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Review

Developmental sculpting of social phenotype and plasticity

Jon T. Sakata[a,*], David Crews[a,b]

[a]*Institute for Neuroscience, University of Texas at Austin, Austin, TX 78712, USA*
[b]*Section of Integrative Biology, University of Texas at Austin, Austin, TX 78712, USA*

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Abstract

Early developmental variables engender behavioral and neural variation, especially in species in which embryonic environment determines gonadal sex. In the leopard gecko, *Eublepharis macularius*, the incubation temperature of the egg (IncT) determines gonadal sex. Moreover, IncT affects the sexual differentiation of the individual and, consequently, within-sex variation. Individuals hatched from eggs incubated at an IncT that produces predominantly males are more masculinized than same-sex counterparts from IncTs that produce predominantly females. Here we review how gonadal sex and IncT interact to affect behavioral, endocrinological, and neural phenotype in the leopard gecko and influence phenotypic plasticity following hormone administration or social experience. We discuss the hormonal dependence of sex- and IncT-dependent behavioral and neural morphological and metabolic differences and highlight the parallels between IncT effects in geckos and intrauterine position effects in rodents. We argue that the leopard gecko is an important model of how the process of sex determination can affect sexual differentiation and of selection forces underlying the evolution of sex ratios.

Keywords: Lizard; Sexual differentiation; Incubation temperature; Sex difference; Intrasexual variation; Cytochrome oxidase; Plasticity; Preoptic area; Hypothalamus; Sexual behavior; Aggressive behavior; Intrauterine position

Contents

1. Introduction

A central pursuit in behavioral biology is to understand the mechanisms underlying behavioral variation. This pursuit is characterized by multiple levels of investigation including the identification of genetic, endocrine and neural

* Corresponding author. Present address: Keck Center for Integrative Neuroscience, Department of Physiology, Box 0444, University of California, San Francisco Medical Center, 513 Parnassus, San Francisco, CA 94143-0444, USA. Tel.: +1-415-502-3279; fax: +1-415-476-4929.
E-mail address: jsakata@phy.ucsf.edu (J.T. Sakata).

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differences among individuals. Variation can be partitioned into two categories: intersexual, or between-sex differences and intrasexual, or within-sex differences. Whereas intersexual variation has received much attention, relatively little research has been devoted to understanding intrasexual variation.

Biological mechanisms underlying behavioral variation in sexual, agonistic and stress-related behaviors have been extensively explored [1]. Sexual differentiation, the process that sculpts the masculinity and femininity of the individual, is dependent on gonadal sex steroid exposure perinatally and in adulthood [2–4]. This framework, known as the organization–activation concept, has been the major infrastructure guiding research into the mechanisms underlying the display of social behavior. Moreover, this perspective has been integral in advancing knowledge on individual differences in behavior, particularly intersexual differences. In brief, the organization–activation paradigm posits that sex differences in gonadal hormone secretion perinatally cause the differential development of the neuroendocrine system in males and females, which in turn establishes differences in circulating concentrations of steroid hormones in adulthood. These differences in the levels of hormones in adulthood elicit different behaviors in males and females. Furthermore, sex differences in early sex steroid exposure organize neural circuits to react differently to sex steroid hormone exposure in adulthood. For instance, males display more copulatory behavior in response to androgen treatment in adulthood, whereas females display more female-typical proceptive and receptive behaviors in response to estrogen treatment in adulthood. Phenotypic differences that persist following gonadectomy and equalization of hormone concentrations in adulthood are considered to be organized by early sex steroid exposure, whereas differences that are dependent on variation in circulating steroid hormone concentrations in adulthood are considered to be activated by hormones.

The extension of the organization–activation hypothesis into within-sex variation in behavior, however, has received less attention [5,6], and it is important to emphasize that differences in both perinatal and adult steroidal stimulation can cause intrasexual variation. For example, in rodents, the position of the embryo in the uterine horn (i.e. intrauterine position) significantly affects within-sex differences in both physiology and behavior [7,8]. Individuals located between two male fetuses (2M) are exposed to more androgens during prenatal development than those situated between two female fetuses (2F), and generally speaking, 2M individuals are more masculinized than 2F individuals. For example, 2M adult female gerbils are more aggressive, have lower estradiol (E2) and higher testosterone (T) concentrations, and have shorter reproductive cycles than 2F females [7,8]. Furthermore, manipulations of maternal steroid environment have been found to affect intrasexual variation in social behaviors [9–11].

Whereas the majority of research on social phenotype deals with mammalian species, particularly rodents, relatively little attention has been devoted to 'alternative' model systems such as reptiles. Reptiles occupy a pivotal position in the evolution of birds and eutherian mammals and are particularly well suited for investigations into intrasexual variation in social behavior because there is substantial variation in reproductive tactics [12]. For example, in tree lizards, *Urosaurus ornatus*, there are orange-blue males (orange dewlap with a blue spot), which are territorial and aggressive, and orange males, which are nonaggressive and nomadic. Evidence suggests that progesterone (P) and T stimulation early in development can affect which reproductive tactic is adopted [13].

Here we review our recent investigations into how embryonic experience affects adult behavioral, endocrinological and neural phenotypes in the leopard gecko, *Eublepharis macularius*. Our studies highlight both inter- and intrasexual differences in phenotype and in plasticity following steroidal or experiential manipulations in adulthood. We focus primarily on social behaviors, sex steroid hormones, and limbic brain areas and measure changes in brain area volume as well as cytochrome oxidase (CO) activity in limbic brain areas. Cytochrome oxidase (otherwise known as ferrocytochrome c or cytochrome aa3) is a rate-limiting enzyme in oxidative phosphorylation, the major cellular energy-producing mechanism used by the central nervous system [14], and consequently, the abundance and activity of CO reflects metabolic capacity [15,16]. Cytochrome oxidase activity is intimately related to baseline metabolic rate and the amount of excitatory and inhibitory innervation [17,18]. In most of our studies, we focus on the phenotypes of naïve individuals; individuals are raised in isolation until time of testing so that dominance–subordinate relationships, for example, are not confounds. Our aim is to produce an appreciation for the complexity with which ecologically relevant embryonic experiences can shape the brain and to motivate similar pursuits in other model organisms. We first introduce our model system, then review between- and within-sex differences in phenotype, followed by between- and within-sex differences in plasticity. Thereafter, we discuss the parallels between the leopard gecko and other model systems, evolutionary considerations and future directions. Some of these results have previously been reviewed [19] so are presented in brief.

2. Effects of incubation temperature on gonadal sex in the leopard gecko

In a variety of reptiles, the temperature experienced by the embryo (incubation temperature, or IncT) during a critical window in development determines the gonadal sex of the individual (temperature-dependent sex determination, or TSD) [20–23]. Incubation temperature during this critical period triggers a molecular cascade that transduces temperature into a signal that regulates, for example,

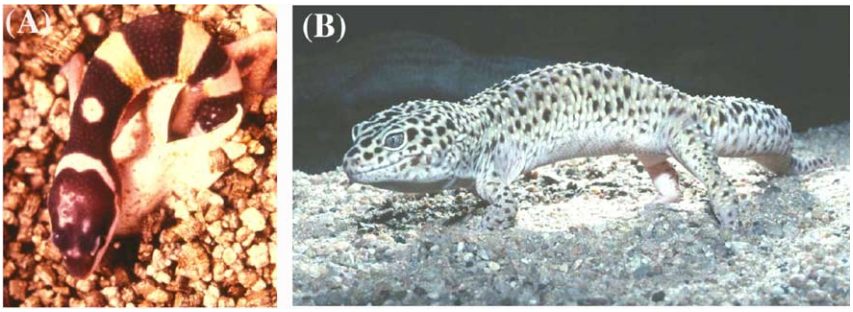

Fig. 1. (A) Picture of a leopard gecko hatchling. (B) During development, the strips break up, forming a leopard-like pattern. This male is showing an aggressive display.

the expression of genes encoding for steroidogenic enzymes and the receptors that bind steroid hormones [22,23]. Estrogens seem to be the physiological equivalent of an IncT that produces all or predominantly females. Applying exogenous E2 to red-eared slider turtle eggs that are incubating at an IncT that generates a male-biased sex ratio results in female hatchlings, and treating eggs with estrogen receptor antagonists or aromatase inhibitors, which block the production of E2, results in male hatchlings. Therefore, as in the process of sexual differentiation, sex steroid hormone exposure during a sensitive period in development influences the process of sex determination in TSD species.

The leopard gecko is a medium-sized, solitary lizard (Fig. 1) whose habitat ranges from western India to Afghanistan. It is a TSD species in which only females are produced at an IncT of 26 °C (Low IncT), and sex ratios (percent male) of ~30% and ~70%, respectively, are produced at the IncTs of 30 °C (female-biased, or Fb IncT) and 32.5 °C (male-biased, or Mb IncT) (Fig. 2) [19,24]. At an IncT of 34 °C (high IncT) <5% of the individuals are male. Therefore, sex ratio follows an inverted U-shaped curve in this species. As in other TSD species, sex ratios are affected by exogenous steroid manipulations during embryogenesis in the leopard gecko. For example, treating eggs incubated at the Mb IncT with E2 increases the number of females produced [25].

The leopard gecko offers a valuable model to assess how the process of sex determination affects the sexual differentiation of the organism. Reproductive maturity is attained between 40 and 50 weeks of age, and individuals breed readily in captivity. Because both sexes are produced at the Fb and Mb IncTs, sex differences in phenotype can be readily studied at these IncTs (Fig. 2). (Sex differences are more difficult to study at the high IncT because of the paucity of males produced at this IncT). Moreover, we can assess how the direction and magnitude of sex differences vary between individuals from different IncTs. Intrasexual differences among males can be readily studied at the Fb and Mb IncTs, and intrasexual differences among females can be studied at all IncTs. An interesting aspect of this model system is that we can correlate behavioral phenotypes with sex ratios produced at each IncT, and in this review we will highlight how the masculinization of sex ratio is often correlated with a masculinization of phenotype in both sexes. Finally, there are interesting parallels between IncT effects on phenotype in the leopard gecko and intrauterine position effects in rodents [7,8]; geckos from the Mb IncT resemble 2M rodents whereas geckos from the Fb IncT resemble 2F rodents. This suggests that the mechanism underlying behavioral variation in these species could be similar.

3. Between-sex differences in phenotype

There are many sex differences in morphological, behavioral, endocrinological, and neural phenotypes in the leopard gecko that resemble sex differences found in other species [1]. At both Fb and Mb IncTs, male leopard geckos are larger than females [25]. At all time points, even upon hatching, males have higher androgen and lower E2 concentrations relative to females [26–29; Crews, unpublished data]. Males are more aggressive than females,

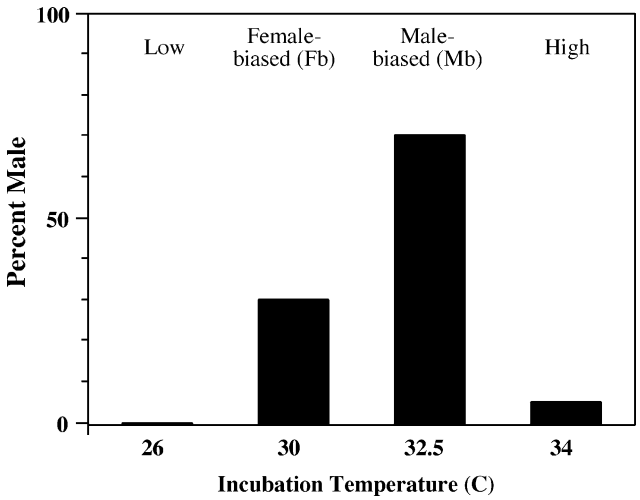

Fig. 2. Sex ratio as a function of incubation temperature in the leopard gecko, *Eublepharis macularius*. Intersexual differences can be readily studied at the female- and male-biased incubation temperatures. Intrasexual differences among females can be readily studied across all incubation temperatures, whereas intrasexual differences among males can be readily studied only at the female- and male-biased incubation temperatures.

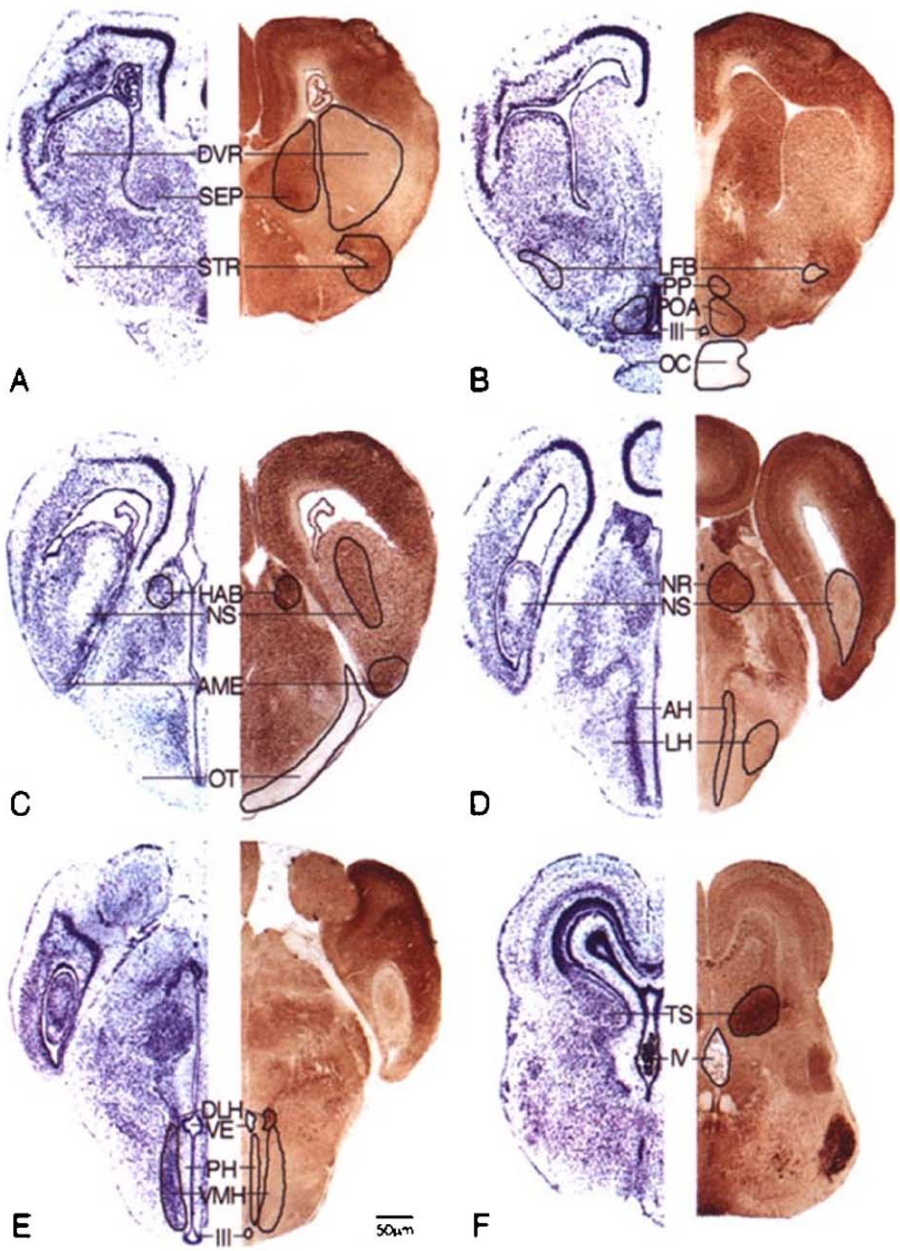

Fig. 3. Brain areas of interest in the leopard gecko. On the left are sections stained with cresyl violet, and on the right are the sections stained from cytochrome oxidase (CO) activity. Darker regions on the right have higher CO activity. III = 3rd ventricle; IV = 4th ventricle; AH = anterior hypothalamus; AME = external nucleus of the amygdala; DLH = dorsal lateral hypothalamus; DVR = dorsal ventricular ridge; HAB = habenula; LFB = lateral forebrain bundle; LH = lateral hypothalamus, 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 hypothalamus. Copied with permission.

and aggression is androgen-dependent [30–34]. Gonadally, intact individuals do not display heterotypical sexual behaviors (i.e. males do not display female-typical receptive behaviors and females do not display courtship behaviors), and, while some females show heterotypical courtship behavior following androgen treatment, sex differences in the display of sexual behaviors persist following gonadectomy and hormone treatment [32]. This indicates that these behavioral differences are organized.

The expression of social behaviors is controlled by limbic brain nuclei that are shaped by the process of sexual differentiation and are often sexually dimorphic (Fig. 3). The preoptic area (POA) and ventromedial hypothalamus (VMH) are two critical hypothalamic brain areas underlying the display of male-typical and female-typical sexual behavior, respectively, and in species such as rats, males have larger POA and VMH volumes than females [1–4, 35–37]. However, in the leopard gecko there are no

significant differences in POA and VMH volumes between males and females within an IncT, though there is a trend for males to have larger POA volumes (Figs. 4 and 5). A lack of sex differences in the size of hypothalamic nuclei has also been found in mice [38].

Unlike neuromorphology, CO activity (metabolic capacity) is sexually dimorphic (Figs. 4 and 5), and interestingly, the pattern and extent of dimorphism depends on IncT (Table 1) ([27] for more comprehensive list of differences). At both the Fb and Mb IncTs, males have elevated CO activity in the nucleus sphericus (NS), an amygdaloid area that receives pheromonal information [39], and females have elevated metabolic capacity in the VMH and dorsolateral hypothalamus (DLH) (Table 1, Fig. 5).

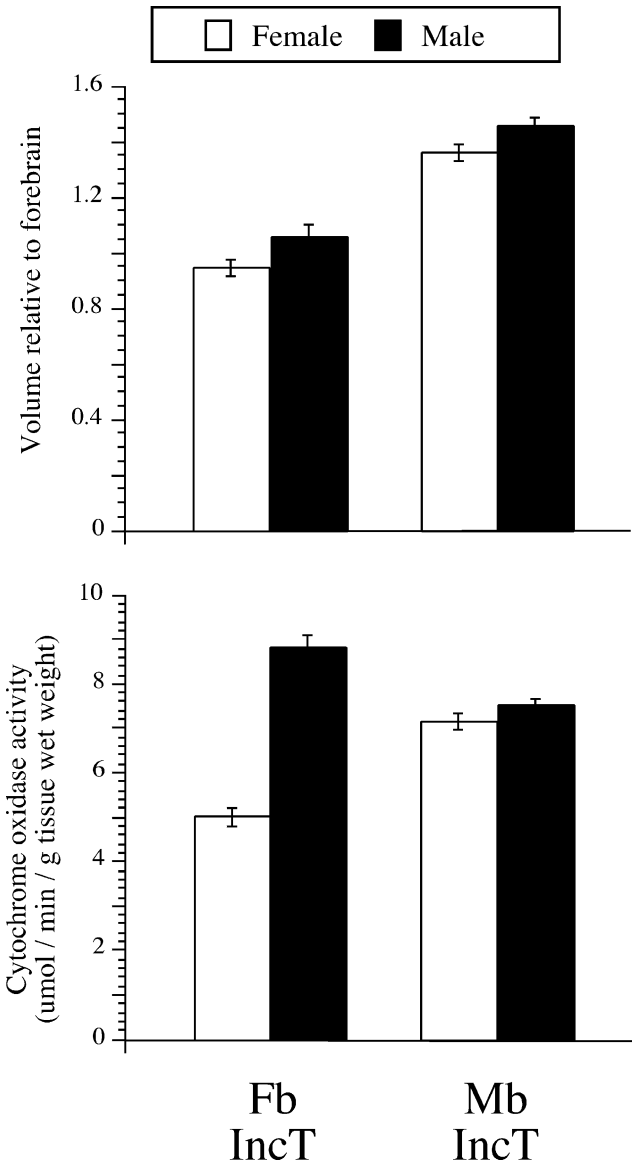

Fig. 4. The volume of the POA is predominantly affect by IncT not gonadal sex, whereas metabolic capacity in the POA is governed by an interaction between gonadal sex and IncT [27].

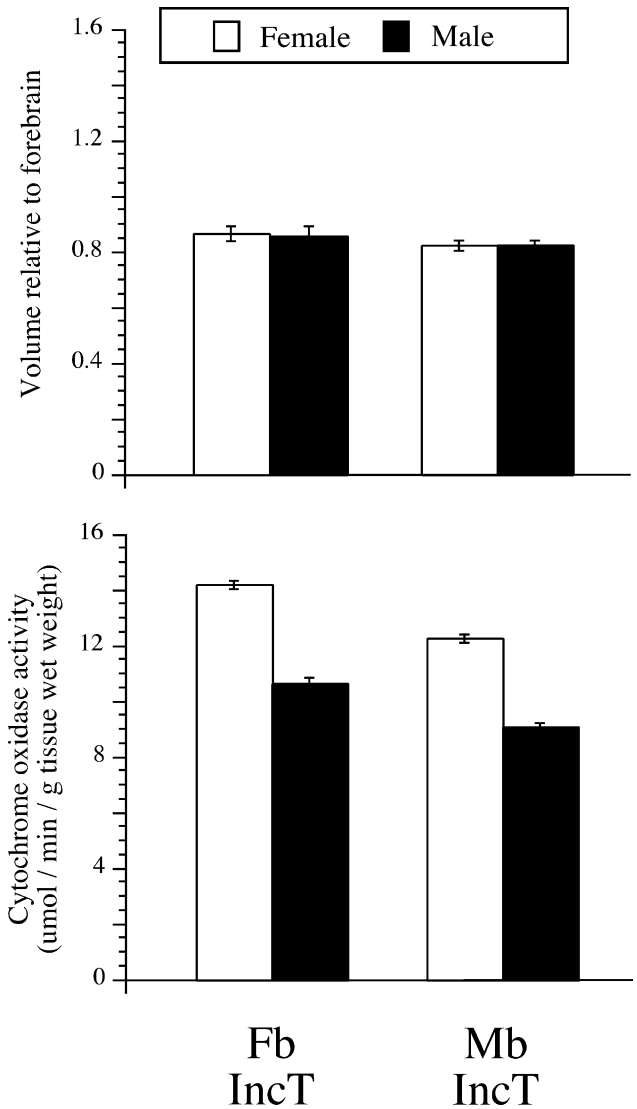

Fig. 5. Among Fb and Mb individuals, both gonadal sex and IncT affect metabolic capacity in the VMH (bottom), whereas neither affects VMH volume (top) [27].

Table 1
Sex differences in metabolic capacity vary at the female- and male-biased incubation temperatures (IncTs)

Region	Fb IncT	Mb IncT
AH	M > F	=
AME	M > F	=
DLH	F > M	F > M
LH	=	=
NS	M > F	M > F
POA	M > F	=
PP	M > F	F > M
SEP	F > M	=
VMH	F > M	F > M

Note: '=' denotes no sex difference. 'M > F' denotes males have greater metabolic capacity than females. 'F > M' denotes females have greater metabolic capacity than males. Differences are $P < 0.01$ (see Ref. [27] for other nuclei), see Fig. 3 for abbreviations.

Table 2
Dependence of sex differences in neurometabolic capacity on hormonal state among Mb individuals (intact, gonadectomized (GNX), and GNX and testosterone (T) treatment)

Region	Intact	GNX	GNX + T
AH	=	=	=
AME	=	F > M	=
DVR	=	=	=
LH	=	=	=
NS	M > F	=	M > F
POA	=	M > F	=
SEP	=	M > F	=
VMH	F > M	F > M	F > M

Note: '=' denotes no sex difference. 'M > F' denotes males have greater metabolic capacity than females. 'F > M' denotes females have greater metabolic capacity than males. Differences are $P < 0.01$ (see Ref. [34] for other nuclei), see Fig. 3 for abbreviations.

On the other hand, metabolic capacity in the POA (Fig. 4), anterior hypothalamus (AH), the external nucleus of the amygdala (AME), and septum (SEP), for example, is sexually dimorphic only among Fb individuals. Though the reason for this is unknown, there are fewer sexually dimorphic nuclei in Mb individuals [27].

The hormonal dependence of sex differences within Mb individuals varies across brain nuclei [34] (Table 2). For example, sex differences in metabolic capacity in the VMH (Fig. 6) and DLH persist following gonadectomy and following androgen replacement therapy, suggesting that these differences are organized. On the other hand, sex differences in the NS (Fig. 6) and periventricular preoptic area (PP) exist when intact and following T treatment but not following gonadectomy, suggesting that this is due to a difference in the sensitivity to androgens (Fig. 6). In other areas such as the AME, POA, and SEP, sex differences exist only following gonadectomy (Table 2). The hormonal dependence of sex differences in Fb individuals has not been investigated, and this will be important, especially given that there are more sexually dimorphic nuclei among Fb individuals.

It is not surprising that areas with sexually dimorphic neural metabolism tend to express androgen receptor (AR) or estrogen receptor (ER) mRNA [40]. For example, AR mRNA is found in the AME, POA, SEP, AH, and VMH, while ER mRNA is expressed in the SEP and VMH. Neural metabolic capacity is sexually dimorphic among intact individuals in all of these nuclei, at least among Fb individuals (Table 1). This is consistent with findings in rats and Japanese quail that androgen manipulations change neural activity in androgen-sensitive areas [41,42]. Some brain areas with sexual dimorphic CO activity do not express AR or ER mRNA, and it will be interesting to assess the contribution of afferent areas on this dimorphism (i.e. trans-synaptic contribution) [43].

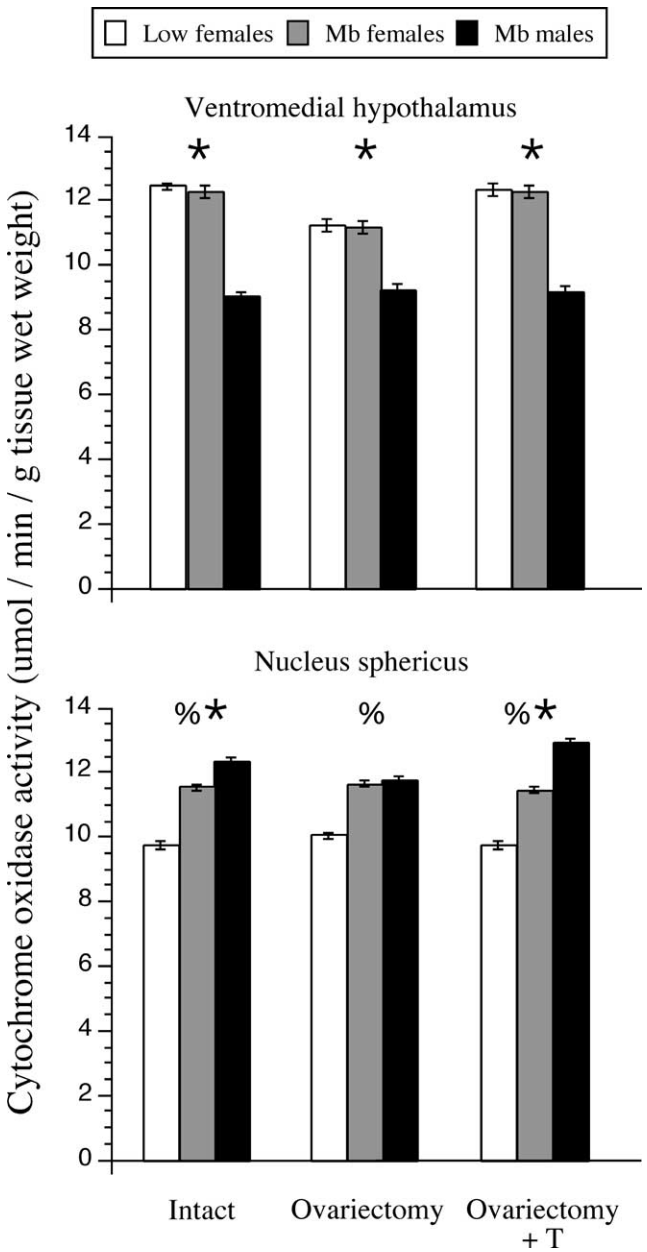

Fig. 6. Intersexual difference in metabolic capacity does not vary with hormonal condition in the ventromedial hypothalamus (top) but does in the nucleus sphericus (bottom). On the other hand, the lack of intrasexual differences in the ventromedial hypothalamus and the presence of intrasexual difference in the nucleus sphericus remain constant across hormonal condition. * denotes significant sex differences, and % denotes significant intrasexual differences ($p < 0.01$) [34].

4. Within-sex differences caused by IncT

In Section 3, we highlighted that, across both Fb and Mb IncTs, males are larger, have elevated androgen concentrations and lower E2 concentrations, show more aggression and courtship behavior, and have elevated CO activity in the NS and lower CO activity in the VMH relative to females. Generally speaking, Mb individuals have a more masculinized phenotype relative to same-sex individuals from

the Low and Fb IncTs. For example, relative to their same-sex Fb counterparts, adult Mb males and females are larger and heavier at sexual maturity, are more aggressive, have lower E2 concentration and have elevated CO activity in the NS and lower CO activity in the VMH [25, 27, 30]. The difference between Fb and Mb males in agonistic behavior persists when sex steroid hormone concentrations are equalized [32], suggesting that this difference is organized by IncT. Furthermore, Mb females have elevated androgen concentrations relative to Fb and Low females and are more likely to show male-typical courtship behavior following androgen treatment relative to Low females [34]. However, one finding that is inconsistent with the trend toward more masculinized phenotypes of Mb individuals is that Fb males are more sexually vigorous than Mb males, even following castration and equalization of sex steroid concentrations [32; Sakata and Crews, unpublished data].

When comparing IncT-dependent behavioral differences in males and in females, we find that a heightened propensity to display agonistic behavior and male-typical courtship behavior are correlated in females but not in males. Specifically, Mb females are both more aggressive and more likely to display male-typical courtship behaviors, whereas Fb males are more sexually vigorous but Mb males are more aggressive. Taken together, these results suggest that the mechanisms underlying intrasexual differences in behavior might differ across the sexes, and the fact that the pattern of IncT-dependent differences in neural metabolic capacity varies between the sexes supports this notion (see below).

Though POA and VMH volumes are not sexually dimorphic, they are affected by IncT [27]. For instance, Mb males have larger POAs than Fb males, and Mb females have larger POAs than Fb and Low females (Fig. 4). The difference between Mb and Low females persists following ovariectomy and following identical androgen treatments [34], and though further evidence is warranted (e.g., does the difference in POA size between Fb and Mb males depend on hormonal status?), this suggests that IncT organizes differences in POA volume in adulthood. Regarding the VMH, Mb females have smaller VMHs than Low females but not Fb females (Fig. 5), and because this difference is found when gonadally intact and following T treatment but not following ovariectomy, this intrasexual variation seems to be caused by differences in androgen sensitivity [34].

Incubation temperature affects metabolic capacity, and there are similarities and differences in the pattern with which IncT affects CO activity within males and within females (Table 3) [27]. In both sexes, Mb individuals have elevated CO activity in the AH, SEP and NS and lower CO activity in the VMH relative to same-sex Fb individuals. Because Mb individuals are generally more aggressive and because the AH, SEP and NS have been implicated in the expression of aggression in a variety of vertebrates [44–47], it is possible that elevated CO activity in these nuclei predisposes the display of aggressive behavior. On the other hand, IncT affects CO activity in the AME in females but not males. Furthermore, metabolic capacity in the POA is greater in Fb males than in Mb males but greater in Mb females than in Fb females. Interestingly, both Fb males and Mb females show more male-typical courtship behavior following T treatment than Mb males and Fb females, respectively [32, 34], suggesting that heightened POA metabolism could predispose the display of courtship behavior in the presence of androgens. This is consistent with the fact that the POA is an evolutionarily conserved nucleus controlling male-typical sexual behavior [35].

The hormonal dependence of intrasexual differences in CO activity has only been studied among Low and Mb females to date. When intact, Mb females have elevated metabolic capacity in areas like the NS, AME, SEP, AH and POA relative to Low females, and differences in the NS, AME, and SEP persist following gonadectomy and following androgen treatment (Table 4). Because the difference

Table 3
The direction and magnitude of intrasexual differences (individuals from the female-biased (Fb) or male-biased (Mb) IncTs) in males and females

Region	Males	Females
AH	Mb > Fb	Mb > Fb
AME	=	Mb > Fb
DLH	Mb > Fb	=
LH	=	=
NS	Mb > Fb	Mb > Fb
POA	Fb > Mb	Mb > Fb
PP	Fb > Mb	Mb > Fb
SEP	Mb > Fb	Mb > Fb
VMH	Fb > Mb	Fb > Mb

Note: '=' denotes no difference across IncTs. 'Mb > Fb' denotes Mb individuals have greater metabolic capacity than Fb individuals. 'Fb > Mb' denotes Fb individuals have greater metabolic capacity than Mb individuals. Differences are $P < 0.01$ (see Ref. [27] for other nuclei), see Fig. 3 for abbreviations.

Table 4
Dependence of within-sex differences (females from the low (Low) and male-biased (Mb) IncTs) in metabolic capacity in limbic brain areas on hormonal state (intact, gonadectomized (GNX), and GNX and testosterone (T) treatment)

Region	Intact	GNX	GNX + T
AH	Mb > Low	=	Mb > Low
AME	Mb > Low	Mb > Low	Mb > Low
DLH	Mb > Low	Mb > Low	Mb > Low
LH	=	=	=
NS	Mb > Low	Mb > Low	Mb > Low
POA	Mb > Low	Low > Mb	=
PP	=	=	=
SEP	Mb > Low	Mb > Low	Mb > Low
VMH	=	=	=

Note: '=' denotes no difference across IncTs. 'Mb > Low' denotes Mb females have greater metabolic capacity than Low females. 'Low > Mb' denotes Low females have greater metabolic capacity than Mb females. Differences are $P < 0.01$ (see Ref. [34] for other nuclei), see Fig. 3 for abbreviations.

between Low and Mb females exists only when intact and following ovariectomy and T treatment, the difference in CO activity in the AH seems to be due to a difference in the sensitivity to gonadal steroid hormones. On the other hand, the difference in POA metabolism is found only when intact (when gonadal steroid hormones differ between Low and Mb females) and, thus, activated by gonadal steroids.

Finally, we have begun studying how differences in the dopaminergic system could contribute to behavioral variation among male leopard geckos. Dopamine has been found to be an important neurotransmitter regulating the display of courtship and copulatory behavior in a variety of vertebrates, including lizards [48–51]. In a preliminary study, we injected castrated, T-implanted (10 mm) males from the Fb and Mb IncTs ($n = 12$ from each IncT) with different doses (0, 2, 4, and 8 mg/kg) of the D1 receptor antagonists, SCH 23390, and after 15 min, we tested them for courtship behavior. All males were injected with each of the four doses, each test separated by five days, using a Latin-squares design, and Fb and Mb males were matched for sexual vigor. We found that only the 8 mg/kg dose decreased the proportion of males displaying courtship behavior in Fb males, whereas both 4 and 8 mg/kg doses decreased courtship behavior in Mb males. This indicates that Fb males are more resilient to the detrimental effects of D1 receptor antagonism on courtship behavior and suggests that there may be IncT-dependent differences in D1 receptor expression in areas like the POA in which dopamine reception has been found to be important in the display of male-typical sexual behavior [48–50].

5. Between-sex differences in plasticity

It is important to study not only how developmental processes can shape phenotype in adulthood but also how these processes can shape phenotypic plasticity. Given that there exist neural differences caused by sex and IncT, it is plausible that the brains of males and females from different IncTs are 'set up' to respond differently to exogenous manipulations.

5.1. Response to sex steroid hormones

Sex differences in the capacity for androgens and estrogens to elicit different social behaviors have been extensively studied in a variety of vertebrates [1], and similar differences exist in the leopard gecko. Following gonadectomy and T replacement, males are much more likely to display agonistic and courtship behaviors than females [32–34]. On the other hand, following gonadectomy and E2 administration, only females display receptive behavior in response to a courting male [32]. These behavioral differences are likely to be organized by early sex steroid exposure and linked to differences in the expression of sex steroid hormone receptors and/or neural reactivity to gonadal steroids.

Table 5
Differences in neurometabolic plasticity following gonadectomy (GNX)

Region	Low females	Mb females	Mb males
AH	=	Intact > GNX	Intact > GNX
AME	=	Intact > GNX	Intact > GNX
DLH	Intact > GNX	Intact > GNX	Intact > GNX
LH	=	=	=
NS	=	=	Intact > GNX
POA	=	Intact > GNX	Intact > GNX
PP	Intact > GNX	Intact > GNX	=
SEP	Intact > GNX	Intact > GNX	Intact > GNX
VMH	Intact > GNX	Intact > GNX	=

Note: '=' denotes no difference between intact and gonadectomized (GNX) individuals given cholesterol. 'Intact > GNX' denotes intact individuals have greater metabolic capacity than GNX individuals. Differences are $P < 0.01$ (see Ref. [34] for other nuclei), see Fig. 3 for abbreviations. Gonadal sex seems to dominate neurometabolic plasticity in the NS and VMH, whereas IncT seems to dominate neurometabolic plasticity in the AH, AME, and POA.

Sex-dependent differences in behavioral response to T are paralleled by differences in the plasticity of neural metabolism [34] Metabolic capacity in the NS decreases following gonadectomy and increases following T treatment in Mb males but not Mb females (Tables 5 and 6), and metabolic capacity in the VMH and PP decreases following gonadectomy and increases following T treatment in Mb females but not Mb males (Fig. 7). In the DLH, Mb males and females show similar declines in CO activity following gonadectomy, but only males show CO activity increases following androgen treatment.

Interestingly, four nuclei with sex differences in metabolic plasticity following hormone manipulations—NS, VMH, PP and DLH—are also the only nuclei (of the 16 examined) in which CO activity is sexually dimorphic among intact Mb individuals [27,34]. Metabolic capacity in the NS is greater in Mb males when intact, and only males show significant

Table 6
Differences in neurometabolic plasticity following testosterone (T) treatment

Region	Low females	Mb females	Mb males
AH	=	T > GNX	T > GNX
AME	T > GNX	T > GNX	T > GNX
DLH	T > GNX	=	T > GNX
LH	=	=	=
NS	=	=	T > GNX
POA	T > GNX	T > GNX	T > GNX
PP	T > GNX	T > GNX	=
SEP	T > GNX	T > GNX	T > GNX
VMH	T > GNX	T > GNX	=

Note: '=' denotes no difference between gonadectomized (GNX) given cholesterol and GNX individuals given T. 'T > GNX' denotes T-treated individuals have greater metabolic capacity than GNX individuals. Differences are $P < 0.01$ (see Ref. [34] for other nuclei), see Fig. 3 for abbreviations. Gonadal sex seems to dominate neurometabolic plasticity in the NS and VMH, whereas IncT seems to dominate neurometabolic plasticity in the AH.

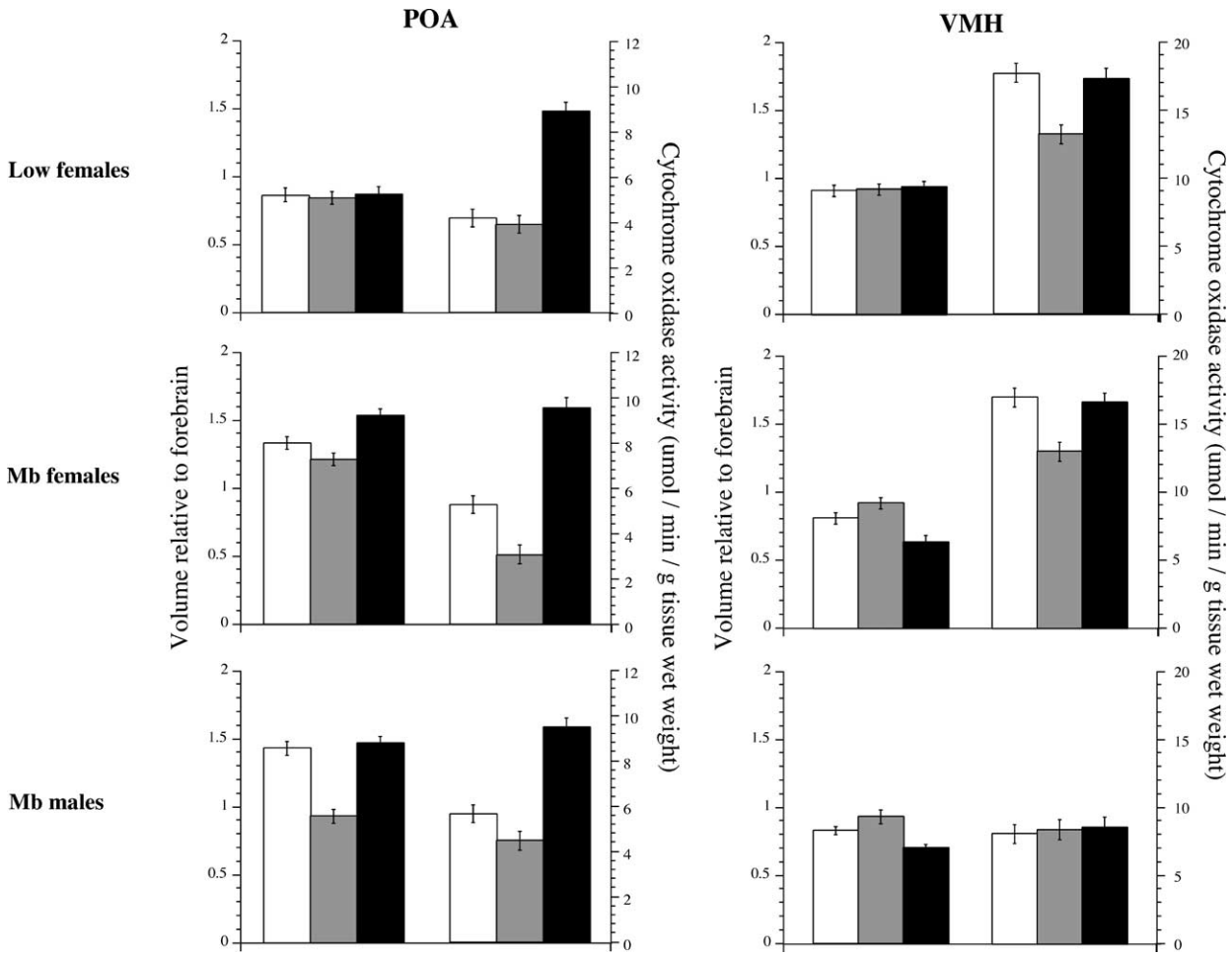

Fig. 7. There exist both inter- and intrasexual differences in neural responsiveness in nucleus volume (left side of each column) and cytochrome oxidase (CO) activity (i.e. metabolic capacity)(right side of each column), though the two parameters can show independent changes. In the POA and VMH, volume changes following hormone manipulations are found in Mb males and Mb females but not in Low females. On the other hand, CO activity in the POA changes in similar ways following testosterone (T) treatment in all individuals examined here [34]. In the VMH, CO activity changes in Low and Mb females but not in Mb males.

changes in CO activity following gonadectomy and following T treatment. Among Mb individuals, metabolic capacity in the VMH and PP is greater in females, and only females show significant changes in CO activity in these areas following gonadectomy and following T treatment. However, in the periventricular hypothalamus and torus semicircularis (TS) CO activity is not sexually dimorphic while intact but a sex difference in neural metabolic plasticity exists. Taken together, because CO activity while intact is sexually dimorphic in four of the six nuclei with sex differences in neural metabolic plasticity, this suggests that a difference in CO activity while intact is a good predictor of a difference in metabolic plasticity following hormone manipulations. If this is true, we predict that sex differences in neural metabolic plasticity following hormone manipulations will be more numerous among Fb individuals because there exist more differences in CO activity when intact (Table 1).

Volumetric plasticity was assessed only in the POA and VMH, and, just as there is no significant sex difference when intact, there is no sex difference in volumetric plasticity following hormone manipulations [34]. However, the VMH and POA show opposite patterns of change following hormone manipulations. In both sexes VMH volume increases following gonadectomy and decreases following T treatment, whereas POA size decreases following gonadectomy and increases following T treatment (Fig. 7).

5.2. Response to sociosexual experience

Among Mb individuals, the sexes show differences and similarities in endocrine and neurometabolic changes following heterosexual housing, though neither sex shows changes in POA and VMH volumes [52]. Just as in rodents, heterosexual housing increases androgen concentrations in males but not females [37]. Experience-dependent increases in CO activity in the POA and AME are found only in females, whereas increases in metabolic capacity in the AH, DLH and lateral hypothalamus (LH) are found only in males (Table 7). The male-specific increase in CO activity in the AH and DLH could be due to male-specific increases in androgen concentrations, as both areas show metabolic changes following androgen manipulations (Tables 5 and 6).

Table 7
Sex and IncT-dependent differences in neurometabolic changes following heterosexual social experience

Region	Low females	Mb females	Mb males
AH	=	=	E > N
AME	E > N	E > N	=
DLH	=	=	N > E
LH	E > N	=	E > N
NS	=	=	=
POA	E > N	E > N	=
PP	=	=	=
SEP	=	=	=
VMH	=	E > N	E > N

Note: '=' denotes no difference between experienced and naïve individuals. 'E > N' denotes experienced individuals have greater metabolic capacity than naive individuals. 'N > E' denotes naive individuals have greater metabolic capacity than experienced individuals. Differences are $P < 0.01$ (see Ref. [52] for other nuclei), see Fig. 3 for abbreviations. Gonadal sex seems to dominate neurometabolic plasticity in the AH, DVR, and POA, whereas IncT seems to dominate neurometabolic plasticity in the VMH.

It is surprising that metabolic capacity in the POA and AME does not increase with heterosexual housing in male leopard geckos as has been found in other species [53,54]. On the other hand, the fact that heterosexual housing increases CO activity in the VMH of males and females is consistent with results in rats and whiptail lizards [53,54].

We have yet to discern the factors underlying sex differences in neural metabolic changes following heterosexual experience, but it is plausible that these experiences activate different populations of neurons across males and females. Following copulation, females undergo a suite of hormonal changes that are responsible for yolk synthesis, egg formation, and egg laying that are not experienced by males [28]. It should be mentioned that experience effects were studied in males that were 2–3 years old whereas experience effects were studied in females that were one year old, and males were housed with females for at least one year whereas females were housed with males only for several months (one reproductive season during which fertile eggs were laid). Therefore, differences in age and/or duration of experience might have contributed to sex differences in metabolic plasticity.

6. Within-sex differences in plasticity

6.1. Response to sex steroid hormones

Intrasexual differences in behavioral response to T treatment in adulthood have been found in males and females, particularly with regard to male-typical courtship behavior. Testosterone treatment following castration leads to a significantly greater induction of courtship behavior in Fb males than Mb males, though the same manipulation leads to a greater induction of scent marking (territorial behavior) in Mb males than Fb males [32]. In females, T treatment following ovariectomy leads to greater induction of male-typical courtship behavior in Mb females than in Low females [34], and Fb females also show low levels of courtship behavior following T administration [32]. Therefore, IncT-dependent differences in the induction of courtship behavior following T vary between males and females: in males, Fb individuals show more courtship behavior, whereas in females, Mb individuals show more courtship behavior following T treatment. As mentioned above, both Fb males and Mb females have elevated metabolic capacity in the POA, which supports the notion that elevated neural activity in the POA might prime the display of male-typical courtship behavior.

Neurometabolic and volumetric plasticity following hormonal manipulations are also affected by IncT (between Mb and Low females; Tables 5 and 6) [34]. Metabolic capacity in the POA, AME, AH, and TS decreases following ovariectomy in Mb but not Low females (Table 5). Testosterone treatment significantly elevates metabolic capacity in the AH and TS only in Mb females and in the DLH only in Low females (Table 6).

When gonadally intact, of the 16 nuclei examined, Mb females have elevated CO activity relative to Low females in the POA, AME, AH, TS, DLH, NS, and SEP as well as in the dorsal ventricular ridge and striatum [27]. All five of the nuclei with intrasexual differences in metabolic plasticity following hormone manipulations—POA, AME, AH, TS and DLH—show intrasexual difference while intact, suggesting that intrasexual differences in metabolic plasticity are intimately related to intrasexual differences in neural metabolism while intact. Interestingly, when comparing intersexual differences in plasticity with intrasexual differences in plasticity, it is evident that there are few nuclei in which both gonadal sex and IncT affect plasticity; only in the DLH and TS do gonadal sex and IncT affect CO activity changes following hormone manipulations. Therefore, it is plausible that gonadal sex and IncT organize plasticity through different mechanisms.

Though POA and VMH volumes do not change with hormonal condition in Low females, both fluctuate in Mb females [34]. The POA decreases and increases, respectively, following gonadectomy and following T treatment whereas the VMH increases and decreases, respectively, following gonadectomy and following T treatment (Fig. 7). Combined with the lack of sexual dimorphism in volumetric plasticity following hormonal manipulations in Mb individuals, this suggests that the sensitivity of POA and VMH size to hormonal manipulations is organized by IncT not gonadal sex.

6.2. Response to sociosexual experience

Behavioral phenotype changes following social experiences [37,55–59], and recently we investigated whether behavioral change following interactions with females differed between Fb and Mb males [60]. We found that sociosexual experience (10 copulatory interactions) increases the display of territorial behavior in Fb but not Mb males. Further, Fb males are more likely to display arousal-related behaviors in response to cues that predict

the introduction of a female, suggesting that more sexual conditioning occurred in these males [60]. Because the POA has been implicated in sexual conditioning [57,61] and because intact Fb males have elevated CO activity in the POA relative to intact Mb males [27], we propose that heightened POA metabolism might facilitate experience-dependent behavioral change. However, neither Fb nor Mb males showed experience-dependent increases in the retention of courtship behavior following castration, a type of plasticity found in other species like cats, rats, mice, and whiptail lizards [37,54,62, Sakata and Crews, unpublished data].

Neurometabolic plasticity following heterosexual housing differs between Low and Mb females [52]. Increases in metabolic capacity in the VMH are found in Mb but not Low females, whereas increases CO activity in the LH are found in Low but not Mb females (Table 7). It is possible that males react differently to Low and Mb females in this context, thereby changing the experiences of each female and, hence, neural activity patterns. The number of clutches laid by the two groups of females when housed with males was not reported, and it would have been interesting to compare their reproductive output. In females from both IncTs, heterosexual housing increases metabolic capacity in the POA and AME but does not affect the volumes of the POA or VMH. Relative to the number of nuclei in which Mb males and females differed, the number of nuclei in which Low and Mb females differed in this plasticity is small, and this could be due, for example, to the fact that the differences in the degree to which reproductive experiences alter physiology is greater when comparing males and females than when comparing females from different IncTs.

7. Model for effects of IncT and gonadal sex on phenotype in naïve individuals

Across a suite of phenotypes, it appears that both IncT and gonadal sex can have organizing effects on phenotype. Just as organizational effects in other species are linked to sex steroid hormone exposure during a critical period in development, we propose that the effects summarized here are similarly influenced by sex steroid hormone exposure during embryonic development. There is considerable evidence showing that sex steroid hormone synthesis and reception occurs during embryonic development [63–67]. We propose that IncT affects the amount of sex steroid hormones produced by the embryo or absorbed from the yolk, which in turn affects both the gonadal sex of the individual as well as the differentiation of the brain (Fig. 8). Specifically, embryos of eggs incubated at the Mb IncT are exposed to more androgens and less estrogen than embryos in eggs incubated at the Low, Fb or High IncTs. Further, we propose that there is a threshold of androgen and estrogen exposure that determines gonadal sex: embryos that are

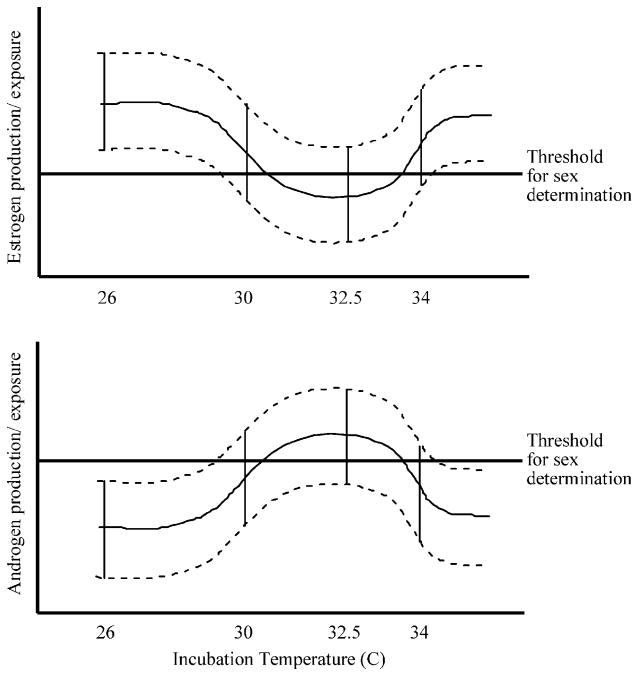

Fig. 8. Proposed mechanism by which incubation temperature (IncT) and gonadal sex organizes the brain of leopard geckos. According to this model, IncT affects the embryo's production of or exposure to sex steroid hormones during development, which, in turn, affects the gonadal sex of the organism. Those exposed to suprathreshold amounts of estrogen and subthreshold amounts of androgens develop into females, whereas those exposed to subthreshold estrogen and suprathreshold androgen develop into males. On average, males are exposed to more androgens and less estrogens than females, and on average, individuals hatched from eggs incubated at 32.5 °C (male-biased IncT) are exposed to less estrogen and more androgens relative to individuals from other IncTs. These differences could account for the masculinized phenotype of individuals from the male-biased IncT, for example. Plotted are the mean (solid line) and variation (hatched lines) of hormone production or exposure across IncTs.

exposed to subthreshold amount of estrogen and suprathreshold amounts of androgens develop into males, whereas those exposed to suprathreshold amounts of estrogen and subthreshold amounts of androgens develop into females. According to our model (Fig. 8), males are exposed to less estrogen and more androgens on average during development relative to females, which could organize sex differences in social behaviors and in responsiveness to hormone manipulation in adulthood. Furthermore, Mb individuals are exposed to more androgens and less estrogens on average than Fb individuals during development, and this may underlie the differences in agonistic behavior among naïve individuals as well as in plasticity following hormone manipulations or sociosexual experience. It will be important to test this model by manipulating the embryonic environment and assessing neural and behavioral phenotype.

On the other hand, it is possible that IncT affects sexual differentiation independent of sex steroid hormones. There exist temperature-sensitive neurons in limbic brain areas such as the POA [68,69], and it is possible that IncT directly influences the development of these neurons. Evidence

suggests that some IncT effects are independent of sex steroid hormones. For example, females hatched from estrogen-manipulated eggs incubated at the Mb IncT show identical growth rates and aggressiveness as females hatch from unmanipulated eggs from the same IncT, and both groups of females grow faster than Low females [25]. It is important to highlight, however, that these two hypotheses are not mutually exclusive; there might exist traits affected by sex steroid hormones and traits unaffected by hormones. Furthermore, because some temperature-sensitive neurons are also sensitive to sex steroid hormones [70], it is possible that IncT and steroid hormones interact or synergize to affect sexual differentiation as they do to determine sex in TSD species [71].

8. Other considerations

Differences in neural metabolic change in response to sex steroid hormone exposure or social experience could be due to a variety of factors. Metabolic capacity is related to, for example, the amount of excitatory innervation [17,18], and it is possible that differences in the degree to which these manipulations alter excitatory inputs underlie variation in CO activity plasticity. Sex steroid receptors as well as immediate early genes act as transcription factors [72–74], and differences in these parameters could also modulate variation in the degree to which the expression of CO genes change with manipulations [17,75].

When looking at plastic changes in brain area volume and metabolic capacity in response to manipulations, it is clear that their regulation can be independent (Fig. 7) [27,34,52]. This is important because changes in neural morphology are thought to be mediated, in part, by changes in neural activity. For example, in Low females, POA and VMH volumes are not affected by hormonal condition, but metabolic capacity in the POA and VMH fluctuates with T treatment (Fig. 7) [34]. Among Mb females, VMH volume increases following gonadectomy and decreases following T treatment but CO activity follows the inverse pattern, and among Mb males, VMH volume but not CO activity varies with hormonal state [34]. The volumes of the POA and VMH do not change with heterosexual housing in these three groups but CO activity increases with experience in the POA of Low and Mb females and in the VMH of Mb males [52].

There are other examples comparing volumetric and metabolic plasticity, and instances of association and dissociation exist. For example, in white-crowned sparrows, seasonal changes in androgens are paralleled by increases in the volume and metabolic capacity of brain nuclei controlling singing behavior [76]. The higher vocal center, robust nucleus of the archistriatum, and Area X show increases in volume as well as CO activity following androgen treatment. On the other hand, metabolic capacity in the lateral magnocellular nucleus of the anterior neostriatum does not increase following T, despite the significant increase in volume. Another dissociation is seen following binaural ablation of the ear ossicles in rats: CO activity in the anteroventral cochlear nucleus decreases while volume remains unchanged [77]. It will be interesting to discern the factors that affect the linkage between these two neural traits.

It is important to note that the direction of causality between changes in neural phenotype and changes in behavior following particular manipulations is unknown. For example, experienced males may be more likely to display territorial behavior because they have elevated metabolic capacity in the AH, or it could be that CO activity in the AH is elevated because experienced males display more territorial behavior [52,62]. On the other hand, the fact that naïve individuals show differences both in neural phenotype and behavior suggest that some of these neural differences drive behavioral differences (Sections 3–6). Thus, parallel studies of CO activity and of behavior highlight the predictive value of differences in neural metabolism on behavioral phenotype.

9. Comparisons with other species

As mentioned before, there exist many parallels between the effects of IncT on phenotype in leopard geckos and the effect of intrauterine position (IUP) in mammals [7,8]. An effect of IUP was first reported in rats [78] then subsequently in mice, gerbils and other mammals [7,8]. Overall, Mb leopard geckos resemble 2M rodents (situated between two males in utero) whereas Fb leopard geckos resemble 2F rodents (Table 8). Though species differences exist, 2M males and females have been found to have higher androgen concentrations in adulthood and to be more sensitive to the activational effects of testosterone on aggression and male-typical mounting behavior than their same-sex 2F

Table 8
Similarities and differences between the effects of intrauterine position (2M vs. 2F) in gerbils and incubation temperature (Fb vs. Mb) in leopard geckos

	Gerbils	Leopard Geckos
Males		
Body size	2M > 2F	Mb > Fb
Aggression	2M > 2F	Mb > Fb
Male-typical sexual behavior	2M > 2F	Fb > Mb
Testosterone concentrations	2M > 2F	Mb > Fb
Females		
Aggression	2M > 2F	Mb > Fb
Male-typical sexual behavior	2M > 2F	Mb > Fb
Testosterone concentrations	2M > 2F	Mb > Fb

Note: '2M > 2F' denotes that individuals that were situated between two males in utero have higher means than individuals that were situated between two females in utero. 'Mb > Fb' denotes Mb individuals have greater metabolic capacity than Fb individuals. 'Fb > Mb' denotes Fb individuals have greater metabolic capacity than Mb individuals, see Refs. [7,8] for reviews on gerbils.

counterparts. Like Fb male geckos [60], 2F male mice are more active than 2M males, and like Mb male geckos [32], 2M male gerbils scent mark more than their 2F brothers, even when T concentrations are equalized. Regarding neural metabolic differences, 2M female gerbils have elevated CO activity in the AH relative to 2F females [79], similar to the difference between Mb and Fb female geckos [27].

There are, however, some differences between IUP and IncT effects. For example, 2M male gerbils impregnate more females and show shorter copulation latencies than 2F males, whereas Mb male leopard geckos show less sexual behavior than Fb males [32, Sakata and Crews, unpublished data]. Though 2F male mice show more receptive behavior following E2 treatment than 2M males, no difference between Fb and Mb male geckos in the capacity of E2 to elicit female-typical receptive behavior has been found [32].

It will be interesting to test the generality of other findings across these two systems. For example, it will be interesting to assess whether IncT affects anxiety levels, novelty seeking, and susceptibility to environmental estrogens in the leopard gecko as IUP does in some mammals [8]. It will also be interesting to assess whether 2F and 2M males and females differ in their behavioral and neural changes following heterosexual experiences (Table 7), and whether IUP and gonadal sex interact to affect neural metabolism and neuromorphology. For example, does the direction of sex difference in POA metabolism vary between 2F and 2M rodents as it does between Fb and Mb geckos (Table 3)? In natural field settings, 2M mice of both sexes maintain larger home ranges than 0M individuals [8,80], and it will be important to assess differences in territory sizes between Fb and Mb male geckos. The sex ratio of litters produced by 2F and 2M female mice and gerbils differs: 2M females give rise to more male-biased litters whereas 2F females give rise to more female-biased litters. Because the nesting preferences of female leopard geckos will affect the sex ratio of their offspring, it will be important to assess whether females from different IncTs prefer different nesting habitats.

Effects of IncT on behavior are not limited to species with TSD but have also been found in species with genotypic sex determination (GSD) [81]. Incubation temperature affects endocrine profiles, running and swimming speeds, thermal preferences, and morphology in a number of reptiles with GSD. However, we have not found a report demonstrating that IncT affects the social behavior of adult lizards with GSD. It is possible that IncT effects on social behaviors have not been examined in GSD species, but it is also plausible that IncT affects sex steroid hormone exposure during development in TSD but not GSD species. If the latter is true and if IncT-dependent differences in sex steroid hormone exposure (versus direct IncT effects) are paramount in engendering differences in social behavior in adulthood, then IncT should have less of an effect on sexual differentiation in GSD species. It would be interesting to compare IncT effects in GSD species that are closely versus distantly related to TSD species, as IncT might have greater effects on sexual differentiation in GSD species that gave rise to or branched off from TSD species.

10. Evolutionary considerations

There have been many discussions on the selection pressures underlying the evolution and maintenance of environmental sex determination (ESD), of which TSD is a specific subset [82–88]. Some hypotheses emphasize phylogenetic inertia (i.e. no current advantage to TSD) or inbreeding avoidance, but the hypothesis that is the most consistent with available data deals with differential fitness, a hypothesis first proposed by Charnov and Bull [82]. In brief, this hypothesis claims that ESD will evolve when the environment is patchy (e.g. resource distribution, predation) and the sexes differ in the relative benefits gained by specific niches. Research on the evolution of TSD has focused on how specific IncTs produce offspring with traits (analogous to 'patches' in the Charnov–Bull model) that are differentially advantageous to one sex over the other [87,88]. Consequently, there will be selection pressure for the sex that benefits the most from the trait to become more abundant at that specific environment. For example, in snapping turtles, individuals from cooler IncTs have elevated growth rates [86,89], and cooler IncTs produce males. This suggests that there might have been selection pressure to produce males at cooler IncTs because males differentially benefit from larger body size due to the high degree of male–male competition for females [90–92]. In species in which large body size translates into increased fecundity in females and in which this benefit exceeds that for larger body size in males, females may be selected for at environmental conditions that increase growth rates [83]. It is the balance of such evolutionary pressures that dictates sex ratios at particular conditions.

In the leopard gecko, male-biased sex ratios are produced at the IncT of 32.5 °C and female-biased sex ratios are produced at 30 °C (Fig. 2). From an evolutionary perspective, it is possible that males benefit more from the phenotype produced by 32.5 °C and that females benefit more from the phenotype produced by 30 °C. In general, post-hatching growth (body size, weight and head width) is greater in individuals incubated at 32.5 °C [25], and individuals from 32.5 °C are more territorial and aggressive [30,32,34]. Because of the high level of male–male aggression in this species [32,33,93], it is possible that large body size and head width benefit males more than females. This is consistent with the fact that clutch size is fixed at one or two eggs in female leopard geckos and other Eublepharid lizards [93]; in other words, increased size does not translate into increased fecundity.

On the other hand, the selective benefit of being female when incubated at 30 °C remains unknown, and it will be important to assess fitness parameters in females across

the range of IncTs. A related question is how and why males are maintained at 30 °C, particularly when males from 32.5 °C seem to be at a selective advantage. One reason could be that, in general, Fb males are more sexually vigorous [32] and more active than Mb males [60]. It is possible that the increased activity and sexual vigor of Fb males allows them to better find and copulate with females, a sexual strategy similar to sneaker males seen in other species [6]. This increased propensity to find and copulate with females could allow for increased reproductive success of Fb males when competing with larger, more territorial Mb males and allow for the maintenance of males at 30 °C.

In trying to understand the evolutionary forces underlying sex ratio evolution, it is also critical to consider maternal nesting behavior. Two studies to date have examined nesting behavior of female leopard geckos, and both studies find that females avoid laying eggs at the extreme IncTs [94,95]. Bragg et al. [95] found that the mean IncT at which eggs were buried was 28.7 °C, an IncT at which ~10% of the offspring are male (assuming constant temperature across incubation period). The population sex ratio for leopard geckos in the wild is unknown, but sex ratios are often significantly female-biased in other TSD species [20,96,97]. Only two of the nine females studied laid eggs at IncTs exceeding 32 °C [95], suggesting that very few Mb males might be found in the field. Furthermore, there was a significant effect of time within the laying season on nest-site selection: eggs laid later in the season tended to be buried at warmer IncTs. (The ambient thermal environment was held constant in the experiment.) This suggests that more females are born at the beginning of the season whereas males tend to be produced later in the season, and it is plausible that females benefit more from the increased time for growth relative to males as suggested in other species [83]. Whereas this is contrary to our earlier statement that body size does not increase clutch size, the amount of growth prior to the first breeding season could affect the time to sexual maturity in females and the number of clutches laid during their first season of reproduction. If survivorship is low in the field, an increase in the number of eggs laid during the first breeding season could yield substantial fitness benefits. Assuming that egg-laying behavior in the laboratory resembles that in the field, it is also possible that the accelerated growth rate conferred by warmer IncTs, particularly for males, partially compensates for the shorter growth period of individuals hatched later in the season.

Finally, because a central tenet of the differential fitness hypotheses is that IncT affects hatchling survivorship, it will be important to discern how hatchling phenotypes such as running speed and foraging skills are affected by the interaction of IncT and gonadal sex in this species. Furthermore, it will be important to assess how thermal variance, which has been shown to affect sex ratio in other TSD species [98–100], affects gonadal sex, hatchling phenotype, and sexual differentiation in leopard geckos.

11. Conclusions and future directions

Here we reviewed our recent studies demonstrating that an ecologically relevant parameter—incubation temperature—dramatically affects both between- and within-sex variation in phenotype and plasticity, and we highlight the following points:

(1) Generally speaking, males are more aggressive, have higher androgen and lower estrogen concentrations, and have elevated CO activity (metabolic capacity) in amygdaloid areas and lower CO activity in the VMH than females.
(2) Relative to their same-sex counter parts from the cooler IncTs (e.g. Low and Fb IncTs), males and females from the Mb IncT have masculinized phenotypes; in other words, with a masculinization of sex ratio is a concomitant masculinization of phenotype.
(3) Incubation temperature is a significant source of within-sex variation in both sexes (Table 3), and thus, the pattern of sex differences varies across IncTs (Table 1).
(4) Some between- and within-sex differences in behavior and neural morphology and metabolism persist following the equalization of hormone concentrations in adulthood (Tables 2 and 4), indicating that both gonadal sex and IncT have organizing effects on behavioral phenotype, whereas other phenotypic differences are reliant on differences in circulating sex steroid hormone concentrations.
(5) Differences in neural phenotype correlate with differences in behavioral expression. For example, elevated CO activity in the POA is correlated with increased display of courtship behaviors (e.g. Fb males and Mb females), and elevated metabolic capacity in the amygdaloid, septal and hypothalamic are correlated with heightened aggressiveness (Mb males and females).
(6) Neurometabolic plasticity following hormone manipulations (Tables 5 and 6) and sociosexual experience (Table 7) is dominated by IncT in some areas and by gonadal sex in others. Between- and within-sex differences in neural metabolic capacity while gonadally intact are predictive of differences in neural metabolic plasticity following hormone manipulations.
(7) Neural morphology and neural metabolic capacity can show independent fluctuations following various manipulation.
(8) It is plausible that sex- and IncT-dependent differences in gonadal steroid hormone concentrations during development (Fig. 8) could organize differences in phenotype and plasticity, or that IncT has direct effects on phenotype.
(9) The phenotypes of Fb and Mb leopard geckos, respectively, resemble that of 2F and 2M rodents. Therefore, the mechanisms by which IncT and intrauterine position affect behavioral, endocrinological and neural phenotypes could be similar.

(10) The leopard gecko offers an excellent evolutionary model to study the selective pressures underlying sex ratio evolution.

As with all pursuits in biology, there are many questions left unanswered. For example, sex differences in behavioral and neural changes following androgen manipulations and following sociosexual experience have only been studied in Mb individuals, and intrasexual differences in hormonal and experiential neural plasticity have only been studied between Low and Mb females. It will be important to assess sex differences in behavioral and neural plasticity among individuals from the Fb IncT, for example. Because males can be created by inhibiting the E2 synthesis during embryonic development [25,89,101], it would also be interesting to assess sex differences at IncTs that typically produce only females (e.g. Low IncT); for example, are POA and VMH volumes the same between females and 'sex-reversed males' from the Low IncT just as they are in individuals from the Fb and Mb IncTs? Because IncT affects behavioral plasticity following sociosexual experiences in males [60], it is possible that IncT affects neural responses to sociosexual experience in males, just as they do in females (Table 7). In this respect, it would be interesting to juxtapose not only Fb and Mb males but also 'sex-reversed males' from the Low IncT. We should analyze the relationship between sex- and IncT-dependent differences in sex steroid receptor expression and differences in neural plasticity following manipulations of sex steroid hormone levels or social experience. We should investigate the intergenerational consequences of IncT. For example, because T concentrations are elevated in High females across the follicular cycle [28] and because maternal steroid hormones are deposited in the yolk [102–105], it is also possible that the eggs of High females could contain more androgen and, thus, these offspring might be more likely to develop into males and/or be more masculinized (for maternal effects on sex ratio in other TSD species: [81,106–108]). In order to understand the selection pressures underlying sex ratio evolution in leopard geckos, it will be important to conduct field studies. Unfortunately, given the current political climate, such field studies in Pakistan and Afghanistan are difficult. In this respect, it will be very profitable to focus on some Australian lizard species—e.g. rock dragon (*Ctenophorus decressii* and *C. ornatus*), frillneck lizard (*Chlamydosaurus kingii*), and jacky dragon (*Amphibolurus muricatus*)—that are more amenable to field studies, have similar sex ratio curves across IncTs, and have similar changes in nest temperatures during the mating season [109–111].

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