Cytochrome Oxidase Activity in the Preoptic Area Correlates With Differences in Sexual Behavior of Intact and Castrated Male Leopard Geckos (Eublepharis macularius)

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Although the utility of analyzing behavioral experience effects on neural cytochrome oxidase (CO) activity is well recognized, the behavioral correlates of endogenous differences in CO activity have rarely been explored. In male leopard geckos (Eublepharis macularius), the incubation temperature experienced during embryogenesis (IncT) and age affect CO activity in the preoptic area (POA), an area that modulates copulatory behavior. In this study, the authors assessed whether differences in POA CO activity correlate with differences in sexual behavior in intact and castrated geckos. Males with IncT- and age-dependent increases in POA CO activity mounted females with shorter latencies while intact and after castration and ejaculated more frequently after castration. The authors discuss the predictive value of CO activity and propose similar parallels in other species.

Cytochrome oxidase (CO) is a rate-limiting enzyme in oxidative phosphorylation and reflects metabolic capacity (Gonzalez-Lima, 1992; Wong-Riley, 1989). CO activity is intimately related to the metabolic history of neural populations (Wong-Riley, Nie, Hevner, & Liu, 1998), and consequently, many have studied changes in CO activity after behavioral (e.g., classical conditioning; Potenza, Jones, & Gonzalez-Lima, 1998) or anatomical manipulations (e.g., lesions, monocular deprivation; Balthazart, Stamatakis, Bacola, Aribot, & Derman, 2001; Wong-Riley et al., 1998). However, few have analyzed how differences in CO activity correlate with differences in behavioral phenotype. This is a worthy endeavor because differences in CO activity are likely to reflect differences in the amount of excitatory and inhibitory innervation and baseline activity (Wong-Riley, 1989; Wong-Riley et al., 1998), which, in turn, can induce variation in behavioral predispositions.

The leopard gecko, Eublepharis macularius, offers an excellent system in which to investigate the predictive value of differences in CO activity. This is because variables such as the incubation temperature (IncT) at which the embryo develops and the age of the individual significantly affect CO activity in limbic brain areas that are critical for the display of social behaviors, particularly the preoptic area (POA). Young adult (1 year old) male geckos hatched from eggs incubated at 30 °C (30M) have elevated metabolic capacity in the POA relative to age-matched males hatched from eggs incubated at 32.5 °C (32.5M; Coomber, Crews, & Gonzalez-Lima, 1997). Older (2–3 years old) 32.5Ms have elevated POA metabolism relative to young 32.5Ms and similar POA metabolism relative to young 30Ms (Crews, Coomber, & Gonzalez-Lima, 1997). Because the POA is an evolutionarily conserved nucleus critical for the display of male-typical sexual behavior (Crews & Silver, 1985; Meisel & Sachs, 1994), we hypothesized that young adult 30Ms and older 32.5Ms might show more sexual behavior than young adult 32.5Ms. In other words, elevated CO activity in the POA might reflect increased excitatory drive into the POA, and males with elevated POA metabolism might be more primed to display sexual behavior.

Here we analyzed the courtship and copulatory behaviors of intact and castrated males with different POA metabolism (young 30Ms, young 32.5Ms, and older 32.5Ms). We studied only older 32.5Ms because the effect of age on CO activity was studied only in these males (Crews et al., 1997). We investigated behavioral differences in castrated males to assess whether behavioral differences in intact males depend on the presence of gonadal steroids. This is important because young 30Ms have elevated estradiol concentrations relative to young 32.5Ms (reviewed in Crews, Sakata, & Rhen, 1998), which could contribute to behavioral variation while intact. Finally, because sociosexual experience increases the display of sexual behavior after castration and affects CO activity in the POA in other species (Meisel & Sachs, 1994; Sakata, Gonzalez-Lima, Gupta, & Crews, 2002; Sakata, Gupta, Gonzalez-Lima, & Crews, 2002), we also investigated the effects of sociosexual experience on postcastration sexual behavior.

Method

We used adult male leopard geckos (Eublepharis macularius) that were hatched from eggs incubated at either 30 °C (30Ms: n = 24) or 32.5 °C (32.5Ms: n = 48). The leopard gecko is a species with temperature-dependent sex determination (reviewed in Crews et al., 1998). At 30 and...
32.5 °C, respectively, a sex ratio of approximately 30% and 70% (percent male) is produced. All males were raised in isolation in polypropylene containers (30 cm long × 12 cm wide × 6 cm high) and thus were socially naive at the beginning of the experiment. All subjects were housed in 14:10-hr light–dark photocycle, and, for the first 10 weeks after hatching, maintained at 30 °C and given water and crickets 5 days a week. Thereafter, temperature was cycled from 18 °C at night to 30 °C during the day, and subjects were given water and mealworms three times a week. Crickets and mealworms were dusted with vitamin supplements. After reaching sexual maturity (40–50 weeks), some males (1.0–1.5 years: young males, n = 24 from each IncT) were placed in larger cages (45 cm long × 25 cm wide × 20 cm high) and remained in isolation. Some 32.5Ms were kept in the smaller polypropylene containers until 2–3 yrs of age, (older 32.5Ms: n = 24), then transferred to the larger cages and kept in isolation. Because leopard geckos commonly live up to 10 years, we were not studying the effect of senescence in these older males.

**Behavioral Testing**

After acclimating to the larger home cage for at least 1 week, all males were allocated to two groups. One group was kept sexually naive (young 30Ms and 32.5Ms: n = 12; older 32.5Ms: n = 11), whereas the other group was given 10 opportunities to copulate with sexually receptive females, each separated by 3–4 days (younger 30Ms and 32.5Ms: n = 12; older 32.5Ms: n = 13). We first screened females for sexual receptivity with sexually vigorous males and used only females that remained motionless and did not bite back in response to courtship. Before copulation opportunities, we removed cage materials (brick, shelter, and water dish) and, after a 5-min habituation period, exposed the males to a receptive female. We terminated the test if a male failed to body-grip the female within 5 min. If a male body-gripped the female, we extended testing for, at most, another 5 min to allow for mounting; if a male mounted the female, we extended the test for, at most, another 5 min to allow for ejaculation. Males in the naive group underwent a similar manipulation without the opportunity to copulate (removal of cage material followed by a 5-min habituation period, followed by a 5-min control period). After the last test, we gonadectomized all males under cold anesthesia and returned them to their home cage. Beginning 1 week after surgery, we tested all males for sexual behavior 10 times, with each test separated by 3–4 days, using the same protocol as before gonadectomy.

In all courtship tests, we recorded the occurrence and time at which males body-gripped, mounted, and ejaculated with stimulus females. Using these data, we calculated latency scores. **Body-grip latency** was defined as the interval from when the female was placed into the cage to when the male body-gripped the female. **Mount latency** was defined as the interval from body-grip to mount, and **ejaculation latency** was defined as the interval from mount to ejaculation. Latencies were defined as such because courtship behavior follows a hierarchical pattern (males first body-grip, then mount, then ejaculate), and each behavior is contingent on the preceding behavior (e.g., a male cannot ejaculate without mounting). Similar latencies are calculated when analyzing data in other species (Meisel & Sachs, 1994). If a male did not show a particular behavior during a given test, we gave no latency score for that test. Because some males never displayed particular behaviors, sample sizes are reduced in these analyses (e.g., mount latencies after castration).

Three to four weeks after the last test with females, we administered two or three consecutive tests to ensure that our castrations were effective in eliminating courtship behavior. In these tests, only 13% of males body-gripped at least once, 9% mounted at least once, and 4% ejaculated at least once. Thereafter, we deeply anesthetized all males using ice then rapidly decapitated them and checked for residual testes. One young naive 30M and one older experienced 32.5M had residual testes and were excluded from the analysis of postcastration behavior (young naive 30Ms: n = 11; older experienced 32.5Ms: n = 12).

**Statistical Analysis**

For data from intact males, we analyzed group differences in the proportion of tests in which males body-gripped, mounted, and ejaculated with a female and differences in body-grip, mount, and ejaculation latencies. We compared group differences in behavior using a univariate analysis of variance (ANOVA) with group (younger 30M, young 32.5M, and older 32.5M) as the independent variable. If the effect of group was significant, we performed post hoc Tukey’s honestly significant difference tests.

We replicated the same set of statistical analyses for postcastration data. However, because both experienced and naive males were tested after castration, we used a two-way ANOVA with group and experience (naive and experienced) as the independent variables. We performed post hoc Studentized t tests when significant effects were found.

Because differences in postcastration behavior were found, we wanted to assess the contribution of baseline differences in behavior while intact. We ran an analysis of covariance (ANCOVA) with group as the independent variable and behavioral score while intact as the covariate. Only experienced males were used in these analyses.

Finally, to assess the effect of castration on courtship latencies and frequency, we used a repeated measures MANOVA. The two dependent variables were behavior scores before castration and behavior scores after castration, and because only experienced males were analyzed (only they had scores pre- and postcastration), the only independent variable was group.

All analyses were done with JMP 3.2 (SAS Institute, 1995) for the Macintosh, and for all analyses, unless otherwise stated, α = .05. All proportion data were arc-sine square-root transformed, and latencies were log transformed to improve normality (Stevens, 1996).

**Results**

In intact males, we found a group difference in mount latencies, $F(2, 26) = 4.38, p = .02$, but not in any other parameter (see Table 1). Older 32.5Ms mounted females sooner than younger 32.5Ms ($p < .05$).

In castrated males, we found a similar difference in mount latencies (but not other latencies), $F(2, 42) = 4.22, p = .02$. Young 30Ms and older 32.5Ms mounted females sooner than younger 32.5Ms ($p < .05$, Table 1). These differences persisted even after we used an ANCOVA (only experienced males) to control for mount latency differences while intact, $F(2, 16) = 6.21, p = .01$. There was no difference between castrated experienced and naive males in courtship and copulatory latencies.

We also found a group difference in the proportion of tests in which castrated males ejaculated with females, $F(2, 64) = 5.14, p < .01$. Young 30Ms and older 32.5Ms ejaculated with females more often after castration than young 32.5Ms (see Figure 1). These differences persisted even after we controlled for the proportion of tests with ejaculations while intact (ANCOVA), $F(2, 32) = 6.36, p < .01$. There was no difference between experienced and naive males in the frequency of body-grips, mounts, or ejaculations after castration.

Castration increased body-grip latencies, $F(1, 23) = 7.82, p = .01$; and mount latencies, $F(1, 17) = 16.12, p < .01$; but not ejaculation latencies (Table 1). There was a significant interaction between group and gonadal state on mount latencies, $F(2, 17) = 3.67, p < .05$, and post hoc contrasts revealed that mount latencies significantly increased after castration only in young 32.5Ms, $F(1, 4) = 104.07, p < .01$. We emphasize, however, that the number of males that showed mounting behavior both before and after castration was relatively small (young 30Ms: n = 7; young 32.5Ms: n = 12).
Individual differences in neural phenotype are often accompanied by differences in behavioral phenotype. In the leopard gecko, differences in neurometabolic phenotype are caused by embryonic experiences (incubation temperature: IncT) and age. Young adult (50 weeks) males hatched from eggs incubated at 30 °C (30Ms) have elevated CO activity in the POA, an area critical for the display of male-typical sexual behavior across all vertebrates studied to date (Crews & Silver, 1985; Meisel & Sachs, 1994), relative to same-age males incubated at 32.5 °C (32.5Ms; Coomber et al., 1997). Metabolic capacity in the POA increases significantly with age in 32.5Ms such that older (2–3 years) males have similar CO activity as young adult 30Ms (Crews et al., 1997).

Table 1

<table>
<thead>
<tr>
<th>Condition and latency measure</th>
<th>Young 30M</th>
<th>Young 32.5M</th>
<th>Older 32.5M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact Body-grip</td>
<td>59.1 ± 11.2</td>
<td>76.9 ± 9.9</td>
<td>71.0 ± 6.6</td>
</tr>
<tr>
<td>Mount</td>
<td>145.0 ± 12.0</td>
<td>169.1 ± 16.6*</td>
<td>118.6 ± 6.5</td>
</tr>
<tr>
<td>Ejaculation</td>
<td>82.8 ± 12.1</td>
<td>126.1 ± 14.7</td>
<td>104.2 ± 20.2</td>
</tr>
<tr>
<td>Postcastration Body-grip</td>
<td>77.1 ± 15.4</td>
<td>112.0 ± 12.6</td>
<td>107.6 ± 19.0</td>
</tr>
<tr>
<td>Mount</td>
<td>146.8 ± 10.2</td>
<td>189.4 ± 11.5#</td>
<td>128.8 ± 10.2</td>
</tr>
<tr>
<td>Ejaculation</td>
<td>105.3 ± 28.1</td>
<td>115.0 ± 33.0</td>
<td>93.1 ± 25.6</td>
</tr>
</tbody>
</table>

Note. Data are from experienced males. 30M = incubated at 30 °C; 32.5M = incubated at 32.5 °C; *p < .05, significantly longer latencies relative to older 32.5Ms.  #p < .05, significantly longer latencies relative to young 30Ms and older 32.5Ms.

Discussion

Individual differences in neural phenotype are often accompanied by differences in behavioral phenotype. In the leopard gecko, differences in neurometabolic phenotype are caused by embryonic experiences (incubation temperature: IncT) and age. Young adult (50 weeks) males hatched from eggs incubated at 30 °C (30Ms) have elevated CO activity in the POA, an area critical for the display of male-typical sexual behavior across all vertebrates studied to date (Crews & Silver, 1985; Meisel & Sachs, 1994), relative to same-age males incubated at 32.5 °C (32.5Ms; Coomber et al., 1997). Metabolic capacity in the POA increases significantly with age in 32.5Ms such that older (2–3 years) males have similar CO activity as young adult 30Ms (Crews et al., 1997).

Here, we report that IncT- and age-dependent differences in POA metabolism are accompanied by quantitative and qualitative differences in courtship behavior in intact and castrated male leopard geckos. When intact, older 32.5Ms were quicker to mount females relative to young 32.5Ms, and after castration, young 30Ms and older 32.5Ms mounted females sooner relative to young 32.5Ms. Further, after castration, both young 30Ms and older 32.5Ms were more likely to ejaculate with females relative to young 32.5Ms. The persistence of behavioral differences after castration is important because 30Ms have elevated estradiol concentrations relative to 32.5Ms while intact (reviewed in Crews et al., 1998). Taken together, these data suggest that males with elevated CO activity in the POA are more primed to display sexual behavior, regardless of hormonal state. This is consistent with the facts that 30Ms show more courtship behavior after castration and treatment with androgens than 32.5Ms (Rhen & Crews, 1999) and that females incubated at 32.5 °C have elevated CO activity in the POA and are more likely to show male-typical sexual behavior after androgen treatment relative to females incubated at 26 °C (Crews, Coomber, Baldwin, Azad, & Gonzalez-Lima, 1996).

Elevated CO activity is linked to greater glutamatergic, excitatory input (Nie & Wong-Riley, 1995, 1996; Zhang & Wong-Riley, 1999). For example, in the primariate striate cortex, CO-rich zones (puffs) in the supragranular layer have more glutamatergic innervation than CO-poor regions (interpuffs; Nie & Wong-Riley, 1996). It is possible that young 30Ms and older 32.5Ms have more abundant glutamatergic inputs into the POA relative to young 32.5Ms. Putative glutamatergic inputs into the POA have been found from areas such as the medial amygdala (MeA), bed nucleus of the stria terminalis (BSNT), lateral septum, and hypothalamic nuclei in male rats (Kocsis, Kiss, Csáki, & Halász, 2003); if similar functional connections exist in male leopard geckos, it is possible that these inputs are more robust in young 30Ms and older 32.5Ms. Males with more robust glutamate innervation into the POA from areas like the MeA and BNST might have a greater enhancement of POA activity after exposure to a female and a more rapid display of sexual behavior.

Metabolic activity in limbic nuclei that regulate the expression of social behavior, such as the POA, has been found to decrease after castration and increase after androgen replacement (Balthazart et al., 2001; Crews et al., 1996; Guerra, Rodriguez del Castillo, Battaner, & Mas, 1987). Because glutamate increases neural activity in the POA (Hoffman, Wuarin, & Dudek, 1994; Karlsson, Sundgren, Näsström, & Johansson, 1997), we propose that castration-induced decreases and androgen-induced increases in POA metabolic activity are linked to decreases and increases, respectively, in the amount of glutamatergic stimulation into the POA. Further, we propose that one of the mechanisms underlying the decline of sexual behavior after castration is decreased glutamatergic innervation into the POA, and that when the amount of glutamatergic innervation (and CO activity) drops below a threshold, sexual behavior is no longer displayed (see Sakata, Gupta, & Crews, 2001). Males with elevated POA metabolism before castration could have more glutamatergic innervation, and it is possible that it takes longer for this innervation to fall below the threshold after castration in these males. Therefore, males with elevated POA metabolism (young 30Ms and older 32.5Ms) retain sexual behavior longer after castration. This notion is consistent with the fact that in species in which copulatory interactions increase the retention of sexual behavior, such as rats and whiptail lizards, sociosexual experience increases CO activity in the POA (Larsson, 1978; Sakata, Gonzalez-Lima, et al. 2002; Sakata, Gupta, et al., 2002).
We did not find that 10 sociosexual interactions with females while intact increased postcastration sexual behavior in male leopard geckos. Consequently, we hypothesize that these interactions do not increase CO activity in the POA in these males. This is plausible given that housing 32.5Ms with females for 1–2 years, a more dramatic social manipulation, neither increases the retention of courtship behavior following castration nor increases CO activity in the POA (Crews et al., 1997; Sakata, Gupta, Chuang, & Crews, 2002).

A suite of other factors could contribute to behavioral differences among young 30Ms, young 32.5Ms, and older 32.5Ms while intact and after castration. For example, dopamine release into the medial POA (mPOA) is critical for the expression of copulatory behavior in rats and birds (Balthazart, Castagna, & Ball, 1997; Dominguez, Riolo, Xu, & Hull, 2001; Hull, Meisel, & Sachs, 2002). Nitric oxide stimulates the release of dopamine from terminals in the mPOA, and nitric oxide synthase (NOS) is regulated by androgen concentrations (Du & Hull, 1999). Therefore, differences in the amount of dopaminergic input or NOS expression could contribute to behavioral differences in male leopard geckos. In preliminary studies, we found that peripheral injections of SCH 23390, a selective dopamine receptor (D1) antagonist, dose-dependently decrease the display of copulatory behavior in castrated, testosterone-implanted 30Ms and 32.5Ms (n = 12 per

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Figure 1. Postcastration courtship behavior in young 30Ms and 32.5Ms and older 32.5Ms. Left: Percentage of males that showed body-grips (A), mounts (B), and ejaculations (C) on Postcastration Tests 1–10. Right: Summary of the percentage of tests in which each behavior was displayed. The left panels depict the decline of sexual behavior after castration, and the line indicates the percentage of all males that displayed the behavior of interest on their final test while gonadally intact (only experienced males; no difference across groups when intact). Because there was no difference between experienced and naive males after castration, data were collapsed across experience within each group (young 30Ms: n = 23; young 32.5Ms: n = 24; older 32.5Ms: n = 23). 30M = incubated at 30 °C; 32.5M = incubated at 32.5 °C. * p < .05.
InC(T). Moreover, courtship behavior was inhibited with the 4- and 8-mg/kg doses in 32.5Ms, but only with the 8-mg/kg dose in 30Ms. Taken together, these results suggest that dopaminergic stimulation is important for the display of sexual behavior in this species and that InC(T) could affect D$_1$ receptor expression in limbic brain areas.

Neural production of steroids has been implicated in the display of social behavior, particularly when circulating concentrations of gonadal steroids are basal (e.g., Soma, Schlinger, Wingfield, & Saldanha, 2003; Soma & Wingfield, 2001). Enzymes requisite for the synthesis of androgens and estrogens have been found in the brains of many vertebrate species, including reptiles (Guennoun, Fiddes, Gouezou, Lombes, & Baulieu, 1995; Holloway & Clayton, 2001; Crews, Bieganski, Baleckaitis, Harada, & Balthazart, 2001; Mensah-Nyagan, Beaunou, Luu-The, Pelletier, & Vaudry, 2001; Soma, Bindra, Gee, Wingfield, & Schlinger, 1999; Ukena et al., 1999; Wade, 1997). It is possible that InC(T) or age affects neurosteroid production in male leopard geckos and that this difference could contribute to behavioral and neurometabolic differences. Whereas circulating androgens and estrogens are undetectable or very low after castration (Crews et al., 1996; Rhen & Crews, 1999), it would have been interesting to assess concentrations of androgen precursors such as dehydroepiandrosterone (DHEA), which can be converted into androgens in the brain. In birds, DHEA has been implicated in the display of social behavior when testes are regressed (e.g., Soma & Wingfield, 2001; Soma, Wissman, Brenowitz, & Wingfield, 2002).

These data highlight the predictive information in CO activity. Differences in CO activity can relate to differences in excitation input, baseline metabolism, and dendritic arborization (Wong-Riley et al., 1998), and such differences in specific neural circuits could prime the display of specific behaviors. Differences in amygdalar, septal, hippocampal, and cortical activity correlate with differences in the predisposition to display learned helplessness in rats (Shumake, Edwards, & Gonzalez-Lima, 2001, 2002, 2003; Shumake, Poremba, Edwards, & Gonzalez-Lima, 2000) and in responsiveness to novel environments (Gonzalez-Lima & Sadile, 2000). We propose that intrauterine position, which affects copulatory behavior in rodents (reviewed in Clark & Galef, 1995; Ryan & Vandenberg, 2002) and has been implicated in the retention of postcastration sexual behavior in mice (Sinchak, Roselli, & Clemens, 1996), might also affect CO activity in the POA of male mammals. Although CO activity has traditionally been used as an endpoint (e.g., the effects of behavioral experience), we propose that neural CO activity can also provide a starting point for investigations into differences in behavioral phenotype.

References


