Embryonic temperature shapes behavioural change following social experience in male leopard geckos, *Eublepharis macularius*

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Individual variation in behavioural expression and in behavioural plasticity exist in all species, and early experiences are critical determinants of both. The leopard gecko is a lizard with temperature-dependent sex determination, and in this species, embryonic incubation temperature (IncT) affects the display of social behaviours. For example, adult males hatched from eggs incubated at an IncT that produces predominantly males (male-biased IncT, 32.5°C) are more aggressive and less sexually active than males hatched from eggs at an IncT that produces predominantly females (female-biased IncT, 30°C). It is not known, however, whether IncT influences behavioural plasticity in adulthood. We assessed whether adult males hatched from eggs incubated at female- and male-biased IncTs showed different changes in territorial behaviour (scent marking in an empty test arena), anticipatory behaviours (activity, scent marking and tail vibrations in response to cues that predict the introduction of a female) and courtship behaviour following social interactions with females. We found that heterosexual social experiences increased territorial behaviour and the display of anticipatory behaviours in males from the female-biased IncT but not in males from the male-biased IncT. This difference was not due to differences in the amount of social experiences acquired, and the greater change in anticipatory behaviour in males from the female-biased IncT suggest that they acquired sexual conditioning more readily. Differences in sex steroid concentrations or neural metabolism caused by IncT could underlie this difference. These results highlight the profound implications of maternal nest site selection for offspring phenotype and plasticity in this species.

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increase might prime Fb males from the female-biased IncT to be more sexually vigorous.

Although it is known that IncT affects the social behavioural phenotype in the leopard gecko, it is not known whether IncT modulates behavioural plasticity in adulthood. This is plausible because individuals from different IncTs have different gonadal steroid milieus in adulthood, and sex steroid hormones can modulate behavioural and neural plasticity (e.g. McEwen et al. 1991; Woolley 1999). Furthermore, differences in neural metabolism caused by IncT could represent differences in baseline metabolism (Gonzalez-Lima 1992), the degree of dendritic arborization (Wong-Riley 1989), excitatory innervation (Nie & Wong-Riley 1996) and/or receptors required for neural plasticity (Zhang & Wong-Riley 1999), all of which could affect behavioural plasticity.

Here we assessed the degree to which adult Fb and Mb males differed in their behavioural plasticity following interactions with females. In a variety of species, interactions with females in adulthood affect agonistic (Albert et al. 1988; Zucker 1994; Sandnabba 1996; Sakata et al. 2002) and courtship behaviours (Dewsbury 1969; Larsson 1978; Rundfeldt & Wingfield 1985; reviewed in Meisel & Sachs 1994; Lumley & Hull 1999; Pfaus et al. 2001). For example, copulatory and courtship experiences in adulthood alter partner preferences in rodents, birds and fish (Inmmelmann et al. 1991; Kruit & Meeuwsen 1991; Marler et al. 1997; Patris & Baudoin 1998) and increase sexual vigour (Crowley et al. 1973; Sakata et al. 2002). In this experiment, we quantified experience-dependent changes in territorial behaviour (scent marking in an empty test arena), anticipatory behaviours (activity, scent marking and tail vibrations in response to cues that predict the introduction of a female) and sexual behaviour in adult Fb and Mb males. We hypothesized that Fb males would show enhanced behavioural changes following interactions with females because these males are more primed to display sexual behaviour (Rhen & Crews 1999) and because they have elevated metabolic capacity in the preoptic area (Coomber et al. 1997), an area requisite for courtship and implicated in sexual learning (Meisel & Sachs 1994; Ritters et al. 1998; Pfaus et al. 2001).

METHODS

Animals

Adult male leopard geckos (1–1.5 years old) that were hatched from eggs incubated either at the female-biased IncT (30°C; Fb males, N=21) or male-biased IncT (32.5°C; Mb males, N=21) were used in this study. At female- and male-biased IncTs, respectively, sex ratios (% male) of approximately 30 and 70% are produced (Crews et al. 1998). All males were raised in isolation in polypropylene containers (30 x 12 x 6 cm) according to procedures described previously (e.g. Sakata et al. 2002). Briefly, for the first 10 weeks after hatching, all individuals were maintained on a 14:10 h light:dark photocycle, at 30°C, and given water and crickets 5 days a week. Thereafter, individuals were maintained on an LD 14:10 h photocycle, with temperatures ranging from 18°C at night to 30°C during the day, and were given water and mealworms three times a week. Crickets and mealworms were dusted with vitamin supplements. After reaching sexual maturity, males (1–1.5 years of age) were placed in larger cages and remained in isolation (45 x 25 x 20 cm).

Figure 1. Experimental design for testing adult male leopard geckos that were hatched from eggs incubated at female-biased (Fb) and male-biased (Mb) embryonic incubation temperatures. Forty-two males (N=21 Fb and Mb males) were first given two tests in an empty test arena (Arena Test–Pre) during which activity and scent marking were recorded. One group of males (N=12 from each IncT; experienced males, ■) was given 10 opportunities to copulate with receptive females in their home cage after a 5-min habituation period (home cage tests). The other group (N=9 from each IncT; Naive males, □) was tested in a similar manner but females were not introduced into their home cage. After these 10 tests, all males were again tested in the empty test arena and watched for activity and scent marking (Arena Test–Post).

Behaviour Testing

Arena Test–Pre

After acclimating for at least 1 week in the larger home cage, all males were observed twice for 5 min in an empty test arena (45 x 25 x 20 cm) lined with a clean paper towel (Fig. 1). During these two observation periods, which were separated by 3 days, we recorded the duration of activity and scent marking. Activity was defined as movement of the body and limbs in any direction (i.e. locomotion or ambulation). Scent marking is a characteristic territorial behaviour in which the preanal pores are pressed down onto the substrate and swiped laterally. Scent marking and activity are considered separate behaviours, and, consequently, activity duration does not include duration of scent marking. If the behaviour of interest was not observed, a duration score of 0 was assigned.

Home cage tests

We allocated males from each IncT to two groups. One group was kept sexually naïve (N=9) and the other group was given the opportunity to copulate with females (N=12). Males were allocated such that, within each IncT, the amounts of activity and scent marking displayed during Arena Test–Pre were equal across sexually naïve and experienced groups (randomized stratified blocking). All testing in this experimental phase occurred in the male’s home cage. Cage materials (brick, shelter and water dish) were removed, and the males were watched...
for 5 min (habituation period). During the habituation period, we noted the occurrence of activity, scent marking and tail vibrating. Although scent-marking behaviour is rarely observed in the home cage after a male thoroughly marks his territory, it is often seen when males are placed in novel areas. Similarly, tail vibration is a stereotypical behaviour usually displayed only in the presence of a female, but here we found that a subset of males displayed tail vibrations during the habituation period. If the behaviour of interest was not observed, a duration score of 0 was assigned.

Following the 5-min habituation period, we observed males in the naïve group in their empty home cages for at least 5 min. For males in the experienced group, we introduced a receptive female into each male’s home cage following the habituation period and observed courtship behaviour. Females were first screened for sexual receptivity with sexually vigorous males, and were considered receptive if they remained motionless and did not bite back in response to courtship. We recorded whether the male body-gripped, mounted and ejaculated in each test. Tests were terminated 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 test was extended again for another 5 min to allow for ejaculation. We administered 10 tests to males in the naïve and experienced groups, each separated by 3–4 days. Males in the experienced group were exposed to the same female at most twice across these 10 tests.

Because males in the experienced group could interact with females for more than 5 min (i.e. if they body-gripped and/or copulated with the test females), the test length for males in the experienced and naïve groups differed. However, this difference in test length should not have affected the group differences reported below, because testing took place in each male’s home cage. It is unlikely that males in the naïve group would have demonstrated tail vibrations and scent-marking behaviour if we had extended the time that cage materials were left out of their home cages.

**Arena Test–Post**

After the home cage tests, males were again exposed for 5 min to a neutral test arena on two occasions, separated by 3 days, and watched for activity and scent marking (Fig. 1). The protocol was identical to that in Arena Test–Pre. These tests were given to assess whether repeated interactions with females altered the behaviour of males and, moreover, whether males from different IncTs reacted differently to social experience.

**Statistical Analysis**

**Behaviour in the empty test arena**

We administered two pairs of tests in the empty test arena, one before (Arena Test–Pre) and one after (Arena Test–Post) the home cage tests. We averaged the durations across the two tests within Arena Test–Pre and Arena Test–Post. Because average activity durations were distributed normally, we analysed group differences using a two-way repeated measures multivariate analysis of variance (MANOVA) with IncT (female-biased versus male-biased) and experience (experienced versus naïve) as the independent variables and time (Arena Test–Pre and Arena Test–Post) as the dependent variable.

Scent-marking behaviour was displayed only by a small subset of males, and there was relatively little variability in duration averages among males that scent-marked. Therefore, we analysed differences in the proportion of males that scent-marked at least once within each phase using likelihood ratio tests. Because this is only a univariate test, we conducted several comparisons instead of a single repeated measures two-way test. First, we analysed overall differences between males from different IncTs during Arena Test–Pre and Arena Test–Post. Then we investigated overall differences between experienced and naïve males during Arena Test–Post as well as differences between experienced and naïve males within each IncT during Arena Test–Post. We did not analyse differences between experienced and naïve males during Arena Test–Pre because males were allocated into groups based on their behaviour during these tests. Altogether, five contrasts were made, and we adjusted our α level to 0.01 to account for the increased number of tests (Bonferroni correction).

**Behaviour during the habituation period (home cage tests)**

We analysed differences in the proportion of tests in which activity, scent marking and tail vibrations were displayed using a two-way repeated measures MANOVA with IncT (female-biased versus male-biased) and experience (experienced versus naïve) as the independent variables, and behaviour (proportion of the 10 habituation tests with activity, with scent marking and with tail vibration) as the dependent variable.

**Courtship behaviour (home cage tests)**

Only males assigned to the experienced group were tested with females following the habituation period, and we investigated behavioural differences between Fb and Mb males. Using likelihood ratio tests, we first analysed differences in the proportion of males that body-gripped, mounted and ejaculated with females on the first test and on the last test. Because of the multiple comparisons we set α to 0.01.

We also analysed group differences in the proportion of the 10 tests in which body grips, mounts and ejaculations were displayed. We analysed courtship behaviour using a one-way MANOVA. The sole independent variable was IncT, and behaviour (proportion of the 10 tests with body grip, with mount and with ejaculation) was the dependent variable.

All analyses were done using JMP 3.2 (SAS Institute 1995) for the Macintosh, and for all analyses, unless otherwise stated, α=0.05. For all multivariate analyses, we used Pillai’s trace as our test statistic because it is the most robust to deviations from multivariate normality and homogeneity of variance–covariance matrices (Olson
Furthermore, all proportion data were first arcsine square-root transformed to improve normality (Sokal & Rohlf 1995).

RESULTS

Behaviour in the Empty Test Arena (Arena Tests–Pre and –Post)

Activity
There was a significant effect of IncT (MANOVA: $F_{1,38}=4.9$, $P=0.033$) but not of experience ($F_{1,38}=0.1$, $P=0.708$) or time ($F_{1,38}=0.6$, $P=0.446$) on average activity duration. Overall, Fb males were more active than Mb males.

Scent marking
Although proportionately more Mb males scent-marked during the Arena Test–Pre relative to Fb males (1/21 Fb males versus 6/21 Mb males: likelihood ratio test: $\chi^2=4.7$, $P=0.032$), this difference was not significant (adjusted $a$ level of 0.01). There was also no significant difference in the proportion of Fb and Mb males that scent-marked during the Arena Test–Post (6/21 Fb males versus 5/21 Mb males: $\chi^2=0.1$, $P=0.726$). During the Arena Test–Post, overall, more experienced males scent-marked (9/24) relative to naive males (2/18), but this difference was not significant at our $a$ level of 0.01 ($\chi^2=4.0$, $P=0.046$). Among Fb males, significantly more experienced males scent-marked (6/12) relative to naive males (0/9) during Arena Test–Post ($\chi^2=8.5$, $P=0.004$; Fig. 2). However, among Mb males, there was no difference between experienced (3/12) and naive (2/9) males during this test ($\chi^2=0.0$, $P=0.882$; Fig. 2).

Behaviour During the Habitation Period (Home Cage Tests)

There was a significant effect of experience (MANOVA: $F_{1,38}=6.3$, $P=0.017$), and experienced males showed overall more behaviour. There was also a significant effect of behaviour ($F_{2,37}=251.5$, $P<0.001$): activity was displayed more frequently than scent marking or tail vibrations. Scent marking was observed only on tests 9 and 10, and tail vibrations were observed mostly on tests 7–10; no male displayed either behaviour on the first test. The effect of IncT approached significance (Fb males>Mb males: $F_{1,38}=3.2$, $P=0.081$). Moreover, the interaction between experience and IncT approached significance ($F_{1,38}=3.4$, $P=0.071$); thus, we analysed the effect of experience in males from each IncT separately using MANOVAs. Among Fb males, experienced males showed overall more activity, marking and tail vibrations relative to their naive counterparts ($F_{1,19}=7.1$, $P=0.011$; Fig. 3). However, among Mb males, there was no difference between experienced and naive males ($F_{1,19}=0.3$, $P=0.616$; Fig. 3).

We also analysed differences in the proportion of males that scent-marked or tail-vibrated at least once across the 10 habituation tests. We restricted the analyses to experienced males because naive males did not show marking behaviour, and only one naive male tail-vibrated. There was a significant difference in the proportion of males that scent-marked at least once...
Here we report that IncT in male leopard geckos shapes behavioural plasticity in response to social experience. Following repeated interactions with females, males from the female-biased IncT (Fb males) but not males from the male-biased IncT (Mb males) showed significant increases in territorial and anticipatory behaviours. Specifically, only in Fb males did the proportion of individuals that scent-marked in the neutral test arena increase with experiences with females. We propose that this increase in scent-marking behaviour in the test arena is analogous to experience-dependent increases in aggressiveness found in male rodents (Albert et al. 1988; Sandnabba 1996). The difference between experienced and naive Fb males is not contingent upon the presence of gonadal steroids as it persists following castration (unpublished data). Furthermore, experienced Fb males showed more activity, scent marking and tail vibrations during the period preceding testing than did naive Fb males, but this difference was not significant among Mb males. The differences in plasticity were not due to differences in the overall amount of sociosexual experience acquired; males from both IncTs showed, overall, comparable amounts of body grips, mounts and ejaculations across the 10 home cage tests. Crews et al. (1997) reported a similar interaction between social experience and IncT on neural phenotype in female leopard geckos. For example, heterosexual housing increased metabolic capacity in the ventromedial hypothalamus in females from the male-biased IncT but not in females from the low IncT (26°C).

The fact that experience with females did not change territorial behaviour in Mb males in the present study was unexpected, because we previously found that Mb males with extensive heterosexual experience are more likely to show territorial behaviour relative to age-matched, socially naive Mb males (Sakata et al. 2002). Procedural differences as well as age differences could have caused this discrepancy. Here, experienced males were housed in isolation and given only 10 opportunities to copulate with females, whereas in our previous study, experienced males were housed with intact females for 1–2 years. Therefore, more social experience may be required to induce increases in marking behaviour in Mb males. Furthermore, males in the present study were 1–1.5 years of age, whereas males in our previous study were twice as old. Given that age and sexual experience interact to affect partner preferences in male rats (Vega Matusczyk et al. 1994), these variables may similarly interact in the leopard gecko to affect territorial behaviour.

We investigated changes in behaviour during the habituation period to assess the degree to which conditioning occurred. Among males with social experience, the removal of home cage material reliably signalled the introduction of a receptive female, and changes in activity, scent marking and tail vibrations of males during the habituation period suggest that males might have been anticipating the opportunity to copulate with females. Activity is pertinent because increases in general activity during the period preceding the introduction of the female have been observed in male rats (Mendelson & Pfau 1989) and Japanese quail, Coturnix japonica (Akins et al. 1994; reviewed in Pfau et al. 2001). In male
Mongolian gerbils, Meriones unguiculatus, scent-marking behaviour seems to be related to sexual behaviour; it is displayed more often in test arenas associated with sexual behaviour than in open fields and is elevated when females are present (Yahr et al. 1980; Pendergrass et al. 1989). In addition, tail vibration is a courtship behaviour in the leopard gecko that is usually directed towards a female, and because females were absent during the habituation period, tail vibrations in this context suggest a level of sexual arousal. Although we have not rigorously tested the idea that both scent marking and tail vibrations in the home cage are linked to sexual arousal, we have not observed either of these behaviours in response to food or to other males (unpublished data), which suggests that they are specific for sexual interactions.

We found that experiences with females led to an overall increase in the amount of activity, marking and tail vibrations during the habituation period and, moreover, that this increase was greater among Fb males. The difference in activity, for example, between experienced and naïve males was much greater among Fb males than among Mb males (Fig. 3). Furthermore, only experienced Fb males scent-marked in the home cage during the habituation period (Fig. 4). Interestingly, males that copulated with females tended to show greater changes in anticipatory behaviours than those that failed to copulate; the same was true for changes in territorial behaviour in the neutral test arena (data not shown). Although more explicit testing is required, this suggests that copulation might be more effective than mere exposure to females at inducing behavioural change.

The mechanism(s) by which IncT modulates social plasticity in male leopard geckos is unknown. It is possible that differences in circulating concentrations of sex steroid hormones or in cytochrome oxidase activity (metabolic capacity) can modulate differences in behavioural plasticity. Because oestrogens modulate behavioural and neural plasticity (e.g. Woolley 1999), Fb males may show greater behavioural plasticity because they have elevated concentrations of 17β-oestradiol, E2 (Tousignant & Crews 1995; Coomber et al. 1997). Cytochrome oxidase activity in the preoptic area of the brain, an area that modulates the expression of sexual behaviour (Meisel & Sachs 1994) and sexual learning (Pfaus et al. 2001), is also elevated in Fb males relative to Mb males (Coomber et al. 1997). Zhang & Wong-Riley (1999) reported a correlation between cytochrome oxidase activity and the expression of NMDA receptors, proteins that modulate neural and behavioural plasticity. Consequently, differences in cytochrome oxidase activity in the preoptic area could represent differences in NMDA receptor expression, and this could explain the heightened behavioural change in Fb males following interactions with females. Interestingly, Powell et al. (2003) showed that the activation of NMDA receptors is important for behavioural change following copulatory experiences with females in male rats. Given the elevated metabolic capacity in brain areas modulating aggressive behaviour in Mb males (Coomber et al. 1997), it would be interesting to test whether these males are more primed to show behavioural changes following agonistic interactions.

We have indirectly tested the importance of differences in cytochrome oxidase activity in the preoptic area versus differences in sex steroid hormone concentrations in generating differences in social plasticity. Relative to young (1 year old) Mb males, older Mb males (2–3 years old) have comparable concentrations of both androgens and E2 but elevated cytochrome oxidase activity in the preoptic area (Crews et al. 1997). In fact, cytochrome oxidase activity in the preoptic area in older Mb males is similar to that in young Fb males. If differences in gonadal hormones are paramount, older and young Mb males should show comparable experience-dependent changes. On the other hand, if differences in cytochrome oxidase activity in the preoptic area are critical, then older and young Mb males should show different social plasticity, and older Mb males and younger Fb males should show similar social plasticity. Following the same protocol used here, we found that older, experienced Mb males showed significantly more anticipatory behaviour (tail vibrations) during the habituation period than older, naïve Mb males (unpublished data); this is similar to the effect of experience in young Fb males. However, older Mb males did not show experience-dependent increases in scent marking in the test arena. This result suggests that enhanced plasticity in anticipatory behaviours is correlated with heightened preoptic area metabolism, whereas increases in territorial behaviour following interactions with females might be constrained by E2 concentrations. These results also highlight the importance of age on phenotypic plasticity.

It has been postulated that males from different IncTs adopt alternative reproductive strategies (Rhen & Crews 2002). For example, Mb male leopard geckos resemble territorial males, whereas Fb male leopard geckos resemble nonterritorial, sneaker males. Consistent with the idea that Fb male leopard geckos adopt a sneaker male strategy is that Fb males were more active in the neutral test arena. It would be interesting to compare behavioural plasticity following social experiences between territorial and nonterritorial males in species with alternative reproductive tactics.

Examples of early effects on plasticity in adulthood have become increasingly evident. For example, prenatal and postnatal sex steroid exposure has been found to affect the degree to which stressful experiences alter learning (Shors & Miesegaes 2002) and the degree to which interactions with females alter partner preferences (Baum et al. 1990; Woodson et al. 2002). In utero hormone exposure also affects the sensitivity of female house mice to cues that modulate the timing of puberty and length of reproductive cycles (vom Saal 1989). The leopard gecko provides a useful model to study how an ecologically relevant parameter, IncT, sculpts behavioural phenotype and plasticity and highlights the long-term consequences of maternal selection of nest sites.

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