the brain areas measured are traced. On the left (A,C,E), the areas measured for volumetrics are traced, whereas, on the right (B,D,F), the areas measured for CO activity are traced. Microscopic images were acquired by video camera and were digitally processed in Adobe Photoshop 2.5.1 (Mountain View, CA). For abbreviations, see list.

To evaluate whether the sex or incubation temperature differences were specific to these two steroid-binding areas (POA and VMH), the volume of areas that do not bind steroids in other vertebrates (HAB and LFB) were also assessed. The HAB is a very cell-dense, oval-shaped cluster located in the dorsomedial corner of the diencephalon, lateral to the third ventricle and dorsal to the distinctly round NR. The LFB is first seen with the initial appearance of the third ventricle lateral to the POA and the medial forebrain bundle. In coronal sections, it is a distinctly round bundle of fibers that becomes continuous with the OT.

Volumetric statistical analysis. All statistical analyses utilized SYSTAT. 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 by using ANOVA, and, if $P \leq 0.01$, then a Tukey's post hoc test was used to determine which groups were significantly different ($P \leq 0.05$).

CO densitometry image analysis. The same image-processing system was used for CO densitometry. The system had a sensitivity to measure 256 gray levels and was calibrated before each measurement session by using a step tablet of absolute optical density (O.D.) standards (Kodak Calibration Tablet no. 2; Kodak, Rochester, NY). The intensity of the CO histochemical reaction product was measured in O.D. units that were transformed to CO activity units. Variability of staining for different incubation batches is a problem with CO histochemistry. To adjust for this variability, adjacent sections of the same rat brain homogenate cut at different thicknesses were stained with each batch of brain sections. The homogenate sections served as internal standards to control for incubation factors affecting stain intensity (Gonzalez-Lima and Garrosa, 1991; Gonzalez-Lima and Cada, 1994; Gonzalez-Lima and Jones, 1994).

For each nucleus, three adjacent sections were measured densitometrically for each animal. Adjacent sections stained with cresyl violet were used to select areas that were difficult to distinguish in the CO-stained slides. Landmarks adjacent to the nuclei were used to ensure that the same portion of each nucleus was measured for each animal. For all measurements, the darkest stained areas (most dense areas) were measured. Four density measurements were taken on each section for a total of 12 measurements/nucleus/animal. These 12 densities were averaged to obtain a mean O.D. for each brain nucleus. By using Sigmaplot (Jandel Scientific, Corte Madera, CA), the mean O.D. values of the rat brain homogenate standards and their CO enzymatic activity, measured spectrophotometrically (Cada et al., 1995), were used to create a regression curve. The brain nuclei O.D. values were then converted to CO activity units (mmol/min/g tissue wet weight) by using this curve.

Sixteen brain regions were analyzed for CO activity (Fig. 2): POA, VMH, AH, AME, DLH, DVR, HAB, LH, NR, NS, OT, PH, PP, SEP, STR, and TS. To evaluate whether the sex or incubation temperature differences were specific to putative steroid-binding areas [POA, VMH, AH, AME, dorsal lateral nucleus of the thalamus (DL), DLH, DVR, LH, NS, PH, PP, SEP, STR, and TS], the metabolic capacity of areas that are unlikely to bind steroids (OT, NR, and HAB) was also assessed.

The DVR, STR, AME, and NS are located in the subcortical region of the telencephalon. The largest delineated neuronal population in the gekkonid telencephalon, the DVR, begins in the far rostral telencephalon and continues into the caudal end of the lateral ventricle, where it merges with the NS at the level of the AH (Smeets et al., 1986). The STR, ventral to the DVR and separated from the DVR by a somata-free area, also begins in the far rostral telencephalon and continues caudally to the level of the anterior commissure. Measurements for CO in the STR were taken in a patch of darkly stained cells just ventral to the somata-free zone. The DVR was homogeneous throughout, with no distinct densely stained areas, so measurements for CO were taken directly dorsal to the measurements taken in the STR. The AME lies lateral to the DVR and dorsolateral to the STR, and it extends rostrally after the posterior end of the STR. The posterior end of the AME forms a triangular point dorsolateral to the NS and surrounded by the lateral ventricle. The darkest stained areas of the AME were found in this triangular tip region. This nucleus is termed the VMN by Greenberg (1982) but is designated AME by others (Smeets et al., 1986; Hoogland and Vermeulen-VanderZee, 1989). The NS, which is considered to be part of the amygdaloid complex (Smeets et al., 1986), contains cells arranged in a cortex-like configuration. The NS begins rostrally with the caudal end of the DVR, forming a cup-shaped structure, and occupies most of the caudal subcortical region of the telencephalon. CO measurements were taken in the inner cortical region that forms an oval-shaped area within the cortical layers of the NS, immediately rostral to the measurement area of the AME.

The SEP forms the medial wall of the hemisphere. Measurements for CO were taken in the medial portion of the posterior septal nucleus, termed the medial septal nucleus by Smeets et al. (1986), which is at the midhemispheric level of the SEP. In sections stained for CO, the medial septal nucleus is a darkly stained circular region that begins just caudal to the anterior end of the POA and ends at the level of the anterior commissure.

In the diencephalon, the PP is a distinct ridge of cells bordering the third ventricle and is located conjointly with the POA. This area stains very dark with cresyl violet but is quite pale in sections stained for CO (see Fig. 2B). The OT appears as two oval or circular regions of white matter ventral to the diencephalon, consisting of fibers tracts that join to form the optic chiasm at the level of the POA. In both cresyl violet-stained sections and sections stained for CO, the OT is distinctly white and free of stain. The AH is found immediately rostral to the POA. In many lizards, this nucleus is continuous with and is often indistinguishable from the POA (Smeets et al., 1986; Crews et al., 1990). However, in the leopard gecko, the columnar shape of the AH distinguishes it from the triangular-shaped POA. The LH is located just lateral to the AH, and, because its cells are more sparse, the LH stains much lighter than the AH in sections stained for CO.

The DLH is composed of medium staining cells (in sections stained with cresyl violet and for CO) and fans out in a dorsolateral direction from the third ventricle, just dorsal to the VMH and PH and dorsolateral to the ventricular ependymal organ (Cruce, 1974; Northcutt, 1978). The PH is found conjointly with the VMH and borders the third ventricle. The NR first appears at the midthalamic level

TABLE 1. Size of Different Brain Areas in Leopard Geckos¹

Region	Incubation temperature (°C)						
	26.0 Females (13.87 ± .60)	30.0 Males (14.13 ± .59)	30.0 Females (13.92 ± .84)	32.5 Males (14.06 ± .90)	32.5 Females (12.79 ± 1.72)	34.0 Males (13.48 ± 1.20)	34.0 Females (13.42 ± .81)
HAB	0.27 ± .02	0.25 ± .03	0.29 ± .04	0.28 ± .03	0.33 ± .06	0.25 ± .06	0.30 ± .05
LFB	0.42 ± .03	0.42 ± .04	0.43 ± .05	0.42 ± .04	0.44 ± .05	0.45 ± .06	0.43 ± .04
POA	0.86 ± .03	1.05 ± .04	0.94 ± .03	1.45 ± .03	1.35 ± .03	1.13 ± .03	1.25 ± .03
VMH	0.91 ± .02	0.83 ± .02	0.86 ± .03	0.80 ± .02	0.80 ± .03	0.89 ± .03	0.93 ± .03

¹The volume of a fixed portion of the forebrain in mm³ (volume) and of different brain areas (region) of adult male and female leopard geckos (*Eublepharis macularius*) from different incubation temperatures (°C) as eggs. Regional values are mean ratios of nucleus volume divided by a fixed portion of forebrain volume × 100 ± standard error. For abbreviations, see list.

TABLE 2. Metabolic Capacity of Different Brain Areas in Leopard Geckos¹

Region	Incubation temperature (°C)						
	26.0 Females	30.0 Males	30.0 Females	32.5 Males	32.5 Females	34.0 Males	34.0 Females
AH	8.72 ± .17	9.65 ± .09	8.83 ± .18	10.40 ± .07	10.12 ± .18	9.27 ± .13	7.93 ± .19
AME	5.88 ± .25	10.14 ± .24	7.16 ± .27	10.12 ± .20	10.42 ± .27	9.86 ± .36	9.15 ± .28
DLH	13.06 ± .17	13.11 ± .23	15.76 ± .18	14.32 ± .19	15.70 ± .18	14.62 ± .35	11.80 ± .20
DVR	6.09 ± .10	9.14 ± .10	5.42 ± .10	8.57 ± .08	8.27 ± .10	8.18 ± .15	6.45 ± .11
HAB	14.98 ± .14	14.92 ± .17	15.15 ± .16	14.74 ± .14	14.87 ± .16	14.53 ± .25	15.18 ± .16
LH	8.50 ± .13	8.45 ± .14	8.49 ± .14	8.55 ± .12	8.58 ± .14	8.20 ± .21	8.46 ± .15
NR	14.19 ± .10	14.19 ± .10	13.99 ± .11	14.15 ± .09	14.22 ± .11	14.19 ± .16	13.96 ± .16
NS	9.65 ± .13	11.69 ± .08	10.06 ± .14	12.25 ± .06	11.54 ± .14	10.73 ± .11	9.70 ± .14
PH	9.26 ± .09	8.64 ± .10	9.75 ± .09	9.20 ± .08	9.02 ± .09	9.13 ± .14	9.85 ± .10
POA	6.14 ± .18	8.80 ± .24	4.96 ± .19	7.46 ± .18	7.11 ± .19	7.32 ± .32	5.56 ± .20
PP	6.18 ± .23	7.38 ± .42	3.94 ± .25	5.08 ± .31	6.32 ± .25	8.45 ± .55	4.56 ± .26
SEP	12.80 ± .13	13.27 ± .14	14.41 ± .14	14.75 ± .12	14.90 ± .14	14.03 ± .20	13.85 ± .14
STR	11.87 ± .08	14.23 ± .08	12.72 ± .08	13.20 ± .07	13.21 ± .08	14.39 ± .13	12.18 ± .09
TS	13.84 ± .13	15.57 ± .17	13.13 ± .14	14.45 ± .14	14.37 ± 1.4	14.81 ± .25	14.44 ± .15
VMH	12.41 ± .14	10.62 ± .17	14.07 ± .16	9.00 ± .14	12.23 ± .16	13.46 ± .25	12.62 ± .17

¹The cytochrome oxidase (CO) metabolic activity in different brain areas (region) of adult male and female leopard geckos (*Eublepharis macularius*) from different incubation temperatures (°C) as eggs. Values are group means ± standard error (μmol/minute/g tissue wet weight). For abbreviations, see list.

and has a distinctly round shape with darkly staining cells (Northcutt, 1978).

In the mesencephalon, the TS is a very large region ventromedial to the optic tectum. In sections stained for CO, the central nucleus is the darkest staining portion of the TS. This large area of highly metabolically active cells begins as an oval patch of cells just ventrolateral to the “arms” of the fourth ventricle as the ventricle forms a Y shape. Rostrally, the central nucleus moves inward, until the left and right central nuclei fuse to form one large central nucleus (Foster and Hall, 1978). Densitometry measurements were taken in the three sections anterior to this fusion.

CO densitometry statistical analysis. All statistical analyses were done by using SYSTAT. The mean CO 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 CO activity unit means and the temperatures and sexes were compared by using ANOVA to verify that there was no significant interaction between covariate (nucleus CO activity) and treatment (temperature and sex). The transformed CO activity unit means were then compared by using ANCOVA, and, if $P \leq 0.01$, then a Tukeys post hoc test determined which individual group comparisons were significantly different ($P \leq 0.05$).

RESULTS

Group means and standard errors of volume and transformed CO 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. All reported differences were significant at $P \leq 0.04$.

Between-sex differences in the effect of gonadal sex at each incubation temperature

Volumetrics. The relative brain volumes were not significantly different between males and females from the female-biased incubation (30°C) and high (34°C) incubation temperatures except at the male-biased incubation temperature (32.5°C), where males were larger than females. The volumes of the HAB and LFB were not significantly different between males and females (Table 1).

The volume of the POA and VMH depended on incubation temperature, not gonadal sex. For example, whereas there were sex differences in the POA when comparing across incubation temperatures, comparison of males and females from the same incubation temperature revealed no significant sex difference (Table 1). In addition, the volume of the POA of males from the female-biased incubation temperature was significantly larger than the POA of females from the low and male-biased incubation temperatures and the high incubation temperature, whereas the volume of the POA of males from the male-biased incubation temperature was larger than the POA of females from the low, female-biased and the high incubation temperatures. The volume of the POA of males from the high incubation temperature was larger than the POA of females from the low incubation temperature but was smaller than the POA in females from the male-biased incubation temperature and tended to be larger than females from the female-biased incubation temperature ($P = 0.09$). Similarly, the VMH volume was not signifi-

cantly different between males and females from each incubation temperature (Table 1).

CO activity. Although sex had a major influence in CO activity, the sexual dimorphisms in metabolic capacity in specific brain nuclei varied with incubation temperature. No significant differences were found between males and females from the female-biased incubation temperature in the metabolic capacity of the HAB and NR (Table 2). The OT measured 0 or less than 10 CO activity units for all animals.

At the female-biased incubation temperature, males had greater metabolic capacity compared with their female counterparts in the POA, AME, DVR, STR, NS, PP, and TS, and AH, whereas females had greater metabolic capacity compared with males in the VMH, SEP, PH, and DLH. Males and females were not significantly different in metabolic capacity in the LH.

At the male-biased incubation temperature, males had significantly greater metabolic capacity compared with their female counterparts in the NS; however, females had greater metabolic capacity compared with males in the VMH and DLH. Males and females were not significantly different in metabolic capacity in the POA, PP, AME, SEP, DVR, STR, AH, LH, PH, and TS.

At the high incubation temperature, males had significantly greater metabolic capacity compared with females in the POA, STR, PP, DLH, DVR, AH, and NS, and, in contrast, females had greater metabolic capacity compared with males in the PH. Males and females from the high incubation temperature were not significantly different in metabolic capacity in the VMH, AME, SEP, LH, and TS.

Hormones. Androgen levels were significantly higher in males than in females. Although mean plasma levels of total estrogens were higher in females than in males from the female-biased and high incubation temperatures, these were not statistically significantly different. However, in animals from the male-biased incubation temperature, females had significantly higher levels of estrogens than males.

Effect of incubation temperature in males

Volumetrics. The volumes of the forebrains were not significantly different between males from the female-biased, male-biased, and high incubation temperatures (Table 1). The volumes of the HAB and LFB were also not significantly different among these males. Males from the male-biased incubation temperature had a larger POA than males from female-biased and high incubation temperatures: The VMH was significantly smaller in males from the male-biased incubation temperature compared with males from the high incubation temperature, but other between-group comparisons were not statistically different due to the large variance (Table 1).

CO activity. The CO activities of the HAB, NR, AME, and LH were not significantly different among males from the female-biased, male-biased, and high incubation temperatures (Table 2). The OT measured 0 or less than 10 CO activity units for all animals. Males from the female-biased incubation temperature had greater metabolic capacity than males from both the male-biased and the high incubation temperatures in the POA, DVR, and TS. Males from the male-biased incubation temperature had greater metabolic capacity than males from the female-biased and high incubation temperatures in the SEP, AH, and NS.

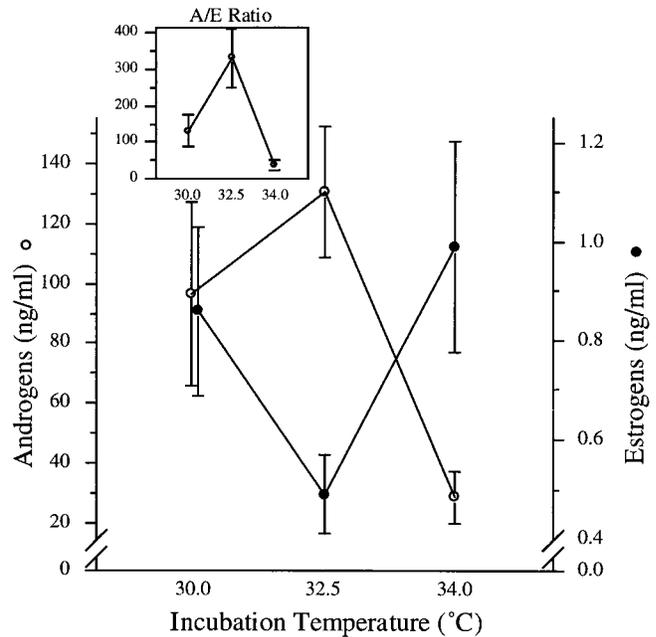


Fig. 3. Plasma levels of androgen and estrogen in male leopard geckos (*Eublepharis macularius*) from different incubation temperatures. Vertical bars represent standard errors. **Inset:** The average androgen/estrogen (A/E) ratio of the same animals.

Males from incubation temperatures that produced mostly females (female-biased and high incubation temperatures) had greater metabolic capacity than males from male-biased incubation temperatures in the VMH, STR, and PP. Males from the warmer incubation temperatures (male-biased and high incubation temperatures) had greater metabolic capacity than males from the cooler, female-biased incubation temperature in the PH and the DLH.

Hormones. Plasma levels of total androgens were significantly correlated with the sex ratio in males. Males from the male-biased incubation temperature had the highest levels, with males from the female-biased and high incubation temperatures having medium and the lowest levels, respectively (Fig. 3). The opposite pattern of significant correlations was found in the circulating concentration of estradiol, with males from the high incubation temperature having the highest levels and with males from the female-biased and male-biased incubation temperatures having medium and the lowest levels, respectively (Fig. 3). Similarly, the androgen/estrogen ratio indicated that males from the male-biased incubation temperature had the highest levels compared with males from the other incubation temperatures (Fig. 3, inset).

Effect of incubation temperature in females

Volumetrics. The volumes of the forebrain, HAB, and LFB did not significantly vary among females from the four incubation temperatures (Table 1). Females from the male-biased incubation temperature had a larger POA than females from low and female-biased incubation temperatures. Females from the high incubation temperature had a larger VMH than females from the male-biased incubation temperature, but other between-group comparisons were not statistically different due to the large variance (Table 1).

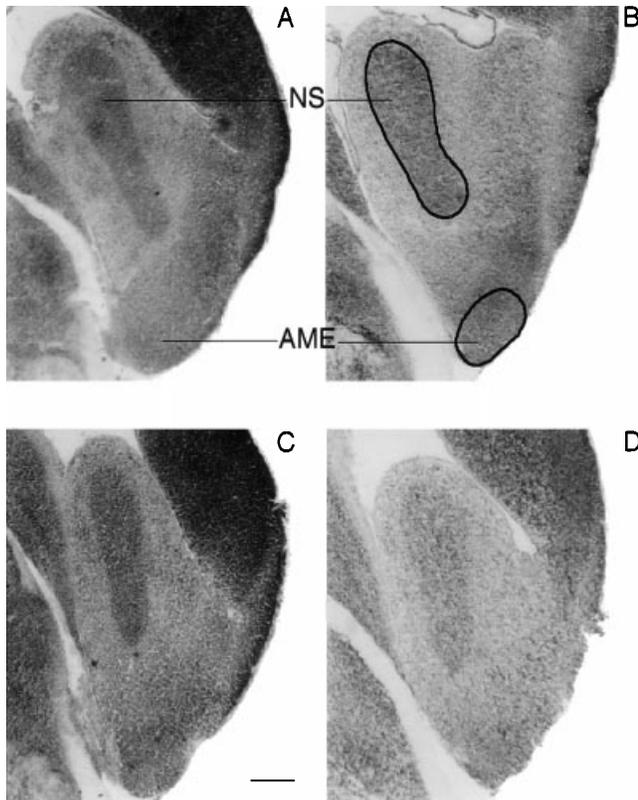


Fig. 4. Coronal sections of the brain of female leopard gecko (*Eublepharis macularius*) from different incubation temperatures: 26°C (A), 30°C (B), 32.5°C (C), and 34°C (D). Dorsal is on the top, and lateral is to the right of each picture. Microscopic images were acquired by video camera and were digitally processed by application of a green separation filter in Adobe Photoshop 2.5.1 (Mountain View, CA). For abbreviations, see list. Scale bar = 50 μ m.

CO activity. The CO activities of the HAB and NR were not significantly different among females from the various incubation temperatures (Table 2). The OT measured 0 or less than 10 CO activity units for all animals. Females from the female-biased incubation temperature had a significantly greater metabolic capacity in the VMH than females from the other incubation temperatures. Females from the male-biased incubation temperature had a significantly greater metabolic capacity than females from the other incubation temperatures in the following brain nuclei: POA, AME, SEP, DVR, STR, AH, and NS (Fig. 4).

The CO activity in the PP, PH, and DLH also varied according to incubation temperature: Females from the low and male-biased incubation temperatures had significantly higher metabolic capacity in the PP and significantly lower metabolic capacity in the PH than females from the female-biased and high incubation temperatures. Females from the female-biased and male-biased incubation temperatures had significantly higher metabolic capacity in the DLH than females from the low and high incubation temperatures (Table 2).

Hormones. There was no significant difference in the circulating concentrations of androgen and estrogen among females from the different incubation temperatures.

Summary of principal findings

The volume of the POA and VMH varies with incubation temperature and not sex. Similarly, within those incubation temperatures that produce both sexes, brain area volumes of males and females do not differ. However, both males and females from the male-biased incubation temperature have a larger POA compared with geckos from the other incubation temperatures.

Metabolic capacity of brain nuclei is influenced by sex and incubation temperature. For example, CO activity is greater in the AH and POA of males and in the VMH of females in geckos from female- and male-biased incubation temperatures. Males from the male-biased incubation temperature have greater CO metabolic activity in the AH, NS, and SEP compared with males from the other incubation temperatures. In contrast, males from the female-biased and high (all-female) incubation temperatures have greater CO metabolic activity in the VMH compared with males from the male-biased incubation temperature. Females from the female-biased incubation temperature have greater CO metabolic activity in the VMH compared with females from the other incubation temperatures, whereas females from the male-biased incubation temperature have greater CO metabolic activity in the AH, POA, and NS compared with females from the other incubation temperatures.

DISCUSSION

Although there are no data currently available on the function of the brain areas measured in the leopard gecko, this and other laboratories have studied the function of some of these areas in other lizard species. These findings in general are consistent with research on other vertebrates; hence, they reflect an evolutionary conservation of structure and function.

Gonadal steroids exert effects on morphogenesis and survival of specific neurons, resulting in marked sex differences in brain nuclei (Arnold and Gorski, 1984; Crews, 1993). Although the results of this study indicate that the volume of the POA and VMH were determined primarily by incubation temperature, the individual's gonadal sex also influenced their metabolic capacity. Changes in CO activity are dynamically coupled with differences in the sustained functional demands of neurons (Wong-Riley, 1989). Sexual dimorphisms have been identified in neurochemical activity, so a similar dimorphism may be expected in oxidative metabolic capacity (Egozi and Sokolovsky, 1986). In male leopard geckos, the higher CO activity measured in the POA and AH compared with that measured in females may be indicative of increased metabolic capacity of these nuclei for male-typical courtship and copulatory behaviors. Conversely, the higher CO activity measured in the VMH of female leopard geckos compared with males may indicate increased metabolic capacity for the VMH in the regulation of female-typical sexual receptivity. In various vertebrates, the higher metabolic capacity in the AH and POA in males parallels their higher androgen receptor levels in these brain areas, and the higher metabolic capacity in the VMH in females parallels their higher estrogen and progesterone receptor levels in this brain area (Young and Crews, 1995). A similar sexual dimorphism in brain oxidative metabolism is exhibited in gray tree frogs (Marler et al., 1992).

The higher CO activity in the AH, AME, POA, DVR, and STR of males compared with females from the female-biased incubation temperature is similar to the sexual dimorphisms in glucose metabolism reported in whiptail lizards. In the little striped whiptail lizards, *Cnemidophorus inornatus*, males exhibit a greater uptake of [^{14}C]2-fluoro-2-deoxyglucose (2DG) in the AH compared with conspecific females. The all-female parthenogenetic *C. uniparens* exhibits a metabolic dimorphism in the POA and VMH, depending upon whether it displays male- or female-like pseudosexual behavior (Rand and Crews, 1995). Because *C. uniparens* induced to pseudocopulate does not show elevated 2DG uptake in the AH, this suggests that the sexual dimorphism in this brain area is strictly related to genetic sex and not to hormone treatment or behavior. Another sexual dimorphism is found in the red-sided garter snake, *Thamnophis sirtalis parietalis*, in which males have a larger NS than females (Crews et al., 1993). In the present study, male leopard geckos had greater metabolic capacity in the NS than females at all incubation temperatures.

Sexual dimorphisms in the metabolic capacity of the PP in leopard geckos was found to be the opposite of what would be expected: CO activity in the PP was greater in males than in females except in animals from the male-biased incubation temperature, in which males and females were not significantly different. In other vertebrates, this brain region may be more active in females than in males. For example, in the rat, the anteroventral periventricular nucleus of the POA (AVPvN-POA) is larger and more cell dense, and it contains greater numbers of tyrosine hydroxylase- and cholecystokinin-containing neurons in females than in males (Simerly et al., 1985; Micevych et al., 1987). However, the greater CO activity found in leopard gecko males may not be related to cell number but, rather, to cell communication. In rats, synaptic connections in the AVPvN-POA are greater in males than in females. Perhaps increased neuronal transmissions result in increased metabolic demands. The most energy-demanding function in neurons is the operation of the sodium/potassium pumps (Sokoloff et al., 1989; Gonzalez-Lima, 1992).

Within-sex differences are also instructive. In rats, mice, and gerbils, the position that a fetus develops in utero relative to the other developing fetuses determines the hormonal milieu it experiences during development (Clark et al., 1991; vom Saal, 1991). Androgens excreted by male fetuses expose female fetuses located between two males (2M females) to higher levels of exogenous androgen compared with female fetuses located between two females (2F females; Clark et al., 1991). The relative position of the fetus is correlated to the sociosexual behavior displayed by the adult animal: 2M females have a masculinized phenotype, mature later, have lower estrogen levels and higher testosterone levels, are more aggressive, and are less attractive to males compared with 2F females (Clark et al., 1991; vom Saal, 1991). Similarly, 2F males have higher levels of estradiol, are less aggressive, mount females less quickly, require more intromissions to ejaculate, and sire fewer offspring than 2M males (vom Saal et al., 1983; Clark et al., 1990, 1991). Intrauterine position effects can also influence brain metabolism. CO activity in the area equivalent to the sexually dimorphic area of the POA (SDA-POA) and in the posterior AH is greater in 2M compared with 2F female gerbils (Jones et al., 1997). The

SDA-POA is critical in the regulation of male-typical behaviors in gerbils (Yahr, 1995), and the posterior AH is an area with neurons containing gonadotropin-releasing hormone (Everett, 1994; Silverman et al., 1994), which may explain the physiological differences between 2M and 2F females.

The effects of incubation temperature on the behavior, physiology, and neurobiology of leopard geckos parallel those found in rodents affected by the intrauterine position phenomenon. Incubation temperature in the leopard gecko regulates attractivity and aggression (Flores et al., 1994; Flores and Crews, 1995), growth rate and age of sexual maturity (Tousignant and Crews, 1995), and endocrine state and morphological characteristics (Gutzke and Crews, 1988; Tousignant et al., 1995). The masculinizing effects of the male-biased incubation temperature may be indicated by 1) the larger POA and higher metabolic capacity in the SEP, AH, and NS of males from this incubation temperature compared with males from the other incubation temperatures and 2) the greater metabolic capacities in the POA and AH observed in female leopard geckos from this incubation temperature compared with females from the other incubation temperatures. In those lizards in which the neural control of behavior has been studied, the AH, POA, SEP, AME, and NS are important in the control of sexual and aggressive behaviors (Crews, 1979; Crews and Silver, 1985; Distel, 1977; Greenberg et al., 1984).

Conversely, possible feminizing effects of incubation temperatures were evident in the pattern of brain morphology and metabolic physiology in males. The volume of the POA and the CO activity in the VMH were correlated with the plasma levels of estrogen in males from different incubation temperatures. Males from the high incubation temperature had the highest circulating level of estrogen and the greatest metabolic capacity in the VMH compared with males from the other incubation temperatures. Males from the female-biased incubation temperature had the next highest circulating levels of estrogens (see also Tousignant and Crews, 1995; Tousignant et al., 1995) and the next greatest metabolic capacity in the VMH. Males from the male-biased incubation temperature had the lowest estrogen levels, the largest POA, and lowest metabolic capacity in the VMH. Steroid hormones, such as estrogens, affect nerve cell structure and function by acting through receptors to alter gene product expression (Nabekura et al., 1984; Kawashima and Takagi, 1994; McEwen, 1994). Thus, in males from the female-biased and high incubation temperatures, the smaller volumes of the POA, higher metabolic capacities in the VMH, and higher plasma levels of estrogen compared with males from the male-biased incubation temperature may be indicative of the feminizing effects of this incubation temperature.

A difference in the level of estrogen receptor (ER) could also affect metabolic capacity of certain brain nuclei. ER level during development is the earliest hallmark of brain sexual differentiation in rats, and, in male rats, testosterone reduces ER abundance in the mPOA, AVPvN-POA, and VMH (Kühnemann et al., 1994). Perhaps a similar phenomenon occurs in male leopard geckos. This may account for the finding that males from the male-biased incubation temperature (which had the highest plasma level of androgen), compared with males from the other incubation temperatures, had lower CO activity in the PP and VMH: CO activity in the POA was lower than that of males from the female-biased incubation temperature

only. These brain areas are known to contain high levels of ER mRNA in lizards and in other vertebrates (Morrell and Pfaff, 1978, 1982; Watson and Adkins-Regan, 1989; Young and Crews, 1995).

Nerve cell structure and function are also affected by androgens. Similar to the effects of estradiol in the VMH, testosterone in neonatal rats enhances the survival of neurons and increases dendritic growth in the POA and hypothalamic cell cultures from neonatal rats (Kawashima and Takagi, 1994). These effects of testosterone on neuronal growth could be one explanation for the large POA in male leopard geckos from the male-biased incubation temperature that have significantly higher levels of circulating androgens than males from the other incubation temperatures. Exogenous androgens also increase dopamine (DA) content in hypothalamic neurons. In neonatal rats, testosterone stimulates DA synthesis in hypothalamic cells but has no effect on neurons in the AVPvN-POA (Kawashima and Takagi, 1994). These androgen effects may contribute to the high level of metabolic capacity in the AH and low metabolic capacity in the PP of male leopard geckos from the male-biased incubation temperature compared with males from the other incubation temperatures (Crews et al., 1997).

In males from the female-biased incubation temperature, compared with males from the male-biased incubation temperature, the small POA plus the low metabolic capacity in the SEP and AH may be factors in their reduced aggression toward female stimulus animals (Flores and Crews, 1995). The AH and SEP are involved in the regulation of dominance behaviors in lizards (Crews, 1979; Greenberg et al., 1984) and in hamsters (Kollack-Walker and Newman, 1995). Thus, males from the female-biased incubation temperature that exhibit reduced levels of aggression might be expected to exhibit low metabolic capacity in those brain nuclei involved in the control of agonistic behaviors. A similar correlation between neuroanatomy and metabolic neurophysiology and behavior is evidenced in females from the low, female-biased, and high incubation temperatures (small POA and low metabolic capacities in the AH and SEP). These relationships may be attributable to the feminizing effects of these incubation temperatures.

Females from the low incubation temperature had lower CO activity measurements than females from the other incubation temperatures. Otherwise, females from the female-biased incubation temperature showed brain morphologic and CO activity measurements that were usually intermediate to that of females from the other incubation temperatures. These females had a larger POA than females from the low incubation temperature but smaller than that of females from the male-biased and high incubation temperatures. In addition, CO activity of the AME, SEP, STR, AH, NS, and PH in females from the female-biased incubation temperature was also intermediate to the CO activity of these nuclei in females from the other incubation temperatures.

The behavioral, neuroanatomical, and physiological differences in females from the male-biased incubation temperature are indicative of the masculinizing effects of this incubation temperature. That is, the small size and reduced CO activity of the POA and the high metabolic CO activity of the VMH may directly affect the endocrine physiology and sociosexual behavior of females from the low, female-biased, and high incubation temperatures

compared with females from the male-biased incubation temperature. Populations of neurons containing gonadotropin-releasing hormone have been described in the area of the POA in all vertebrates studied to date (Silverman et al., 1994). A previous study (Gutzke and Crews, 1988) found that females from the male-biased incubation temperature had higher circulating levels of testosterone and lower levels of estrogen relative to females from the other incubation temperatures, an observation that may relate to the pattern of brain morphology and metabolic activity exhibited by these females. However, in the present study, androgen and estrogen levels were not correlated with incubation temperature among females. This discrepancy may be related to differences in reproductive state and age of the animals in these studies: Independent research indicates that the circulating levels of androgen in females are affected both by reproductive condition and by age (Tousignant et al., 1995; our unpublished data).

The AME, NS, and SEP are critical neural substrates for sexual and aggressive behaviors in reptiles (Crews, 1979; Greenberg et al., 1984). The high metabolic capacity in these brain regions in females from the male-biased incubation temperature compared with females from the other incubation temperatures could be linked to these females' greater aggression toward males (Gutzke and Crews, 1988; Flores et al., 1994). Conversely, the low metabolic capacity in the AME, NS, and SEP of females from the low incubation temperature may be a factor in these females' reduced aggression toward male and female stimulus animals (Flores and Crews, 1995).

This experiment has determined for the first time how factors other than gonadal sex hormones arising from an individual's genetic constitution can affect the sexual differentiation of a vertebrate brain. Incubation temperature determines much of the individual and sexual variation in behavior, endocrinology, and morphology of the leopard gecko. These differences, in turn, are reflected in differences in the size and metabolic capacity of specific regions of the brain. Although gonadal sex did not affect the size of the brain nuclei measured, it did influence the metabolic capacity of specific nuclei in the adult brain. It remains to be determined exactly how this organization is accomplished. In the leopard gecko and in other reptiles with temperature-dependent sex determination, the embryo is sensitive to temperature only during the midtrimester of development (Tousignant and Crews, 1994). Because eggs were incubated at a constant temperature throughout embryogenesis in the present experiment, it is not known whether the effects of temperature on the brain are also restricted to the midtrimester. In this study, the only time individuals differed was during incubation; thereafter, all animals experienced the same rearing regimens. It would also be useful to know whether the brain is dimorphic at birth or whether these differences emerge during postnatal development.

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