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Effects of age and sociosexual experience on the morphology and metabolic capacity of brain nuclei in the leopard gecko (*Eublepharis macularius*), a lizard with temperature-dependent sex determination

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Abstract

In vertebrates having sex chromosomes, sexual behavior is influenced by steroid hormones throughout life as well as by the cumulative experiences of the individual. Because males and females differ genetically as well as hormonally, it would be valuable to distinguish the contribution of sex-specific genes from hormones. In addition, since animals age as they gain sociosexual experience, but do not necessarily gain sociosexual experience as they age, it is important to separate the effects of age from those attributable to experience. The leopard gecko is a lizard lacking sex chromosomes, depending instead upon the temperature during incubation to establish gonadal sex. This effectively removes sex-specific genetic influences from any study of sexual differentiation. Eggs were incubated at either 26°C or 32.5°C, temperatures that produce only female hatchlings or a male-biased sex ratio, respectively. By raising geckoes in isolation and then housing some animals together in breeding groups at different ages after they attained sexual maturity, it was possible to assess the relative effects of age and sociosexual experience on the volume and metabolic capacity of limbic and non-limbic brain areas. In general, males showed more changes compared to females. For example, there was a decrease with age in the volume of the preoptic area and the ventromedial hypothalamus in males, but not in females. Both age and sociosexual experience influenced cytochrome oxidase activity in these and other brain areas. Experienced animals had greater metabolic capacity in nuclei functionally associated with sociosexual behavior in lizards and other vertebrates. For example, cytochrome oxidase activity was higher in the anterior hypothalamus of males, in the ventromedial hypothalamus of both males and females from the male-biased incubation temperature, and in the preoptic area of females from both incubation temperatures. These differences were not paralleled by differences in circulating levels of sex hormones; only plasma androgen levels differed as a function of experience in males. These data suggest that the volume and metabolic capacity of specific brain regions change as animals age and gain sociosexual experience, but the nature and degree of change depend upon prenatal events.

Keywords: Reptile; Prenatal; Embryo; Differentiation; Brain; Age; Sexual experience

1. Introduction

Gonadal sex hormones are necessary for the expression of sexual behavior in many vertebrates. However, an individual's sociosexual experience not only can modify its mating behavior, but can also change how the adult responds to sex steroid hormones [31,32]. In guinea pigs, the amount of sexual activity experienced as a juvenile affects the level of sexual behavior displayed as an adult, as well as modifies the individual's response to castration and androgen replacement therapy [46]. In cats, males that have had sexual experience before castration persist in displaying sexual behavior, whereas males that are sexually naive prior to castration show an abrupt cessation in sexual behavior; further, castrated, sexually naive, male cats given exogenous testosterone take longer to exhibit sexual behavior compared to castrates who have had sexual experience [40]. In gulls, males reared in social groups until sexually mature and then subjected to social isolation for 2 weeks exhibit lower levels of sexual behavior in response to exogenous testosterone compared to similarly reared males exposed to unfamiliar conspecifics [22]. If a female zebra finch is raised in a mixed sex group, she will

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prefer the company of males when she reaches adulthood [35]. However, if she is raised in an all-female group, she will associate more with females and, if also treated with exogenous estrogen, will preferentially pair with females.

Behavioral and physiological differences among individuals of different ages and having different experiences are likely to be reflected in the brain [31,45]. While sociosexual experience influences an individual's behavior, to the best of our knowledge, little is known how such experience might influence the morphology and metabolic capacity of brain nuclei involved in the regulation of these behaviors [47]. Studies with a variety of mammals indicate that social and sexual behaviors as well as pheromones and genital stimulation can influence the expression of the immediate early gene c-*fos* in those brain areas involved in aggression and mating [3,16,18,29,30,39,41]. In whiptail lizards, the behavior of the partner regulates the abundance of steroid receptor in the brain independently of the gonads [24]. Thus, sociosexual experience may mediate the effects of steroid hormones by affecting the individual's sensitivity to hormone, perhaps by influencing receptor density and/or the metabolism of the hormone. The best information available comes from research on the telencephalic neural system that underlies song in birds [38]. In male canaries and zebra finches, singing behavior varies according to seasonal and hormonal fluctuations, which in turn is paralleled by changes in the structure and size of brain nuclei involved in song [5,15]. This does not appear to be a causal relationship, however. If marsh wrens are given the opportunity to learn large or small repertoires of song, the size of associated song control nuclei does not increase in birds that learned the larger song repertoire [4].

Nervous tissue function depends on oxidative metabolism for energy production. Cytochrome oxidase (CO) is a mitochondrial enzyme and has a rate-limiting role in oxidative metabolism. Thus, CO activity is an excellent marker of cellular metabolic capacity: sustained increases or decreases of neuronal activity in a brain nucleus lead to corresponding increases or decreases in CO activity [48]. The histochemical quantification of CO activity provides a useful functional index of regional metabolic capacity in the brain [21]. Further, age-related modifications of CO activity have been found in brain structures of rats [14], cats [26], and humans [49].

This study investigated the effects of age and sociosexual experience on the sexual differentiation of the brain in adult leopard geckos (*Eublepharis macularius*). In the leopard gecko, the sex ratio varies with incubation temperature: 26°C produces only female hatchlings, whereas 32.5°C produces a male-biased sex ratio. Experiments indicate that the incubation temperature of the egg has profound effects on the growth [42,43], morphology [43], and physiology [23,42–44] of the adult. Leopard geckos from different incubation temperatures also differ significantly both between and within a sex in their aggressive and sexual behavior [19,20,23]. Further, the volume of specific brain nuclei known to control these behaviors in lizards and other vertebrates is determined by the temperature of the egg experienced during development, while the metabolic capacity of these brain nuclei are affected principally by incubation temperature, and secondarily by gonadal sex [9,12]. This study examined whether the volume and metabolic capacity of brain nuclei of animals that differ in their prenatal environment (incubation temperature) can be modified further by their postnatal environment (age or sociosexual experience).

2. Materials and methods

General procedures regarding animal care, histology, and radioimmunoassay are detailed in ref. [9]. In the leopard gecko, sexual maturity occurs at 45 weeks and animals typically live for 5–7 years. Thus, the terms "young" (= 1 year of age) and "old" (= 2–3 years of age) are relative and do not imply immaturity and senescence. Inexperienced animals were housed in isolation from egg to sacrifice. Experienced animals were housed at sexual maturity (45 weeks of age) in breeding groups consisting of one male and four females and had completed one or more reproductive seasons.

To examine the effects of age and sociosexual experience (both separately and combined) in male and female leopard geckos from an all-female (26°C) and a male-biased (32.5°C) incubation temperature, 12 experimental groups would be required. While it was not possible to establish all of the necessary groups, a subset of nine groups provided a valid test of the hypotheses. Two groups of females, one from each of the two incubation temperatures, and one group of males from the male-biased incubation temperature, representing the following four conditions (young, old, inexperienced, experienced) were used in this study.

2.1. Young, inexperienced

Females ($n = 15$) from 26°C incubation temperature and females ($n = 13$) and males ($n = 13$) from the 32.5°C incubation temperature.

2.2. Old, inexperienced

Males from the 32.5°C incubation temperature ($n = 13$). Old, inexperienced females from either the 26°C or the 32.5°C incubation temperatures were not available for this study.

2.3. Young, experienced

Females from the 26°C ($n = 6$) and 32.5°C incubation temperature ($n = 6$) taken during a breeding season. Young, experienced males were not available for this study.

2.4. Old, experienced

Females ($n = 12$) from the 26°C incubation temperature, and females ($n = 8$) and males ($n = 10$) from the 32.5°C incubation temperature.

Thus, the groups compared for *age* effects in *males* consisted of inexperienced animals, whereas in *females* they consisted of experienced animals. The groups compared for *sociosexual experience* effects in *males* consisted of old animals, whereas in *females* they consisted of young animals. All statistical analyses were done using SYSTAT software (Evanston, IL).

2.5. Qualitative cytochrome oxidase histochemistry

Cytochrome oxidase standards were generated from the brains of 12 adult male rats. Two sets of brain paste standards were included with each incubation. Slides were processed for CO quantitative histochemistry using previously described procedures [6,21]. Briefly, slides were treated in 10% sucrose phosphate buffer (0.1 M, pH 7.6) containing 0.5% glutaraldehyde for 5 min to facilitate adherence of the sections and standards to the slides. Four changes of 10% phosphate buffer at 5 min each were then used. Slides were preincubated for 10 min in Tris buffer (0.05 M, pH 7.6) containing 275 mg/l cobalt chloride, 10% sucrose, and 0.5% dimethylsulfoxide. The slides were then rinsed in the phosphate buffer and incubated at 37°C for 60 min in 700 ml of an oxygen-saturated reaction solution containing 350 mg diaminobenzidine tetrahydrochloride, 52.5 mg cytochrome *c*, 35 g sucrose, 14 mg catalase, and 1.75 ml dimethylsulfoxide in phosphate buffer. To stop the reaction and fix the tissue, a 30 min immersion in 10% sucrose phosphate buffer with 4% formalin (v/v) was used before dehydrating, clearing, and cover-slipping with Permount.

To spectrophotometrically measure CO activity, a solution of 1.0% cytochrome *c* in 0.05 M potassium phosphate buffer (pH 7.0) was reduced with sodium ascorbate. The excess reducing agent was removed by dialysis against 0.05 M potassium phosphate buffer for 24 h (3 changes) and was diluted to 0.07% cytochrome *c* in 0.05 M potassium phosphate buffer and chilled on ice. A 20% homogenate consisting of 1.00 g of tissue paste was suspended in 4 ml isolation buffer (0.32 M sucrose, 1 mM dipotassium EDTA, 10 mM Tris, pH 7.4) and chilled on ice. The assay mixture had final concentrations of 0.25% tissue and 0.5% sodium deoxycholate; it was equilibrated at room temperature (22°C) for 5 min with frequent gentle mixing and then placed on ice.

For the assay, 10 ml of the assay mixture and 1 ml 0.07% cytochrome *c* were reacted together at 37°C. The change in absorbance at 550 nm was taken for 1 min beginning immediately after placement of the cuvette in the spectrophotometer. Activity units were defined at pH 7 and 37°C where 1 unit oxidizes 1 mmol of reduced cytochrome *c* per min (μmol/min/g tissue wet weight).

2.6. 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). The outline of the preoptic area (POA), ventromedial nucleus of the hypothalamus (VMH), habenula (HAB) and lateral forebrain bundle (LFB) were traced in one hemisphere; the side being consistent for all nuclei in an animal. Volumes of the forebrain were measured at 80 × and the volumes of the POA, VMH, HAB and LFB at a magnification of 320 ×. To determine the volume of specific brain areas, each brain image from the slide was projected onto the computer monitor through the microscope and camera. 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.

2.7. Brain area volume statistical analysis

Forebrain volumes (ratio of nucleus volume divided by forebrain volume × 100) and the incubation temperature and sex were compared using analysis of covariance (ANCOVA) to verify there was no significant interaction (parallelism or "homogeneity of slopes") between covariate (forebrain volumes) and treatment (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 only if $P \leq 0.01$ was Tukey's post-hoc test used to determine which groups were significantly different ($P \leq 0.05$). This rigorous threshold was established to insure a conservative interpretation of the data.

2.8. 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 using a step tablet of absolute optical density (OD) standards (Kodak Calibration Tablet No. 2, Kodak, Rochester, NY). The intensity of the CO histochemical reaction product was measured in OD 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 [9].

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 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 OD for each brain nucleus. Using Sigmaplot (Jandel Scientific, Corte Madera, CA), the mean OD values of the rat brain homogenate standards and their CO enzymatic activity measured spectrophotometrically [6] were used to create a regression curve. The brain nuclei OD values were then converted to CO activity units (μmol/min/g tissue wet weight) using this curve.

Sixteen brain regions were analyzed for CO activity: anterior hypothalamus (AH), external amygdala (AME), dorsal lateral nucleus of the hypothalamus (DLH), dorsal lateral nucleus of the thalamus (DL), dorsal ventricular ridge (DVR), HAB, lateral hypothalamus (LH), nucleus rotundus (NR), nucleus sphericus (NS), periventricular nucleus of the hypothalamus (PH), POA, periventricular nucleus of the preoptic area (PP), septum (SEP), striatum (STR), torus semicircularis (TS), and VMH. To evaluate whether the sex or incubation temperature differences were specific to putative steroid-binding areas (POA, VMH, AH, AME, 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.

2.9. CO densitometry statistical analysis

All statistical analyses were done 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; re-analysis with Bartlett's test showed the data for all nuclei to be homogeneous after transformation. Transformed CO activity unit means and the temperature and sex were compared using ANCOVA 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 using ANOVA, and only if $P \leq 0.01$ was a Tukey's post-hoc test used to determine which individual group comparisons were significantly different ($P \leq 0.05$). This rigorous threshold was established to insure a conservative interpretation of the data.

3. Results

Volume indices and transformed CO activity unit means were compared for effects of age and sociosexual experience within and between male and female leopard geckos from the two incubation temperatures (Tables 1 and 2). The significant ($P \leq 0.01$) effects of age and sociosexual experience on brain regions are summarized in Tables 3 and 4. The relative forebrain volumes were significantly different between young and old animals, with old animals having larger forebrain volumes (mean $mm^3 \pm$ S.E. = 17.33 ± 0.27 vs. 14.06 ± 0.27; $P = 0.001$ for males from the 32.5°C incubation temperature and 14.72 ± 0.37 vs. 12.66 ± 0.40; $P = 0.008$ for females from 32.5°C incubation temperature for young vs. old, respectively). The relative forebrain volumes were not significantly different between inexperienced and experienced young animals. The white matter of the optic tract (OT) measured zero or less than one CO activity unit for all animals.

Interassay coefficients of variation were 14% for androgen and 19% for estrogen assays. Intra-assay coefficients

Table 1
Male group statistics

	Young, inexperienced	Old, inexperienced	Old, experienced
Fb volume			
	14.06 ± 0.27	17.33 ± 0.27	17.04 ± 0.31
Region			
HAB	0.28 ± 0.03	0.27 ± 0.04	0.27 ± 0.05
LFB	0.42 ± 0.03	0.40 ± 0.05	0.41 ± 0.04
POA	1.45 ± 0.03	1.09 ± 0.03	1.16 ± 0.03
VMH	0.80 ± 0.02	0.61 ± 0.02	0.63 ± 0.02
CO metabolic activity			
AH	10.4 ± 0.1	10.4 ± 0.1	11.2 ± 0.1
AME	10.1 ± 0.2	11.5 ± 0.2	11.4 ± 0.2
DL	12.1 ± 0.3	10.7 ± 0.3	19.0 ± 0.3
DLH	14.3 ± 0.2	10.1 ± 0.2	14.1 ± 0.2
DVR	8.6 ± 0.1	9.5 ± 0.1	9.0 ± 0.1
HAB	14.7 ± 0.1	14.8 ± 0.1	15.2 ± 0.2
LH	8.6 ± 0.1	8.6 ± 0.1	9.3 ± 0.1
NR	14.2 ± 0.1	14.3 ± 0.1	14.1 ± 0.1
NS	12.3 ± 0.1	12.8 ± 0.1	12.8 ± 0.1
PH	9.2 ± 0.1	10.1 ± 0.1	7.9 ± 0.1
POA	7.5 ± 0.2	9.2 ± 0.2	9.0 ± 0.2
PP	5.1 ± 0.3	6.8 ± 0.3	7.6 ± 0.4
SEP	14.8 ± 0.1	15.8 ± 0.1	15.0 ± 0.1
STR	13.3 ± 0.1	14.1 ± 0.1	13.1 ± 0.1
TS	14.5 ± 0.1	14.9 ± 0.1	13.8 ± 0.2
VMH	9.0 ± 0.2	7.5 ± 0.1	11.0 ± 0.2

The volume of a fixed portion of the forebrain in mm^3 (Fb volume) and of different brain areas (region) of young and old, inexperienced and experienced, adult male leopard geckos (*Eublepharis macularius*). Volume measurements are mean ratios of nucleus volume divided by a fixed portion of forebrain volume $\times 100 \pm$ S.E. Values of CO are in activity units (μmol/min/g of tissue wet weight). All males were incubated at 32.5°C.

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 preoptic area; SEP = septum; STR = striatum; TS = torus semicircularis; VMH = ventromedial nucleus of the hypothalamus.

Table 2
Female group statistics

	Young, inexperienced (26)	Young, experienced (26)	Old, experienced (26)	Young, inexperienced (32.5)	Young, experienced (32.5)	Old, experienced (32.5)
Fb volume						
	13.87 ± 0.25	14.14 ± 0.40	15.14 ± 0.28	12.79 ± 0.27	12.66 ± 0.40	14.72 ± 0.37
Region						
HAB	0.27 ± 0.03	0.28 ± 0.03	0.27 ± 0.04	0.33 ± 0.05	0.31 ± 0.03	0.29 ± 0.04
LFB	0.42 ± 0.03	0.43 ± 0.03	0.42 ± 0.04	0.44 ± 0.05	0.43 ± 0.03	0.43 ± 0.04
POA	0.86 ± 0.03	1.04 ± 0.05	0.92 ± 0.04	1.35 ± 0.04	1.28 ± 0.05	1.24 ± 0.05
VMH	0.91 ± 0.03	0.86 ± 0.04	0.74 ± 0.03	0.80 ± 0.03	0.91 ± 0.04	0.93 ± 0.04
CO metabolic activity						
AH	8.7 ±0.2	9.5 ±0.2	9.4 ±0.2	10.1 ±0.2	10.6 ±0.2	10.7 ±0.2
AME	5.9 ±0.2	9.2 ±0.3	9.3 ±0.2	10.4 ±0.2	11.1 ±0.3	11.7 ±0.3
DL	11.0 ±0.2	11.1 ±0.3	10.0 ±0.2	11.3 ±0.2	12.4 ±0.3	11.4 ±0.2
DLH	13.1 ±0.1	12.5 ±0.2	12.5 ±0.2	15.7 ±0.2	15.6 ±0.2	15.6 ±0.2
DVR	6.1 ±0.1	6.2 ±0.2	7.9 ±0.1	8.3 ±0.1	8.3 ±0.2	8.6 ±0.1
HAB	15.0 ±0.1	15.0 ±0.2	14.7 ±0.1	14.9 ±0.1	14.6 ±0.2	14.6 ±0.2
LH	8.5 ±0.2	9.2 ±0.3	9.2 ±0.3	8.6 ±0.2	9.3 ±0.3	9.2 ±0.2
NR	14.2 ±0.1	14.0 ±0.1	13.9 ±0.1	14.2 ±0.1	14.0 ±0.1	14.0 ±0.1
NS	9.7 ±0.1	9.7 ±0.1	10.9 ±0.1	11.5 ±0.1	11.6 ±0.1	12.1 ±0.1
PH	9.3 ±0.1	9.3 ±0.1	8.4 ±0.1	9.0 ±0.1	9.2 ±0.1	8.5 ±0.1
POA	6.1 ±0.2	7.8 ±0.3	7.8 ±0.2	7.1 ±0.2	8.7 ±0.3	8.7 ±0.3
PP	6.2 ±0.2	6.3 ±0.3	7.1 ±0.2	6.3 ±0.2	6.4 ±0.3	6.5 ±0.3
SEP	12.8 ±0.1	12.7 ±0.2	14.0 ±0.1	14.9 ±0.1	14.8 ±0.2	14.8 ±0.2
STR	11.9 ±0.1	12.7 ±0.1	11.6 ±0.1	13.2 ±0.1	13.0 ±0.1	13.1 ±0.1
TS	13.8 ±0.1	13.8 ±0.2	12.6 ±0.1	14.4 ±0.1	13.4 ±0.3	13.5 ±0.2
VMH	12.4 ±0.1	12.5 ±0.2	12.2 ±0.2	12.2 ±0.2	13.0 ±0.2	12.9 ±0.2

The volume of a fixed portion of the forebrain in mm^3 (Fb volume) and of different brain areas (region) of young and old, inexperienced and experienced, adult female leopard geckos (*Eublepharis macularius*). Volume measurements are mean ratios of nucleus volume divided by a fixed portion of forebrain volume × 100 ± S.E. Values of CO are in activity units (μmol/min/g of tissue wet weight). All males were incubated at 32.5°C. Number in parentheses indicates the temperature (°C) at which the individuals were incubated as eggs. Units and abbreviations as in Table 1.

of variation were 8% for androgen and 13% for estrogen assays. Using Bartlett's test for homogeneity (SYSTAT, Evanston, IL), hormone values were found to be non-homogeneous. Hormone values were homogeneous after log-transformation and were compared by Analysis of Variance (ANOVA) and subsequent Tukey's post-hoc tests (Table 5). The follicle size of females was noted at sacrifice and hormone levels were transformed using the natural logarithm before statistical analysis. As in previous

Table 3
Effects of age on the volume and cytochrome oxidase (CO) activity of brain nuclei in young (1 year of age) vs. old (2–3 years of age) leopard geckos (*Eublepharis macularius*) within each incubation temperature

Incubation temperature	(A) Males	(B) Females	
	32.5°C	26°C	32.5°C
Volume	POA (decrease)	none	none
	VMH (decrease)		
CO activity			
Increase	AME	DVR	NS
	DVR	NS	
	NS	SEP	
	PP		
	POA		
	SEP		
Decrease	DL	PH	PH
	VMH	TS	

Abbreviations as in Table 1. Summary of significant ($P \leq 0.01$) effects shown; "increase" indicates a greater mean and "decrease" a lower mean in the older group as compared to the younger group.

Table 4
Effects of sociosexual experience on the volume and cytochrome oxidase (CO) activity of brain in leopard geckos (*Eublepharis macularius*) within each incubation temperature

Incubation temperature	(A) Males	(B) Females	
	32.5°C	26°C	32.5°C
Volume	none	none	none
CO activity			
Increase	AH	AME	POA
	DVR	POA	VMH
	LH		
	VMH		
Decrease	PH	DLH	
	TS		

Abbreviations as in Table 1. Summary of significant ($P \leq 0.01$) effects shown; "increase" indicates a greater mean and "decrease" a lower mean in the experienced group as compared to the inexperienced group.

Table 5
Circulating concentrations of androgen and estrogen (ng/ml) in male and female leopard geckos (*Eublepharis macularius*) from different incubation temperatures (26°C or 32.5°C) and of different age (young or old) and sexual experience (inexperienced or experienced)

	Young, inexperienced			Young, experienced			Old, inexperienced			Old, experienced		
	T	E	T/E	T	E	T/E	T	E	T/E	T	E	T/E
Female – 26°C												
Mean	0.69	1.58	0.46	0.63	1.75	0.42				0.63	1.92	0.51
(S.E.)	(0.19)	(0.33)	(0.09)	(0.21)	(0.48)	(0.15)				(0.28)	(0.84)	(0.18)
Female – 32.5°C												
Mean	0.56	1.93	0.41	0.81	0.40	2.46				2.36	1.04	2.96
(S.E.)	(0.13)	(0.35)	(0.10)	(0.17)	(0.11)	(0.73)				(0.42)	(0.24)	(0.73)
Male – 32.5°C												
Mean	87.06	0.60	196.60				70.91	1.07	144.27	139.69	0.63	227.20
(S.E.)	(10.00)	(0.06)	(46.60)				(14.18)	(0.28)	(35.41)	(24.21)	(0.08)	(44.59)

Indicated are the mean with standard error (S.E.) in parentheses.
T = total androgens; E = total estrogens. A ratio of androgen-to-estrogen (T/E) was calculated for each individual and the average for the group is presented.

studies [9,44], circulating concentrations of both total androgens ($P = 0.005$) and total estrogens ($P = 0.04$) were higher in females with larger follicles.

3.1. Effect of age in males

3.1.1. Brain area size within an incubation temperature

The relative volumes of the POA and VMH were lower in old vs. young males ($P = 0.0009$ and 0.0001, respectively) (Tables 1 and 3A). The volumes of the HAB and LFB were not significantly different.

3.1.2. CO activity within an incubation temperature

Metabolic capacity was significantly higher in old vs. young males in the AME ($P = 0.0003$), DVR, NS, POA, SEP (all $P = 0.0001$), and PP ($P = 0.005$), but significantly lower in the DL ($P = 0.007$) and VMH ($P = 0.0001$) (Tables 1 and 3A); CO activity for the AH, DLH, HAB, LH, NR, PH, STR, and TS was not significantly different.

3.1.3. Hormones

Circulating concentrations of total androgens and total estrogens between young and old males from the 32.5°C incubation temperature were not significantly different (Table 5).

3.2. Effect of age in females

3.2.1. Brain area size within an incubation temperature

The volume of the VMH was significantly different between young and old females from the 26°C incubation temperature ($P = 0.002$), but not at the 32.5°C incubation temperature (Table 2). The volume of the POA tended to be larger among young females compared to old females from the 26°C incubation temperature, but this did not reach the statistical criterion ($P = 0.02$). The volumes of the HAB and LFB were not significantly different between young and old females from either incubation temperature.

3.2.2. CO activity within an incubation temperature

Metabolic capacity was significantly higher in old compared to young females from the 26°C incubation temperature in the DVR ($P = 0.0001$), NS ($P = 0.0001$), and SEP ($P = 0.007$), but significantly lower in old females from this incubation temperature in the PH ($P = 0.0001$) and TS ($P = 0.0007$) (Tables 2 and 3B). Opposite trends were evident in the PP (higher in old females) and the DL (lower in old females), but this did not reach the statistical criterion (both $P = 0.02$). There were no significant differences in CO activity between young and old females from the 26°C incubation temperature for the POA, VMH, AH, AME, DLH, HAB, LH, NR, and STR. The PH was significantly lower in metabolic capacity in old females from the 32.5°C incubation temperature ($P = 0.0002$) (Tables 2 and 3B). Metabolic capacity in the NS tended to be higher in old compared to young females from the male-biased incubation, but this did not reach the statistical criterion ($P = 0.05$). There were no significant differences between young and old females from the 32.5°C incubation temperature in the AME, DVR, SEP, and TS.

3.2.3. Brain area size between incubation temperatures

The VMH volume of young and old females from the 32.5°C incubation temperature was larger compared to their counterparts from the 26°C incubation temperature ($P = 0.002$ for both).

3.2.4. CO activity between incubation temperatures

Both young and old females from the 32.5°C incubation temperature had greater metabolic capacity than their counterparts from the 26°C incubation temperature in the AH ($P = 0.02$ and 0.0003, respectively), AME ($P = 0.0002$ and 0.0001 for young and old, respectively), DLH (both $P = 0.0001$), DVR ($P = 0.0001$ and 0.0004, respectively), NS (both $P = 0.0001$), SEP ($P = 0.0001$ and 0.009, respectively), and STR (both $P = 0.0001$) (Table 2).

Metabolic capacity was not significantly different between young and old females between incubation temperatures in the PH and PP. In addition, metabolic capacity was greater in old females from the 32.5°C incubation temperature compared to old females from the 26°C incubation temperature in the DL and TS ($P = 0.0007$ and 0.001, respectively) (Table 2). Both young and old females from the 32.5°C incubation temperature tended to have greater metabolic capacity in the POA than their counterparts from the 26°C incubation, but this did not reach the statistical criterion ($P = 0.02$ for both).

3.2.5. Hormones

Although older, experienced females from the 32.5°C incubation temperature had the highest circulating concentrations of androgens (mean of 2.36 ng/ml), the differences detected were not significant when statistically controlling for follicle size using ANCOVA (Table 5).

3.3. Effect of sociosexual experience in males

3.3.1. Brain area size within an incubation temperature

The volumes of the HAB, LFB, POA and VMH were not significantly different between experienced vs. inexperienced males from the 32.5°C incubation temperature (Tables 1 and 4A).

3.3.2. CO activity within an incubation temperature

Metabolic capacity was greater in experienced vs. inexperienced males in the AH ($P = 0.0001$), DVR ($P = 0.004$), LH ($P = 0.0008$), and VMH ($P = 0.0001$), but lower in the PH ($P = 0.0001$) and TS ($P = 0.0004$) (Tables 1 and 4A). The CO activity was not significantly different in the AME, DL, DLH, HAB, POA, NR, NS, PP, SEP, and STR.

3.3.3. Hormones

There were significant differences in the circulating concentration of total androgens ($P = 0.003$) between males that differed in their sociosexual experience, but not in total estrogens (Table 5).

3.4. Effect of sociosexual experience in females

3.4.1. Brain area size within an incubation temperature

The volume of the POA tended to be larger in experienced vs. inexperienced females from the 26°C incubation temperature, but this did not reach the statistical criterion ($P = 0.05$); no significant differences were found for HAB, LFB, and VMH (Tables 2 and 4B).

3.4.2. CO activity within an incubation temperature

Comparing experienced to inexperienced females from the 26°C incubation temperature, metabolic capacity was greater in the POA ($P = 0.001$) and AME ($P = 0.0001$) (Tables 2 and 4B). There were no significant differences between experienced and inexperienced females from the 26°C incubation temperature in the AH, DLH, LH, VMH, and TS. Metabolic capacity was greater in the POA between experienced vs. inexperienced females at the 32.5°C incubation temperature ($P = 0.003$) (Tables 2 and 4B). There were no significant differences between experienced and inexperienced females from the 32.5°C incubation temperature in the AME, DLH, LH, TS, and VMH, and there were no differences between experienced and inexperienced females from both incubation temperatures in the DL, DVR, HAB, NR, NS, PH, PP, SEP, and STR (Table 2).

3.4.3. Brain area size between incubation temperatures

The volume of the POA tended to be larger in experienced females from the 32.5°C incubation temperature vs. experienced females from the 26°C incubation temperature ($P = 0.02$), but this did not reach the statistical criterion (Table 2).

3.4.4. CO activity between incubation temperatures

Metabolic capacity was significantly greater in experienced females from the 32.5°C incubation temperature vs. the 26°C incubation temperature in the AME ($P = 0.0002$), DLH, DVR, NS, SEP, and STR (all $P = 0.0001$) (Table 2). Experienced females from the 32.5°C incubation temperature tended to have greater metabolic capacity in the AH compared to their counterparts from the 26°C incubation, but this did not reach the statistical criterion ($P = 0.02$). There were no significant differences between experienced females from the 26°C and 32.5°C incubation temperatures in the DL, LH, PH, POA, PP, TS, and VMH.

3.4.5. Hormones

When statistically controlling for follicle size using ANCOVA, the differences detected in the circulating concentration of androgens and estrogens were not significantly different (Table 5).

4. Discussion

Individuals vary in their sexual behavior. Two sources of this variation are experience and age. Sexual experience improves sexual performance and efficiency and, usually, experienced individuals are older than inexperienced individuals. But there are many situations in which this does not occur. In a number of vertebrate species, social factors insure that relatively few males will mate. For example, in territorial and haremic species, the resident or dominant male will fertilize most of the females and non-resident or subordinate males obtain little sexual experience with adult females. Further, in every vertebrate species studied to date, wild and domesticated, some males will not be motivated to mate and may even ignore females.

As much evidence exists refuting [11,17] as supporting [36,37] a correlation between individual differences in the

frequency or intensity of sexual behavior and circulating levels of sex hormones. These contradictory results could be due to the difficulty in determining the age and/or experience of the individuals studied. The present experiment demonstrates that experience can influence plasma levels of androgens and estrogens in males. It is not surprising that age effects were minimal as leopard geckos often live to 15 years in captivity, and the difference in age (1 versus 2–3 years of age) was not large between the groups. However, experience was an important factor. Among the older males, those with sociosexual experience had higher circulating concentrations of androgens than naive males; similar effects were not observed in females.

Although the evidence at present is limited, there appears to be a correlation between an individual's behavior and patterns of gene expression in the brain. High and low sexually performing rams exhibit differences in the abundance of estrogen receptor in the hypothalamus [1]. In the little striped whiptail lizard, males typically exhibit either low or high levels of sexual behavior, and males that exhibit intense courtship are more likely to be sensitive to exogenous progesterone [33,34]. Implantation of progesterone into the AH-POA of castrated, progesterone-sensitive males completely restores sexual behavior, but not in castrated, progesterone-insensitive males [13]. Further, androgen receptor- and progesterone receptor-mRNA expression in specific brain regions is significantly different in progesterone-sensitive versus progesterone-insensitive animals [13]. Such differences in receptor regulation at an individual level are paralleled by differences at a species level [51].

Another source of variation is the prenatal environment. Indeed, the nature of the experiences an individual has later in life depend to a great extent on events experienced earlier in life. In the gerbil, as in many mammals that produce multiple young in a litter, androgens excreted by male fetuses expose fetuses located between two males (2M) to higher levels of exogenous androgen compared to fetuses located between two females (2F). These hormone differences resulting from intrauterine position account for many of the differences among adult individuals of the same sex. For example, in mice and gerbils, 2M females have a masculinized phenotype, are less attractive and more aggressive, become sexually mature at a later date, have lower estrogen levels and higher androgen levels, and produce smaller litters that tend to be male-biased compared to 2F females [8]. In addition, brain CO activity in the sexually dimorphic area of the POA, a region involved in the regulation of sex-typical behaviors in this species [50], is greater in 2M female compared to 2F female gerbils [27]. CO activity is also different in the posterior AH, an area replete with neurons containing gonadotropin hormone-releasing hormone (GnRH), which may explain the physiological differences between 2M and 2F females. The prenatal environment can also be influenced by the experience of the mother which, in turn, may have transgenerational consequences. If mice are crowded while pregnant, the physiology and behavior of at least two generations of progeny will be adversely affected [7].

Current paradigms in behavioral endocrinology will not aid in the search for the bases of individual differences [11]. There are several reasons for this. First and foremost, genetic sex and gonadal sex are linked in species with sex chromosomes, making it impossible to distinguish environmental from genetic contributions to individual differences. Consider resource-based aggression: to what extent are the differences observed between adult males and females due to their differences in sex chromosomes, non-genomic yet heritable factors, the nature and pattern of hormone secretion, or even sex-typical experiences? Second, sexuality resides in the brain, not in the gonads, and what dictates gonadal sex need not dictate sexuality. Third, sex and sexuality are often confused, yet conceptually distinct; gonadal sex is a discrete trait that categorizes the individual, whereas sexuality is a suite of continuously variable traits that is unique to each individual. Fourth, even as adults each individual is capable of displaying the behaviors characteristic of the opposite sex; indeed, to the best of our knowledge there are no behaviors in a vertebrate that are completely sex-specific. The result is that the range of variation among individuals within a sex usually is greater than the difference between the averages for each of the sexes. Thus, although the sexes differ in many traits, this often is a statistical phenomenon.

Because individual variation is the substance of evolutionary change, understanding its organization will require both new paradigms as well as alternative animal model systems that allow separation of the effects of genes and hormones from environmental and experiential stimuli. How the environment induces phenotypic plasticity, resulting in individually distinct patterns of sexuality, results from two distinct levels of organization of the sexual phenotype [11]. The primary level is the process of sexual differentiation that follows the determination of the gonad and is manifest as the morphological, physiological and behavioral traits characteristic of sex. A second, and subsequent, level of organization is the basis of individual variation in sexually dimorphic behaviors. This can be termed secondary organization and involves stimuli from other sources such as (i) the embryonic environment (broadly defined to include hormones within the embryonic environment as well as physical factors such as temperature), (ii) the psychological and physiological condition of the mother during pregnancy and birth or yolk deposition and egg-laying, (iii) the social and sexual experiences during growth and adulthood, and (iv) the aging process. Thus, individual differences in aggressive and sexual behaviors arise out of circumstances other than sex chromosomes and the steroid hormones that are secreted following gonadal differentiation. Studies of the leopard gecko reveal how experiences before and after birth can elicit different phenotypes from a particular genotype.

In species like the leopard gecko in which the temperature of the incubating egg determines the gonadal sex of the hatchling, there are no sex chromosomes. Instead, each embryo has an equal ability to become a male or a female, and temperature serves as the trigger regulating the genetic cascades that lead to the development of testes or ovaries [10]. But incubation temperature does more than establish the gonadal sex of the individual. Indeed, the temperature experienced during embryogeny accounts for much of the individual and sexual variation observed in the morphology, growth, endocrine physiology, and sociosexual behavior of the adult. Recent studies of the organization and activity of different brain areas subserving these behaviors reveal that the POA and VMH of male and female geckos from the same incubation temperature are not dimorphic [9]. There are, however, consistent differences across incubation temperatures, suggesting that the temperature experienced by the embryo has a direct effect on brain organization that is independent of gonadal sex [12]. For example, the volume of the POA is larger in both males and females from the male-biased temperature compared to geckos from the female-biased temperature. Similarly, the volume of the VMH is larger in low-temperature females compared to females from the male-biased temperature.

In terms of metabolism, males on average have greater CO activity in the POA, whereas females on average have greater CO activity in the VMH. As is the case with volume, males and females from the male-biased temperature have greater CO activity in the POA compared to animals from the other incubation temperatures, and females from the female-biased temperature have greater CO activity in the VMH compared to females from the male-biased temperature. Thus, incubation temperature appears to be the major determinant of metabolic capacity in these brain areas of the adult, but unlike the case with volume, the gonadal sex (presumably sex hormones) of the individual is also a factor [12].

It is important to distinguish the contribution of experience from age in any study of individual differences because organisms age as they gain experience, but do not necessarily gain experience as they age. In male rats the density of synaptic input to GnRH-containing neurons increases with age in sexually inexperienced males, but not in aged males that have had breeding experience [47]. As aging is a constant, yet experience is variable, one might expect experience to have less of an effect than age on those brain nuclei mediating sociosexual behaviors. This was the case for the volume of the POA among males as well as for females from the two incubation temperatures.

In both sexes the effects of age and experience were modulated by incubation temperature. For example, both young and old females from the male-biased incubation temperature had a larger POA than their counterparts from the low incubation temperature. On first examination, the volume of the POA appeared to be affected differently by age in male compared to female leopard geckos. That is, the volume of volume of the POA became smaller in males as they aged, but remained unchanged in females. As might be predicted from the literature on sexually dimorphisms in brain nuclei, young inexperienced males had a larger POA than did young inexperienced females from the same incubation temperature. However, older males, whether experienced or inexperienced, had smaller POA volumes than did females of the same age and incubation temperature. Androgens prevent degeneration of neurons in the POA [2,28] and, in guinea pigs, sexually dimorphic regions in the medial POA increase in "somewhat older" males [25]. The fact that in the leopard gecko males showed a decrease in volume of the POA with age, yet females from the male-biased incubation temperature (but not from the lower, all-female incubation temperature) maintained a large POA volume with age, suggests that the increased circulating levels of androgens in females from the male-biased incubation temperature may "rescue" neurons in the POA, resulting in no change in volume when a decrease might be expected.

It was surprising to find that sexual experience and age can actually have opposite effects on brain organization and activity. For example, in sexually inexperienced males, the volume of the POA decreased and CO activity increased with age. That is, the POA was smaller but more active in the older males. Comparison of experienced and inexperienced older males revealed that the POA was larger in experienced males, but not restored to the size found in the younger, inexperienced males. A similar relationship was found in females, but unlike males, volume and CO activity were not concordant. That is, in young females from the 26°C incubation temperature the volume of the POA increased as a function of sexual experience, yet comparing sexually inexperienced with experienced females from this same incubation temperature revealed that POA volume decreased as a function of age. This relationship was not seen in CO activity. These effects were also found to vary according to incubation temperature; that is, the relationships just mentioned applied only to low-temperature females, not to females from male-biased incubation temperatures.

Taken together, these results indicate that the volume and metabolic capacity of specific brain regions are dynamic in adulthood, changing as the individual ages and gains experience. It also indicates that an individuals' embryonic experience will influence how it responds to events later in life; that is, the nature and degree of change are dependent upon prenatal events. A question to be answered now is: why is there an advantage to have such environmentally induced phenotypic plasticity, particularly at the level of the size and activity of certain brain areas? Such phenomena suggest that it might be more profitable to view hormones and other factors during embryogenesis as allowing for the growth of neural connections, whereas participating in particular behaviors later in life sculpting and then maintaining the unique functional integrity of the

attendant neuroendocrine systems within each individual. Such a view is supported by these experiments with the leopard gecko which show that prenatal experience modifies how the individual responds to sex steroid hormones, modulates behavior, and shapes brain organization and activity.

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