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Stress coping styles and singing behavior in the short-tailed singing mouse (*Scotinomys teguina*)

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ABSTRACT

Stress coping styles have been characterized as a proactive/reactive dichotomy in laboratory and domesticated animals. In this study, we examined the prevalence of proactive/reactive stress coping styles in wild-caught short-tailed singing mice (*Scotinomys teguina*). We compared stress responses to spontaneous singing, a social and reproductive behavior that characterizes this species. To establish proactive/reactive profiles for singing mice, we measured exploratory and anxiety behavior using an open-field behavioral test. We examined correlations between open-field behaviors and fecal corticosterone (CORT) metabolites, baseline plasma CORT, and stress-induced CORT. Mice with proactive behavioral responses in the open-field had higher fecal CORT titers than reactive males, but did not differ in baseline or stress-induced plasma CORT. We suggest that individual differences in CORT metabolism may contribute to this surprising pattern. Males that sang in the open-field were behaviorally proactive and had lower stress-induced CORT, indicating a link between stress responses and singing in this species. Overall, the data demonstrate that singing mice offer an interesting model for exploring how stress reactivity can shape social behaviors.

Introduction

In response to stress, vertebrates mount a suite of behavioral and physiological responses to maintain or re-establish homeostasis (Sapolosky et al., 2000). The magnitude of the stress response varies greatly between individuals in many taxa, including rodents (De Boer et al., 2003), pigs (Ruis et al., 2001), birds (Marchetti and Drent, 2000), fish (Dingemanse et al., 2007; Schojolden et al., 2005; Ward et al., 2004), and humans (Tyrka et al., 2006). These individual differences form suites of correlated responses known as *stress coping styles* which are causally linked, predictable, and specific to groups or populations (Koolhaas et al., 1999). Because ecologically important behaviors often require some balance of risks and pay-offs (e.g. Lima et al., 1985; Sih, 1992; Zuk and Kolluru, 1998), coping styles may prove profoundly important in multiple behavioral contexts. We suggest that animal displays offer useful, ecologically meaningful traits for exploring the intersection between coping styles and two major domains of animal behavior: sociality and reproduction. We explore these interactions in a novel rodent model, the short-tailed singing mouse (*Scotinomys teguina*).

Stress coping styles are commonly characterized as proactive or reactive (Koolhaas et al., 1999). Behaviorally, proactive animals are aggressive toward conspecifics, develop rigid learned routines, and avoid or manipulate stressful stimuli (Koolhaas et al., 1999, 2007). In response to stress, proactive animals exhibit high sympathetic activity including increases in blood pressure, heart rate, and the production of catecholamines (Øverli et al., 2007). Conversely, proactive animals exhibit low hypothalamic–pituitary–adrenal (HPA) activity resulting in the production of low levels of glucocorticoids (the main steroids involved in the stress response; Øverli et al., 2007). In contrast, reactive animals display low levels of conspecific aggression, learn more flexible routines, and react passively to stress (Koolhaas et al., 1999, 2007). Physiologically, reactive animals respond to stress with high parasympathetic and HPA activity, resulting in high levels of glucocorticoids (Øverli et al., 2007).

The ecological and evolutionary relevance of stress coping styles is not clear because few studies have examined the occurrence of stress coping styles in wild animals. Studying coping styles in strains of laboratory animals selected for divergent stress responses has illuminated the physiological mechanisms and behaviors ascribed to these correlated responses. Extending studies from laboratory and domesticated animals to their wild counterparts will provide a more complete understanding of the occurrence and frequency of stress coping styles in natural systems, and how these suites of behavioral and physiological characters influence sociality, survival, and reproduction.

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Acoustic signals are excellent candidates for exploring the ecological and evolutionary consequences of stress coping styles because the decision to display to conspecifics must weigh the risks associated with predation against the benefits of communication. In response to stressors, auditory signals are modulated or withheld in Gulf toadfish (Remage-Healey et al., 2006), túngara frogs (Ryan, 1985), and katydids (Faure and Hoy, 2000). Glucocorticoids modulate both alarm vocalizations (Blumstein et al., 2006; Boinski et al., 1999; Swiergiel et al., 2007) and courtship vocalizations (Leary et al., 2006; Remage-Healey et al., 2006). Although vocalization has not been examined in the context of stress coping styles, proactive animals seem more likely than reactive animals to engage in risky displays. Proactive animals are less anxious, more aggressive toward conspecifics, and physiologically less responsive to stress (lower HPA reactivity and activity). If stress coping style relates to an animal's propensity to vocalize, this could profoundly shape the ecological and evolutionary relevance of stress coping styles in wild animals.

We examined stress coping styles in wild short-tailed singing mice (*S. teguina*). Specifically, we explored the association between behavioral and physiological stress responses and the singing behavior that gives these animals their name. We measured behavioral response to novelty (open-field test) and HPA activity and reactivity. By examining the relationships between stress reactivity and vocal communication, we explore whether such individual differences in temperament contribute to variation in social behavior.

Methods

Animal model

Short-tailed singing mice (Muroidea: Cricetidae) are small diurnal rodents that inhabit tropical cloud forests of Central America (Hooper and Carelton, 1976). Singing mice derive their name from the ability to produce long frequency-modulated vocalizations that can be heard at a distance. While many rodent species produce ultrasonic vocalizations, singing mice vocalize between 10,000 and 40,000 Hz (partially within human auditory range) making them a unique and tractable species with which to study rodent vocalizations (Hooper and Carelton, 1976). Like advertisement vocalizations in other species, the song seems to function in both mate attraction (Fernandez-Vargus et al., 2008) and male–male interactions (Pasch, 2009).

Singing mice are abundant where trapped, and reproduce year round (Hooper and Carelton, 1976). Although the short-tailed singing mouse has not been examined in detail, its congener, *S. xerampelinus*, which also sings, exhibits patterns of space use characteristic of Muroid polygamy; male home-ranges overlap one another extensively while female home-ranges are relatively exclusive (Blondel et al., 2009).

Animal trapping and site descriptions

We trapped short-tailed singing mice at two sites near Boquete, Panama, in the summers of 2006 and 2007. Rainfall in the region shows some seasonal variation, with summers being wetter. However, the mice reproduce all year round, as indicated by trapping pregnant and lactating females throughout the year (Hooper and Carelton, 1976). One site, the Peterson's Nature Reserve, is a privately owned nature reserve located in Jaramillo Arriba, 15 km north of Boquete, Panama. This reserve consists of approximately 100 ha of secondary-growth tropical cloud forest bordered on all sides by agricultural lands. This reserve is privately owned and, consequently, there is very little anthropogenic disturbance within its boundaries. The second site, Volcán Baru National Park, is a publicly owned and operated national park located 15 km west of Boquete, Panama. While dominated by secondary-growth cloud forest, this park is heavily fragmented due to extensive agriculture and grazing. These sites are located approximately 12 linear kilometers apart, separated by lowland habitat (975 m), and divided by the Chiriqui River. Preliminary genetic analyses recovered a single mitochondrial haplotype for animals from both sites suggesting that they constitute one genetically indistinct population (Campbell et al., 2010; Pino et al., 2009).

We trapped mice using *Sherman* live traps baited with peanut butter and oats. Although both male and female mice sing, males sing much more often than females (Hooper and Carelton, 1976). We have found that, in the laboratory, all adult males sing, and only a subset of (~10–25%) of females sing. For this reason, we only used males in this experiment. Additionally, because male singing is androgen-mediated (Pasch, 2009), we only used males with fully descended testes. After capture, we weighed mice to the nearest 0.1 g and, using digital calipers, measured head width, tail length, hind foot length, mass and anogenital distance (the distance between the anus and genital opening) to the nearest 0.1 mm. All mice were measured once and, for consistency, by one person (OC). Mice were housed in PVC-coated wire mesh cages $11'' \times 11'' \times 11''$ for seven days in captivity under natural lighting conditions. Animals were provided with dry kitten food, sunflower seeds, and water *ad libitum*. Mice that were inactive or not eating were excluded from this study and immediately returned to the trapping site.

Behavioral stress response: open-field assay

To measure behavioral stress response we conducted open-field trials the morning following capture (day 2) between noon and five PM. The open-field test assays behavioral stress by taking advantage of the natural tendency of rodents to avoid open spaces and maintain contact with vertical surfaces (thigmotaxis) in response to a perceived stressor (Abramsky et al., 1996; Hughes et al., 1994; Jensen et al., 2003). While this assay has primarily been used to assess behavioral stress in nocturnal rodents, it has also been used to quantify anxiety in the diurnal rodent *Octodon degus* which, like nocturnal rodents, is less active in the center of the open-field compared to the periphery (Braun et al., 2003; Popović et al., 2009). In our experiment, the open-field arena consisted of a circular plastic container 50 cm in diameter and 22 cm in depth with an inner circle 35 cm in diameter marked with push pins. We lined the arena with corn cob bedding which we changed between trials after cleaning the arena with ethanol. Prior to the initiation of the ten-minute open-field trial, we placed mice in an opaque covered container in the center of the arena for five minutes. We videotaped trials using a Sony CCD-RTV118 Handycam and used JWatcher (Blumstein et al., 2000) to quantify the following behaviors: latency to first movement (seconds), latency to first cross from inner to outer circle (seconds), singing, jumping, posting, and crossing between the inner and outer circle. Mice defecated during the open-field assay and, after the ten-minute period, we collected these feces to quantify fecal CORT metabolites. We observed peak CORT metabolite levels six hours after an adrenocorticotropic hormone (ACTH) challenge suggesting that stimulation of the HPA-axis during the open-field trials did not influence CORT titers in feces collected 10 min after trial initiation (Fig. 1).

Spontaneous singing

We measured spontaneous singing in two behavioral contexts. The first by documenting the number of songs emitted during the open-field trial. The second was a measure of songs made in visual and acoustic isolation. To measure the latter, we recorded spontaneous singing rate over a two hour period on the day following capture (day 2). Within their home cages, mice were placed in opaque, acoustically insulated chambers (40 cm × 40 cm × 40 cm) and allowed to acclimate for 30 min. Because the chambers accommodate the home cages, mice were not directly handled and had food and water available during the two hour period. Isolated recordings were made between seven

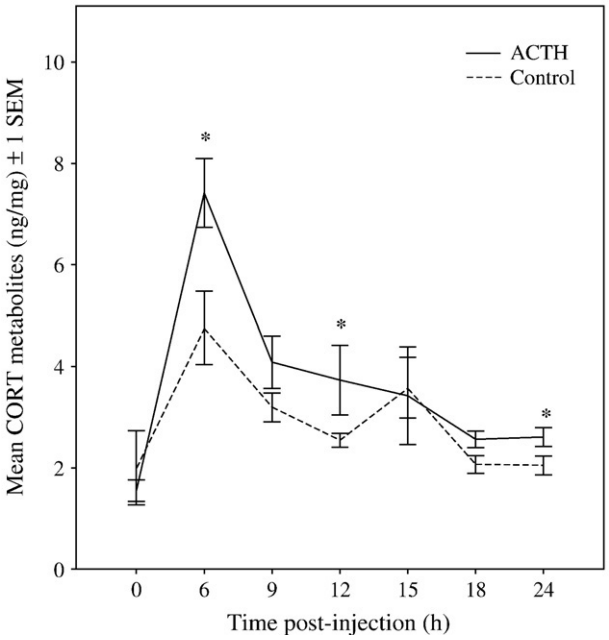

Fig. 1. Mean plasma CORT ± SEM at 0, 6, 9, 12, 15, 18, and 24 h following injection with ACTH or saline. $^{*}p<0.05$.

AM and noon by one observer (OC) for both years and coincided with automated laboratory recordings made from microphones located in the chambers that confirmed appropriate detection by the observer. We quantified *song rate* as the number of songs in the two hour period.

Hypothalamic–pituitary–adrenal (HPA) reactivity: plasma CORT

We measured HPA reactivity between 1200 and 1600 h, seven days after capture to allow mice to acclimate to laboratory housing (e.g. Bhatnagar et al., 2004). Because of their small size, we did not think it was prudent to take iterative blood samples from one individual. For this reason, we exposed mice to a zero (control), or 20 minute restraint stress treatment. We restrained animals by holding them by the scruff on their backs. After restraint we immediately collected 100–150 μl of blood from the retro-orbital sinus. For animals in the control treatment, we collected a blood sample from the retro-orbital sinus in less than two minutes (including capture time). Blood samples collected in less than three minutes are thought to reflect unstressed or baseline CORT measurements because of the delay between HPA stimulation and the release of glucocorticoids (Romero and Reed, 2005). We centrifuged whole blood at 3000 rpm for four minutes and then isolated and stored plasma at −20 °C.

To examine plasma measures of stress reactivity, we compared our various behavioral measures (open-field behavior, singing) to either baseline plasma CORT (zero restraint group) or acute stress-induced plasma CORT (20 minute restraint group).

Fecal CORT extraction and radioimmunoassays (RIA)

Using a standard protocol, we extracted CORT from fecal samples (Mateo and Cavigelli, 2005). Because all fecal pellets were collected within the same time interval (10 min), we extracted directly from wet feces (Wasser et al., 2000). We weighed feces and placed them in individual test tubes with 1 ml of 90% methanol. Using a spatula, we homogenized the samples and agitated them for 24 h. We centrifuged the samples at 2000 rpm for five minutes, decanted the extract, and stored it in glass test tubes at −20 °C until assayed. To account for differences in fecal mass, we divided the total CORT (ng/ml) by fecal mass.

To quantify fecal and plasma CORT, we used the commercially available corticosterone ^{125}I-radioimmunoassay kit for mouse and rat serum (MP Biomedicals, Solon, OH; catalog no. 071200103). We validated the radioimmunoassay for fecal CORT using standard criteria (Good et al., 2003; Touma et al., 2004). Briefly, we serially diluted pooled fecal and plasma samples ten-fold which, after analysis, yielded a parallel standard curve at six points. We used an analysis of covariance (ANCOVA) to test for parallelism between the RIA standard curve and serially diluted pooled fecal extract ($F=2.41$, $p=0.14$, df = 1, 20). A lack of interaction between these two functions has been used to assess assay parallelism (Beehner and McCann, 2008; Mateo and Cavigelli, 2005).

We diluted fecal samples to 1:64 and un-extracted plasma samples to 1:320 in assay diluent prior to radioimmunoassay. We ran each sample in duplicate, quantified gamma emissions on a Hewlett Packard Cobra II Auto Gamma Counter, and calculated CORT values from a standard curve. Intra- and interassay coefficients of variation (CV) were 6.1 and 10.9%, respectively, over five assays. Individual samples exhibited CVs ranging from 0.07% to 11.08%.

To further establish the validity of the fecal CORT assay, we conducted an adrenocorticotropic hormone (ACTH) challenge with captive singing male mice. At the beginning of the experiment, we injected each mouse at 6 am with either 4 IU/kg of ACTH (Sigma ACTH porcine pituitary powder product number: A6303-250IU) dissolved in 0.1 ml saline or 0.1 ml saline ($n=5$ for both treatments). To quantify fecal CORT metabolites, we collected fresh feces prior to injection and then at the following hours following injection: 6, 9, 12, 15, 18, and 24. Feces were stored at −20 °C until assayed. One mouse from the ACTH group did not eat nor defecate for six hours following injection and was excluded from the study resulting in $n=4$ for the ACTH group and $n=5$ for the control group. Additionally, we were unable to obtain fecal samples within 30 min from one mouse in the ACTH group 9 h following injection and one control mouse 15 and 18 h following injection. Thus the respective samples sizes for ACTH and saline injected animals at each time point were: 0 h, (ACTH, $n=4$, control, $n=5$); 6 h (ACTH, $n=4$, control, $n=5$); 9 h ($n=3$, $n=5$); 12 h ($n=4$, $n=5$); 15 h ($n=4$, $n=4$); 18 h ($n=4$, $n=4$), and 24 h (ACTH, $n=4$, control, $n=5$).

All procedures used in these experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida.

Statistical analyses

To assess the stability of stress reactivity profiles across sites and years, we first tested for site and year effects. If site or year effects were present, we accounted for this variation using general linear mixed effects models with site designated a fixed effect and year a random effect. Doing so allowed us to examine both individual differences and site effects in parallel.

All mice were tested for isolated singing rate, open-field behavior, and fecal CORT metabolite levels ($n=82$). In the open-field experiment, animals that did not move for the entire 10 minute open-field trial ($n=6$) were excluded from all analyses. Although freezing is a behavior that is often associated with the reactive phenotype, animals may not have moved for reasons other than a stress coping response such as sickness. For this reason, we thought it appropriate to exclude them from analyses. Of the remaining 76 mice, ultimate sample sizes were reduced to the following: open-field behavior ($n=76$), isolated song ($n=74$), and fecal CORT metabolites ($n=60$). For a subset of these mice ($n=41$), we tested HPA stress response for zero ($n=23$) and 20 min ($n=18$) of stress exposure. Because not all of the 76 mice had fecal or plasma CORT measures available, comparisons among different variables have varied sample sizes. Exact sample sizes for such comparisons are reported with the results.

All open-field parameters were non-normally distributed (Shapiro–Wilk's W test; $p \leq 0.01$ for all behaviors). To account for this, we log transformed behavioral data and used the resulting values in analyses. We then used a principal component analysis (PCA) to reduce open-field behaviors to factor or component scores. PCA has previously been used as a statistical method to evaluate stress response in behavioral assays similar to the open-field test (Roman et al., 2006; Campbell et al., 2003). For the PCA, we only accepted factors with eigenvalues greater than 1.0 and with loading scores greater than 50 (Campbell et al., 2003). Five of the open-field behaviors were used in the PCA (latency to first movement, latency to first cross inner circle, jumps, posts, and number of traverses between inner and outer circle). Singing rate was excluded from the PCA because relatively few animals sang during the open-field trial ($n = 20$). To examine the significance of singing in the open-field assay, we used linear mixed effects models to test if animals that sang at least once (*singers*) had greater behavioral and physiological stress response than animals that did not sing (*non-singers*).

Results

ACTH challenge

A one-way ANOVA comparing fecal CORT metabolites at all time points following injection revealed that mice injected with ACTH had significantly higher overall levels of fecal CORT metabolites compared to control mice ($p = 0.04$, $F = 4.59$, df = 1, 49). Both control and ACTH injected mice had significantly higher fecal CORT metabolite titers six hours following injection (i.e. in the sample interval at 1200 h) than prior to injection (i.e. time zero; $p < 0.01$, $t = -5.30$, df = 14; Fig. 1). Mice injected with ACTH had significantly higher fecal CORT metabolite titers than control mice six hours ($p = 0.03$, $t = 2.64$, df = 7) and 12 h ($p = 0.05$, $t = 2.34$, df = 7), and 24 h following injection ($p = 0.03$, $t = 2.64$, df = 7).

Behavioral stress assay: open-field behavior

Five components or factors emerged from the PCA, but only the first two (Factors 1 and 2) conformed to our previously outlined criteria. Factor 1 (eigenvalue = 3.0) explained 59.4% of total variance. Jumping, posting, and crossing from inner to outer circle loaded highly and positively on Factor 1 while latency to first movement and latency to cross the inner circle loaded highly and negatively. Factor 2 (eigenvalue = 1.2) explained 23.4% of total variance. Latency to first movement and latency to cross the inner circle loaded highly and positively on Factor 2. We considered jumping, posting, and crossing the circle to be exploratory or bold behaviors and both latency scores to indicate anxiety or passive coping. Factor 1 includes both latency and active engagement measures, and can be considered a measure of "pro-activity". Factor 2 captures orthogonal variation in freezing behaviors associated with anxiety, but seems a less complete measure of the proactive/reactive behaviors.

We found significant site differences in open-field behavior in 2006 ($n = 39$), but not in 2007 ($n = 37$). In 2006, mice from the Peterson's Nature Reserve had significantly higher Factor 1 scores compared to mice from Volcán Baru National Park ($p < 0.01$, $F = 10.04$, df = 1, 37). There was no difference in Factor 2 scores between sites ($p = 0.88$, $F = 0.03$, df = 1, 37). However, in 2007, there were no site differences in Factor 1 ($p = 0.75$, $F = 0.11$, df = 1, 35) nor Factor 2 scores ($p = 0.27$, $F = 1.26$, df = 1, 35).

HPA activity and reactivity: fecal, baseline, and stress-induced plasma CORT

There were no site, year, or site-by-year effects for fecal CORT metabolites from the open-field experiment ($p > 0.05$ for all, $n = 60$). There was a significant positive correlation between fecal CORT metabolites and Factor 1 PCA scores (Fig. 2; $p = 0.01$, $F = 6.83$), but no significant correlation between fecal CORT metabolites and Factor 2 PCA scores (Fig. 2; $p = 0.47$, $F = 0.54$).

There was no significant correlation between baseline plasma CORT (control treatment) and Factor 1 (Spearman, $r = -0.27$, $p = 0.22$, $n = 23$) or Factor 2 scores (Spearman, $r = 0.27$, $p = 0.24$). Likewise, there were no significant correlations between stress-induced plasma CORT levels (20 min of restraint stress) and Factor 1 (Spearman, $r = 0.13$, $p = 0.62$, $n = 18$) and Factor 2 scores (Spearman, $r = 0.18$, $p = 0.46$, $n = 18$). There was no correlation between baseline plasma CORT and fecal CORT metabolites ($r = -0.31$, $p = 0.24$, $n = 15$).

Singing behavior and stress responses

Mice that sang in the open-field trial (*singers*, $n = 20$) had higher Factor 1 scores than mice that did not sing (*non-singers*, $n = 56$; Fig. 3; $p = 0.04$, $F = 4.62$, df = 1, 75). There was no significant difference between Factor 2 scores for *singers* and *non-singers* (Fig. 3; $p = 0.87$, $F = 0.03$, df = 1, 75). There was no significant difference between fecal CORT metabolites for open-field *singers* ($n = 18$) compared to *non-singers* ($n = 41$; $p = 0.90$, $F = 0.02$, df = 1, 58).

Compared to mice from 2007, mice from 2006 had significantly lower levels of plasma CORT for both zero and 20 minute treatments ($p < 0.01$, $F = 13.19$, df = 1, 22). Accounting for this variation using mixed linear effects models, we found that animals who did not sing in the open-field had significant rises in plasma CORT in response to restraint stress (Fig. 4; 0 min, $n = 16$; 20 min, $n = 14$; $p < 0.01$, $F = 20.17$), but open-field *singers* did not (Fig. 4; 0 min, $n = 7$; 20 min, $n = 4$; $p = 0.47$, $F = 0.54$). There was no difference in baseline

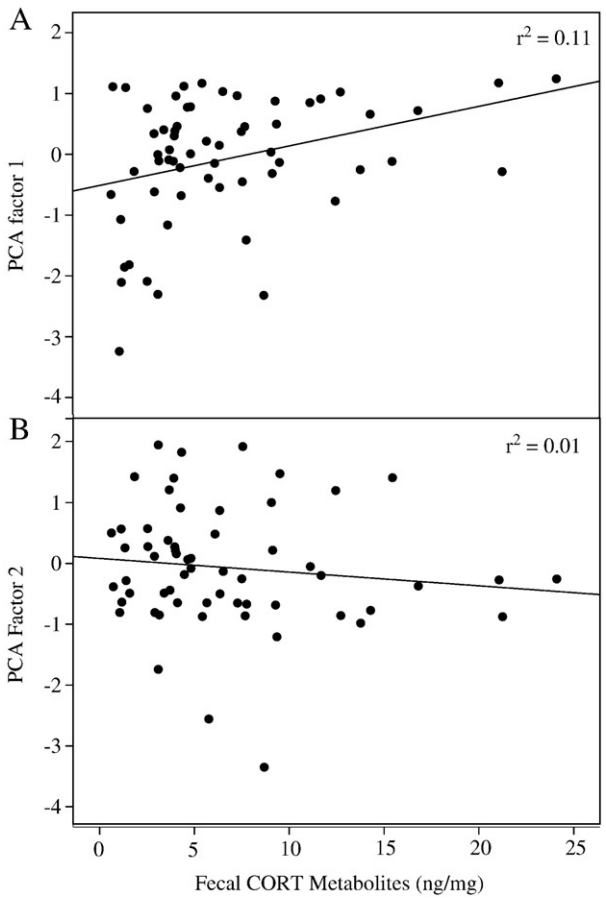

Fig. 2. Scatterplots of fecal CORT (ng/g) titers and (A) PCA Factor 1 ($p = 0.01$, $r^2 = 0.11$) and (B) Factor 2 ($p = 0.47$, $r^2 = 0.01$). $n = 60$.

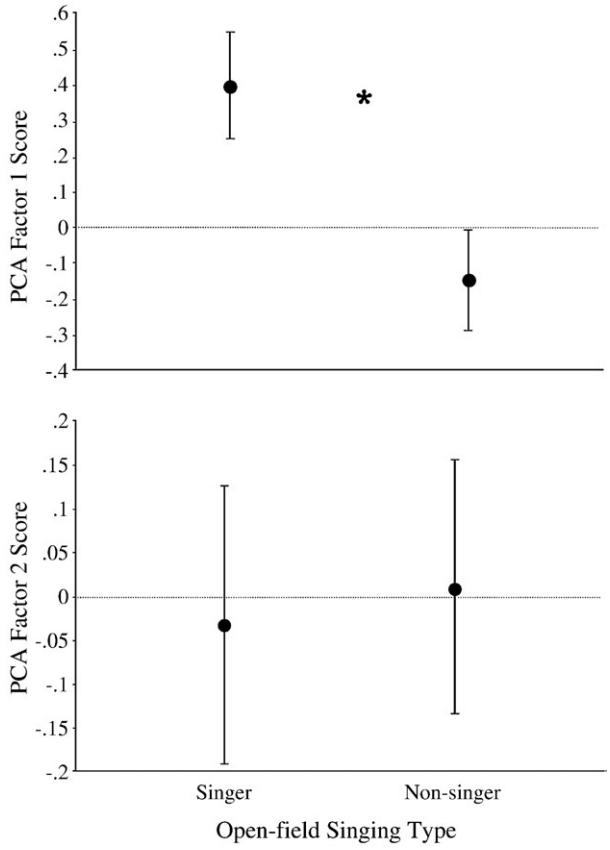

Fig. 3. Mean ± SEM measures for Factor 1 and Factor 2 scores from PCA of open-field behaviors for singers ($n = 20$) and non-singers ($n = 56$). *$p < 0.05$.

plasma CORT between open-field *singers* ($n = 7$) and *non-singers* ($n = 16$; $p = 0.78$, $F = 0.08$) but *singers* ($n = 4$) did have significantly lower plasma CORT following 20 min of restraint stress compared to *non-singers* ($n = 14$; $p = 0.03$, $F = 5.62$).

In the isolated singing trial, we found no significant differences between *singers* and *non-singers* for PCA Factor 1 score ($p = 0.60$, $F = 0.27$, df = 1, 73) or for PCA Factor 2 ($p = 0.41$, $F = 0.68$, df = 1, 73). There was no significant difference in fecal CORT titers for mice that sang in the isolated singing trial (*singers*, $n = 21$) compared to mice that did not sing (*non-singers*, $n = 37$; $p = 0.99$, $F = 0.00$, df = 1, 57). Among isolated *singers*, fecal CORT titers did not predict singing rate ($p = 0.21$, $F = 1.68$, $n = 21$). Interestingly, there was a positive relationship between anogenital distance and isolated song rate (Fig. 5; $p = 0.04$, $F = 4.60$, $n = 25$).

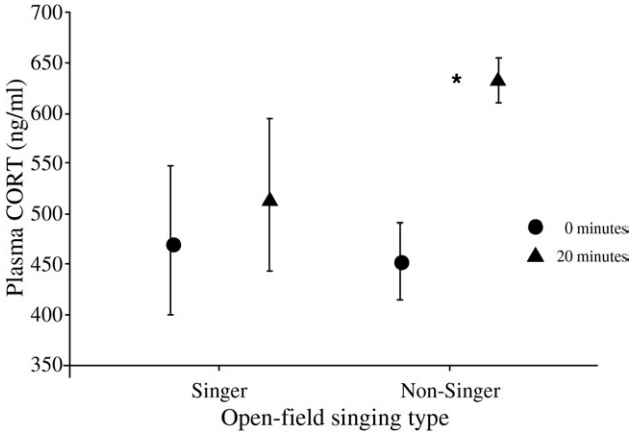

Fig. 4. Mean plasma CORT (ng/ml) ± SEM for open-field *singers* and *non-singers* exposed to either zero or 20 min of restraint stress. * indicates $p < 0.001$.

Discussion

We found significant variation both within and between populations in multiple measures of behavioral stress reactivity, singing behavior, and physiological stress responses. Behavior in the open-field could be separated into two dimensions. Factor 1 scores included strong positive loadings of jumping, posting, and number of crosses between inner and outer portions of the open-field arena, as well as strong negative loadings of latency to move from center of arena, and latency to reach outer portions of arena. Factor 2 scores measured latency to move from center of the arena, and latency to reach the outer portion of the arena. Factor 1 describes how actively animals engage with a novel environment, and captures what is classically considered a proactive phenotype; Factor 2 describes freezing behaviors alone. Only Factor 1 scores were associated with individual differences in HPA function and open-field singing. Interestingly, Factor 1 scores also showed differences between populations in our first study year. This indicates interesting temporal and spatial variation of population-level patterns of stress reactivity; although we cannot identify the origin of such variation, it suggests an exciting avenue of future study.

Our two measures of spontaneous song – open-field and isolated song – showed strikingly distinct patterns. Animals who sang in the open-field had significantly weaker CORT responses to restraint stress than did non-singers, suggesting enhanced negative feedback mechanisms characteristic of proactive animals (Fig. 4, Meaney, 2001). Open-field singers also had higher Factor 1 scores than did non-singers (Fig. 3). These findings demonstrate that open-field singers are less stress reactive behaviorally and physiologically. In contrast, spontaneous song in isolated chambers was a poor predictor of open-field behavior, HPA function, or the likelihood of isolated song. However, among mice that sang in the isolated chambers song rate was well predicted by anogenital distance, a measure of prenatal androgen exposure in other rodents (Fig. 5; Ryan and Vandenbergh, 2002). Males are known to sing significantly more often than females in this species, and we have recently found male vocalization to be strongly regulated by adult androgens (Pasch, 2009). We suggest that the safe context of isolated song measurements – the visually and acoustically isolated recording chambers – reduces the association between singing behavior and stress reactivity. By reducing variability associated with stress reactivity, isolation may facilitate detection of

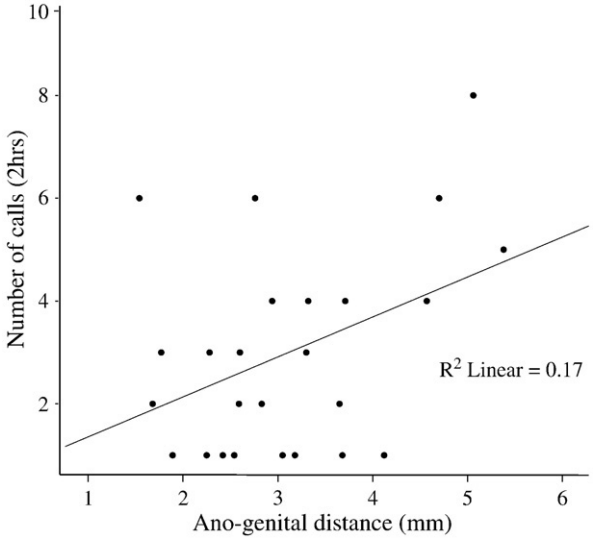

Fig. 5. Singing rate in a two hour period in relation to anogenital distance ($r^2 = 0.14$).

other sources of individual differences, such as behavioral masculinization (reflected in AGD).

To more fully characterize natural variation in HPA function, we examined multiple measures of CORT release. These provided a more complete but also more complex picture. First, fecal CORT was strongly predictive of Factor 1 scores (Fig. 2). We assumed that high levels of fecal CORT would predict a more stress-reactive phenotype. Instead, animals with high fecal CORT levels are more proactive. Although our fecal CORT assays detected CORT induced by an ACTH challenge, it did not predict individual differences in baseline plasma CORT. This suggests a relationship between stress reactivity and fecal CORT levels that is not mediated by individual differences in baseline CORT or negative feedback. Alternatively, it is possible that we failed to see a correlation between baseline plasma CORT and fecal CORT because these samples were collected at different times. However, because the stress response is highly repeatable within individuals, we would expect rank order consistency between mice throughout the day independent of circadiel fluctuations in CORT (Wada et al., 2008). Therefore, we would expect a correlation between these two CORT measurements despite their temporal separation. Although fecal CORT has been used to assess stress response in many species (Cyr and Romero, 2008; Hunt et al., 2006; Thanos et al., 2009), to our knowledge, the current study is the first to directly compare fecal CORT to both physiological and behavioral measures of stress reactivity ascribed to the proactive/reactive dichotomy.

One plausible explanation for the positive relationship between fecal CORT and proactive open-field behaviors is that proactive animals metabolize CORT at a higher rate, perhaps using metabolism as a means of reducing negative impacts of CORT exposure. For example, transcription of the hepatic enzyme Cyp3A is induced by glucocorticoids and, in turn, promotes their metabolism (Burk and Wojnowski, 2004). Fecal CORT may be a better measure of stress responses within an individual, where metabolism is more consistent, than of between-individual differences, where individual variation in stress responses and CORT metabolism can be difficult to disentangle. Given the widespread use of this marker in studies of field populations, manipulative studies will need to examine whether CORT metabolism is shaped by the same developmental events that regulate neural and behavior responses to adult stressors.

Alternatively, mice with higher fecal CORT may have had higher levels of circulating glucose and, hence, more expendable energy (Remage-Healey and Romero, 2001). Such variation could conceivably produce a more "proactive" behavioral pattern that could mask a high stress profile. In this scenario, we would expect behaviorally proactive mice to also have high levels of plasma CORT which are necessary to promote gluconeogenesis and liberate stored energy. However, baseline plasma CORT did not predict either behavioral profiles or fecal CORT, this seems like a less likely explanation.

Despite the prevalence of recent empirical studies of stress reactivity and other behavioral syndromes (Sih et al., 2004), relatively little theoretical work has examined the evolutionary consequences individual differences in temperament (but see Réale et al., 2007; Smith and Blumstein, 2008). In the context of stress reactivity, our work suggests several important considerations for any such effort. First, proactive behavioral differences between nearby sites within the same year, or at an identical site in subsequent years, highlight the highly plastic nature of stress reactivity. Such phenotypic plasticity could facilitate the fine-tuning of individual behaviors to local environmental demands (Mateo, 2006). Second, the fact that proactive animals sing more emphasizes how a given attribute of temperament can influence behaviors in diverse domains, a fact reflected in discussions of behavioral syndromes (Réale et al., 2007; Sih et al., 2004; Smith and Blumstein, 2008). Conversely, differences between singing performance in the open-field and in isolation suggest how multiple dimensions of temperament (stress reactivity and masculinization) can interact to shape a common behavior (singing). In the case of mouse song, the importance of each dimension seems to be highly context-dependent. Lastly, theoretical considerations of the trade-offs associated with stress reactivity should consider how physiological costs (e.g. high CORT and associated energy use, immune suppression; Korte et al., 2005) may be mitigated by a diverse array of physiological mechanisms, including changes in CORT metabolism and the use of alternative signals (e.g. corticotrophin releasing factor). An accurate assessment of ecological patterns and evolutionary consequences of stress reactivity will require assessment of multiple physiological and behavioral measures.

In summary, our data indicate that singing is linked to behavioral and physiological stress responses in the short-tailed singing mouse. The presence of pronounced individual differences in singing and stress reactivity, coupled with the spatial and temporal heterogeneity of populations, suggests that individual differences in coping styles are major contributors to intra-specific variation. Lastly, we found a surprising relationship between behavioral stress responses and fecal CORT that highlights a need to study the role of metabolism in stress reactivity. Together these data offer important insights into the role of coping styles in the performance of ecologically relevant social behaviors, and into the measurement of CORT function in natural settings.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yhbeh.2010.02.011.

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