DIVERGENT STRESS COPING STYLES AND SINGING BEHAVIOR IN THE SHORT-TAILED SINGING MOUSE (*Scotinomys teguina*)

By

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To Drs. Paulette Bierzychudek, Robert Baylor, and Dan Blumstein in recognition of their contributions to my early enthusiasm for research and animal behavior
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In response to stressors, vertebrates experience a suite of behavioral and physiological changes that elicit context-appropriate responses in order to maintain homeostasis. The acuity of the stress response and its associated behaviors vary greatly between individuals in many taxa and form suites of discrete characters described as stress coping styles. Stress coping styles have commonly been described in a proactive/reactive dichotomy. Proactive animal are behaviorally less wary and mount less acute physiological stress responses compared to reactive animals. It has been proposed that divergent stress coping styles give animals a survival or reproductive advantage in certain environmental contexts.

I examined the prevalence of divergent stress coping styles in male short-tailed singing mice (*Scotinomys teguina*) from a national park used for agriculture (Park) and a relatively undisturbed nature reserve (Reserve) in 2006 and 2007. Additionally, I examine the relationship of divergent stress coping styles to singing behavior, a trait presumed to be important for reproductive success in the short-tailed singing mouse. In 2006, males from the Park displayed behavioral and physiological traits associated with the reactive stress coping style while males from the Reserve displayed the proactive stress coping style. Additionally, Reserve males were more likely to vocalize than males from the Park. In 2007 there were no differences in
behavioral stress responses between sites, though the trends in physiological stress responses and singing behavior remained consistent. The proactive/reactive dichotomy does not appear to consistently explain behavioral and physiological variation in male short-tailed singing mice between sites. However, mice that expressed proactive stress coping behaviors were more likely to vocalize than reactive animals. This indicates that propensity to sing can be mapped on to the proactive/reactive stress coping style dichotomy.
In response to external stressors, vertebrates mount a suite of behavioral and physiological stress responses to maintain or re-establish homeostasis (Sapolsky et al. 2000). The acuity of the stress response and its behavioral manifestations has been shown to vary 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; Ward et al. 2004), and humans (Tyrka et al. 2006). These individual differences form suites of correlated behaviors and physiological responses known as stress-coping styles that are causally linked, predictable, and characteristic of a group or population of animals (Koolhaas et al. 1999).

Coping styles have been characterized along a shy-bold continuum and as a proactive/reactive dichotomy (Koolhaas et al. 1999). Boldness has broadly been described as the willingness to engage in risky behaviors in exchange for potential increases in reproductive or foraging success (Ward et al. 2004). Conversely, shy animals are defined as risk-averse and engage in behaviors with lower gains, but may experience higher survival in some contexts (Ward et al. 2004). The proactive/reactive dichotomy describes similar behavioral types, but makes context-specific behavioral and physiological predictions. Behaviorally, proactive animals are highly aggressive toward conspecifics, readily develop rigid learned routines, and actively avoid or manipulate stressful stimuli (Koolhaas et al. 1999, Koolhaas et al. 2007). The reactive stress coping style is characterized by low levels of conspecific aggression, flexible cue-dependent learning routines, and passive behaviors such as immobility in response to stressful stimuli (Koolhaas et al. 1999, Koolhaas et al. 2007).

In addition to these stereotyped behaviors, proactive/reactive stress coping styles are characterized by distinct assemblages of physiological and neuroendocrine stress responses. In
general, proactive animals show a high sympathetic response to stressors including an increase in blood pressure and heart rate and the production of catecholamines including both noradrenaline and adrenaline (Øverli et al. 2007). Conversely, reactive animals show higher parasympathetic reactivity to stressors than proactive animals and have lower hypothalamic-pituitary-adrenal (HPA) axis activity and reactivity (Øverli et al. 2007). Therefore, proactive animals typically have lower levels of basal glucocorticoids (the main hormones involved in the stress response and the ultimate product of HPA axis activation) and lower stress-induced glucocorticoid levels (Øverli et al. 2007).

Summed together, these behavioral and physiological characteristics describe animals that differentially respond to environmental stressors and may experience differential survival and fitness under fluctuating environmental conditions such as population density, social stability, predator density, and resource availability (Korte et al. 2005). For example, in captive great tits (*Parus major*), reactive-like individuals (defined as “slow explorers”) will quickly alter their foraging strategy when the location of food is experimentally manipulated. Conversely, proactive birds (“fast explorers”) will continue to visit the same feeder even after the food has been removed (Marchetti and Drent 2000). In wild populations of great tits, reactive individuals or “slow explorers” may be more likely to locate new food sources during times of resource paucity. Whereas proactive individuals or “fast explorers” may have an advantage during periods of resource stability because their social dominance allows them to dominate food sources (Marchetti and Drent 2000) Stress coping styles in rodents may provide similar survival advantages within an ecological context. In lab studies, proactive rodents aggressively defend their territory against conspecific intruders (de Boer et al. 2003) which could translate into higher fitness in densely populated environments (Korte et al. 2005).
While proactive and reactive animals employ different strategies for coping with stressors, the ultimate result (minimizing further exposure to a stressor and, thus, restoring the animal to homeostasis) can be the same between these two phenotypes. This is exemplified in the shock prod burying test used on laboratory rats (Koolhaas et al. 1999). In this assay, an animal is presented with an electrified prod in its home cage. When exploring the novel object, animals receive a mild but aversive shock. Rats that express the proactive phenotype will actively bury the electric prod with cage bedding and, thus, avoid further shocks. Conversely, reactive rats will hide in the corner of a cage and show immobility behavior. Though these two stress coping styles employ different behavioral responses, the ultimate outcome (avoiding further electrical shock) is identical (Koolhaas et al. 1999). Interestingly, these stress coping styles are highly context dependent. If a proactive animal is tested in the electric prod burying assay in a cage with no bedding, it will employ the same behavioral response as a reactive animal (Koolhaas et al. 1999).

In addition to the context-specific advantages and expression of divergent coping styles, both phenotypes are also associated with context-specific physiological and behavioral costs. Long-term exposure to glucocorticoids has been associated with reduced immunocompetence, growth, and longevity (Cavigelli and McClintock 2003) providing a potential morbidity cost to the reactive stress phenotype. Likewise, proactive animals may face mortality costs associated with their low wariness of novelty and propensity to engage in risky behaviors that increase their exposure to predators (Magnhagen and Staffan 2005). Different environmental contexts may generate tradeoffs in short and long-term survival that influence the frequency and fitness of alternative stress phenotypes in certain populations or environments (Sih et al. 2004).
In addition to tradeoffs between short and long-term survival, divergent stress coping styles may also generate tradeoffs between survival and reproduction. For example, in food-limited contexts, bold, highly aggressive female fishing spiders (*Dolomedes fimbriatus*) will cannibalize all courting male spiders and thus be unable to mate (Arnqvist and Henriksson 1997). Divergent stress coping styles may also influence reproduction by creating tradeoffs between sexually selected traits, such as display vocalizations, and predator or conspecific avoidance. In response to stressors, auditory signals have been shown to be modulated or withheld in Gulf toadfish (Remage-Healey et al. 2006), túngara frogs (Ryan 1985), and katydids (Faure and Hoy 2000). Glucocorticoids have specifically been shown to modulate the prevalence of alarm vocalizations (Boinski et al. 1999, Blumstein et al. 2006, Swiergiel et al. 2007) and display vocalizations (Remage-Healey et al. 2006, Leary et al. 2006). Although the propensity to vocalize has not been differentially ascribed to divergent stress coping styles, it is likely that proactive animals that are less behaviorally wary and have lower levels of glucocorticoids would engage in more display vocalization compared to reactive animals. If stress coping style influences display vocalizations, this characteristic could be a useful behavioral proxy to describe the ecological and evolutionary relevance of stress phenotypes in wild animals.

While stress coping styles have been well described in the laboratory, far fewer studies have examined the occurrence of stress phenotypes in wild animals within natural contexts. Extending studies from the laboratory-housed animals to wild animals will provide a more complete understanding of potential tradeoffs generated by stress coping styles. In addition, studying these coping styles outside the laboratory will allow us to investigate the influence of
ecological factors on stress coping style expression, the putative context-specific reproductive advantages of divergent coping styles, and their long-term evolutionary consequences.

Here, I document the prevalence of proactive/reactive stress coping styles and display vocalizations in two populations of short-tailed singing mice (*Scotinomys teguina*) Muroidea: Cricetidae) in distinctly different habitats. I first describe the sites at which the work was executed and document physiological and morphological differences in animals between sites. Next, I demonstrate the validity of fecal and serum corticosterone (CORT) assays used to measure the physiological stress response of singing mice. Finally, I compare spontaneous and CORT-induced singing behavior to behavioral stress responses (open-field behavior), basal CORT titers (HPA activity), and stress-induced CORT titers (HPA reactivity).
CHAPTER 2  
PHYSIOLOGICAL AND MORPHOLOGICAL VARIATION BETWEEN TWO  
POPULATIONS OF SHORT-TAILED SINGING MICE (SCOTINOMYS TEGUINA) 

Introduction 

Singing mice (Muroidea: Cricetidae) are small diurnal rodents that inhabit tropical cloud forests of Central America (Hooper and Carleton 1972). Singing mice derive their name from their ability to produce long frequency-modulated vocalizations. 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 Carleton 1972, Phelps et al. unpublished data). Although the purpose of singing is not fully known, evidence suggests the primary vocalization functions in mate attraction and male-male interactions (unpublished data).

There are two species of singing mice in the genus Scotinomys: the short-tailed singing mouse (Scotinomys teguina) and the long-tailed singing mouse (Scotinomys xerampelinus). Short-tailed singing mice range from southern Mexico to western Panama and are found at elevations between 1000-3000 meters (Hooper and Carleton 1976). The long-tailed singing mouse is found at elevations between 2150-3300 meters and ranging through Costa Rica and Panama (Hooper and Carleton 1976). Broadly, the two species are considered contiguously allopatric with small local areas of sympatry (Hooper and Carleton 1976). Despite being abundant and widely distributed, relatively little is known about the behavior and life histories of these charismatic species.

I surveyed short-tailed singing mice at two sites located near Boquete, Panama in 2006 and 2007. One site, the Peterson’s Nature Reserve (Reserve) is a privately owned nature reserve located in Jaramillo Arriba, 15 km north of Boquete, Panama. This reserve consists of approximately 100 hectares of secondary-growth tropical cloud forest bordered on all sides by
agricultural lands. Because this reserve is privately owned, there is very little anthropogenic disturbance within its boundaries. The second site, Volcán Baru National Park (Park), is a publicly owned and operated national park located 15 km west of Boquete, Panama with an altitudinal range between 1,840-3,478 meters. 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 (unpublished data).

Here I provide morphological data for male short-tailed singing mice from both sites. I also compared condition of animals between sites by quantifying hematocrit (the percentage of red blood cells to whole blood) and parasite prevalence. Finally, I quantify average vegetation height to examine habitat differences between the two sites.

Methods

Animal Trapping and Housing

I trapped mice from May to July 2006 and May to June 2007 using Sherman live traps baited with peanut butter and oats. I clipped no more than two toes with no more than one toe taken from each foot in a unique pattern to identify individual animals. Mice were housed in the laboratory in wire mesh cages and fed an *ad libitum* diet of dry kitten food and sunflower seeds.

Morphological and Condition Measurements

After capture, I assessed reproductive condition, parasite prevalence, and measured morphological traits including head width (width of head directly rostral to ears), hind foot (from back of heel to tip of middle toe excluding nail), half hind foot (from back of heel to hallux), tail
length (posterior margin of anus to tail tip), ano-genital distance (anus to genital projection), mass, and residual mass (mass/hind foot length).

Singing mice carry several ectoparasites including unidentified species of lice, fleas, and ear mites (personal observation). I categorized parasite prevalence based on the presence or absence of any visible ectoparasites.

To quantify hematocrit, I collected 100 – 150 μL of blood from the orbital sinus of mice using microhematcrit tubes. I centrifuged blood at 3000 rpm for four minutes and measured hematocrit as the percent of red blood cells to whole blood.

**Vegetation Survey**

To examine vegetation differences between sites, I quantified mean maximum vegetation height along eight 20-meter transects at both sites. Excluding trees, I measured maximum vegetation height as the highest vegetation stem at each meter mark for every meter on each 20-meter transect. I chose the transaction location and compass direction randomly within areas where I captured mice.

**Results**

**Morphological Summary**

Interestingly, despite their genetic similarity, I noted overall differences in male body size between sites in 2006 and 2007 (Table 2-1). There were significant site-by-year interactions for the measurements of hind foot length, half-hind foot length, head width, ano-genital distance, absolute mass, and residual mass (p < 0.05 for all). For this reason, morphological traits were analyzed separately for 2006 and 2007. In 2006, male mice from the Reserve were significantly smaller than males from the Park in measures of tail length, hind foot length, head width, absolute mass, and body condition (Table 2-1). Conversely, in 2006 Reserve males had significantly larger ano-genital distances than Park males (Table 2-1, p < 0.01, F = 12.40). In
2007, males from the Reserve were significantly smaller than Park males in tail length only (Table 2-1, p = 0.01, F = 6.74). There were no significant differences between Reserve and Park males in all other morphological measurements including testes mass in 2007 (Table 2-1).

Within the Park, males differed between years. In 2007 males from the Park were significantly smaller (p < 0.01, F = 22.43), half-hind foot (p < 0.01, F = 17.40), head width (p < 0.01, F = 19.93), and weight (p = 0.04, F = 4.48). However, in 2007 Park males had significantly larger ano-genital distances than Park males in 2006 (p = 0.04, F = 4.82). Males from the Reserve were not significantly different in morphological measurements between 2006 and 2007 (0.84 > p > 0.18) for all characters except for having significantly smaller ano-genital distances in 2007 (p = 0.01, F = 6.78).

**Parasite Prevalence**

There were no significant differences in parasite load between males from the Reserve and the Park in 2006 and 2007 (p = 0.24, χ² = 1.38). There were more parasitized males from the Reserve than the Park, though the results are not quite significant (p = 0.052, χ² = 3.77, Figure 1-1).

**Hematocrit**

There was no significant site by year interaction in percent hematocrit between Reserve and Park males for 2006 or 2007 (p = 0.26, F = 1.27). Analyzing years together and combining adult and sub-adult males, I found that males from the Park had significantly higher percent hematocrit than males from the Reserve (p = 0.04, F = 4.25, n = 30 and 41 respectively). Similarly, comparing only adult males from 2006 and 2007, males from the Park had a non-significant trend for higher mean percent hematocrit than males from the Reserve (p = 0.06, F = 3.55, n = 27 and 34 respectively).
Vegetation Survey

I found no significant difference in mean maximum vegetation height between the two sites (Figure 1-2; p = 0.21, F = 1.61, n = 8 for each site). I also found no significant difference in vegetation heterogeneity (measured by comparing the standard deviation of mean vegetation height) between the two sites (p = 0.19, F = 1.89). Although I did not further assess site differences in vegetation or habitat, based on the distinct and obvious visual differences, it is probable that additional measurements would reveal significant site differences.

Discussion

Here I have provided demographic and morphological data for male short-tailed singing mice from two sites in Boquete, Panama. Interestingly, in 2006, I found that males from the Park are larger than males from the Reserve in several morphological traits, including tail length, hind foot, absolute mass, and head width, but had smaller ano-gential distances. In 2007, Park males were of comparable size to Reserve males in all morphological measures with the exception of having longer tails. In addition to size differences, more males from the Reserve had ectoparasites than males from the Park in both 2006 and 2007.

Despite obvious visual differences in habitat, I found no difference in mean vegetation height between the Park and the Reserve. It seems likely that these habitat differences could be quantitatively described in a more biologically meaningful way by measuring habitat brokenness or floral diversity as examples. Habitat differences likely influence other ecological factors such as predator and conspecific density and resource availability. These ecological factors could be easily measured and, if labile, could contribute to the observed variation in morphological data between sites in 2006 but not 2007.

Variation in ecological factors could also explain the anomalous trend in 2006 for Park males to be morphologically larger than Reserve males in all measurements except ano-genital
distance in which they were significantly smaller. The overall larger body size may reflect a higher density of insect prey localized around the agriculture areas in the Park. Ano-gential distance is often used as a proxy for sexual maturity (Ashby et al. 1997, Pocock et al. 2002). It is possible that, despite putatively larger food availability, the males I trapped from the Park were less sexually mature than males from the Reserve. The majority of the animals surveyed from the Park were captured in fringe habitat bordering agricultural areas. If these fringe areas in the Park are marginal in respect to cover for predator avoidance or breeding habitat, it is possible that the mice I surveyed are younger animals that were forced from ideal habitat into this “sink” habitat. However, if this were true, I would also expect to observe a similar trend in body size differences between sites in 2007. Instead, I observed no difference in body size between sites in all measurements except tail length.

Alternatively, the anomaly between size trends for body size and ano-genital distance between sites in 2006 could be attributed to endocrine disrupting agents such as pesticides. Pesticides and other chemicals are used extensively in the agricultural areas at the Park (personal observation). Some of these pesticides, such as Atrazine, have been demonstrated to retard sexual development in rats (Stoker et al. 2000) and male Japanese quail (Wilhelms et al. 2005). If pesticide use is influencing sexual development in the mice at the Park, the inconsistent application of these chemicals (dependent on agriculture schedules) could explain the opposite trends in body size and ano-gential distance between males from the Park and Reserve in 2006 and the absence of these trends in 2007.
Figure 2-1. Percent of parasitized and nonparasitized males from the Reserve and the Park in 2006 and 2007.
Figure 2-2. Percent hematocrit (red blood cells/whole blood) of males from the Reserve (n = 41) and Park (n = 30).
Figure 2-3. Mean ± SEM maximum vegetation height at the Park and Reserve (n = 8 transects)
Table 2-1. Mean morphological traits of adult males from the Reserve and the Park in 2006 and 2007. ANOVA, Fisher’s PLSD used to determine differences between sites in 2006 and 2007.

<table>
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<tr>
<th>Trait</th>
<th>Reserve (n=25)</th>
<th>Park (n=16)</th>
<th>Site Difference Reserve/Park</th>
<th>Volcano Baru (n=19)</th>
<th>Site Difference Volcano Baru</th>
<th>Volcano Baru (n=17)</th>
<th>Site Difference Volcano Baru</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tail (cm)</td>
<td>52.0</td>
<td>51.9</td>
<td><strong>P &lt; 0.01</strong>, F = 9.86</td>
<td>54.9</td>
<td>54.7</td>
<td><strong>P = 0.01</strong>, F = 18.24</td>
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</tr>
<tr>
<td>Hind foot (cm)</td>
<td>15.70</td>
<td>15.63</td>
<td><strong>P &lt; 0.001</strong>, F = 10.81</td>
<td>16.57</td>
<td>15.81</td>
<td><strong>P = 0.41</strong>, F = 0.70</td>
<td></td>
</tr>
<tr>
<td>Half hind foot (cm)</td>
<td>6.59</td>
<td>6.33</td>
<td><strong>P = 0.10</strong>, F = 2.87</td>
<td>6.77</td>
<td>6.10</td>
<td><strong>P = 0.25</strong>, F = 1.38</td>
<td></td>
</tr>
<tr>
<td>Head width (cm)</td>
<td>11.53</td>
<td>11.65</td>
<td><strong>P &lt; 0.001</strong>, F = 14.59</td>
<td>12.74</td>
<td>11.88</td>
<td><strong>P = 0.14</strong>, F = 2.28</td>
<td></td>
</tr>
<tr>
<td>Anogenital distance (cm)</td>
<td>3.74</td>
<td>2.86</td>
<td><strong>P &lt; 0.001</strong>, F = 12.80</td>
<td>2.51</td>
<td>3.04</td>
<td><strong>P = 0.38</strong>, F = 1.10</td>
<td></td>
</tr>
<tr>
<td>Mass (g)</td>
<td>12.6</td>
<td>12.8</td>
<td><strong>P &lt; 0.001</strong>, F = 18.59</td>
<td>14.78</td>
<td>13.4</td>
<td><strong>P = 0.38</strong>, F = 1.12</td>
<td></td>
</tr>
<tr>
<td>Residual mass (gram/hind foot)</td>
<td>0.30</td>
<td>0.32</td>
<td><strong>P &lt; 0.001</strong>, F = 9.43</td>
<td>0.39</td>
<td>0.85</td>
<td><strong>P = 0.41</strong>, F = 0.70</td>
<td></td>
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<tr>
<td>Testes mass (mg)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>38</td>
<td><strong>P = 0.78</strong>, F = 0.19</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>56.6</td>
<td>55.3</td>
<td><strong>P = 0.31</strong>, F = 1.87</td>
<td>58.0</td>
<td>59.0</td>
<td><strong>P = 0.13</strong>, F = 2.48</td>
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</table>

(n = 21) (n = 12) (n = 19) (n = 16) (n = 14) (n = 11)
CHAPTER 3
BIOCHEMICAL AND PHYSIOLOGICAL VALIDATION OF CORTICOSTEROID RADIOIMMUNOASSAY FOR PLASMA AND FECAL SAMPLES IN THE SHORT-TAILED SINGING MOUSE (*Scotinomys teguina*)

**Introduction**

In response to an environmental stressor, animals experience a suite of behavioral and physiological responses that promote a context-appropriate stress response. One physiological component of the stress response involves activation of the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is initiated when a stressor stimulates the release of corticotrophin releasing hormone (CRH) from the hypothalamus. CRH stimulates the release of adrenocorticotropin hormone (ACTH) from the anterior pituitary which causes the release of glucocorticoids (the main steroid hormones involved in the stress response) from the adrenals (Sapolsky et al. 2000). Glucocorticoids regulate many facets of the physiological stress response by increasing available glucose, altering cardiovascular activity, and inhibiting immediate non-essential physiological processes such as reproduction, growth, and immune system function (review in Nelson 2005). Individuals vary greatly in the magnitude of HPA response to stressors (De Boer et al. 2003, Ruis et al. 2001, Marchetti and Drent 2000, Dingemanse et al. 2007; Ward et al. 2004).

An animal’s physiological response to a stressor is often evaluated by assessing glucocorticoid titers or glucocorticoid metabolites in blood and feces. Measuring glucocorticoids in the blood is thought to give an accurate measure of stress response because it evaluates the concentration of glucocorticoids directly available to tissues (Harper and Austad 2000). However, blood hormone assays provide hormone concentration at a single point in time and, therefore, do not capture long-term hormone profiles (Harper and Austad 2000). Additionally, glucocorticoids increase in as little as two minutes in response to handling stress and, thus, the
procedures involved in blood collection are sufficient to illicit a stress response and increase glucocorticoid titers (Romero and Reed 2005).

The use of fecal hormone metabolite measurements is becoming an increasingly common way to assess hormone profiles. Fecal hormone metabolite assays integrate hormonal fluctuations over the period of time that the feces are formed and stored, providing a broad and perhaps more informative hormonal profile than single-point measurements provided by blood samples (Harper and Austad 2000, Touma et al. 2004). Additionally, because of the slow metabolism of glucocorticoids, acute stress associated with handling will have little to no influence on metabolite concentration (Harper and Austad 2000).

Blood glucocorticoids titers are traditionally measured using radioimmunoassay (RIAs) or enzyme immunoassay (EIA). There are pronounced interspecific differences in the metabolic rate and secretion of fecal glucocorticoids that may preclude the use of immunoassays designed for laboratory mice in exotic animals (Palme 2005, Touma et al. 2005). For this reason, it is necessary to biochemically and physiologically validate immunoassays to ensure that they can detect biologically meaningful hormone measurements. Here, I validate the corticosterone (CORT) 125I-radioimmunoassay kit (MP Biomedicals, Solon, OH; catalog no. 071200103) for plasma CORT and fecal CORT metabolites in the short tailed singing mouse (Scotinomys teguina) using a combination of validation methods described by Good et al. (2003) and Touma et al. (2004).

Methods

Physiological Assay Validation of Plasma Corticosterone (CORT)

To assess the short-term effects of handling stress on singing mice, I exposed animals to five treatments of restraint stress (0, 3, 5, 10, and 20 minutes). I restrained animals by hand for the designated restraint time and then immediately collected 100 – 150 μL of blood from the
retro-orbital sinus. For control animals receiving zero minutes of restraint stress, I collected blood in less than two minutes to avoid initiating a stress response. I centrifuged blood at 3000 rpm for four minutes and stored the plasma at -40°C.

**Biochemical Validation**

To ascertain assay parallelism, I compared the slope of the standard curve to the slope of a curve generated using serially diluted fecal samples (1:7, 1:15, 1:31, 1:63). I logit-transformed percent bound and log-transformed CORT titers (ng/ml). I assessed assay precision by calculating inter and intra-assay coefficients of variation of the percent bound of the internal controls. I used variation between duplicate samples within an assay to calculate intra-assay precision and variation between average samples between assays to calculate inter-assay precision.

**Radioimmunoassay (RIA)**

I diluted fecal samples to 1:63 and plasma samples to 1:127 in assay diluent prior to radioimmunoassay. I ran each sample in duplicate, quantified gamma emissions on a Hewlett Packard Cobra II Auto Gamma Counter, and calculated CORT values from the standard curve.

**Results**

**Physiological Assay Validation of Plasma CORT**

Plasma CORT titers significantly increased between 10 and 20 minutes of restraint stress (p = < 0.01, F = 13.36; Figure 2-1). There were no significant differences in plasma CORT titers between any of the restraint treatments between zero and 10 minutes (0.88 < p < 0.23 for all).

**Biochemical Validation**

I found log-logit transformed curves of serially diluted fecal CORT extracts to be parallel to log-logit transformed standard curves (Figure 2-2). Intra-assay variance was 3.67% for the
high control and 1.71% and for the low control. Inter-assay variance for the pooled plasma sample was 8.3% and 6.5% for the pooled fecal extract.

**Conclusion**

My results demonstrate a biochemical and physiological validation of the use of radioimmunography to detect biologically relevant levels of CORT in short-tailed singing mice. Restraint stress produced a significant increase in serum CORT titers between 10 and 20 minutes of restraint that was detected by RIA. Log-logit transformed curves of serially diluted fecal samples were parallel to a similarly transformed standard curve. This demonstrates the validity of using RIA to measure fecal CORT metabolites at a dilution of 1:63 fecal extract to buffer. In summary, these findings allow us to use RIA to determine serum CORT titers and fecal CORT metabolites in a wild animal, the short-tailed singing mouse.
Figure 3-1. Mean serum CORT titer ± SEM following zero, 3, 5, 10, and 20 minutes of restraint stress (n = 24, 8, 9, 22 respectively). Serum CORT significantly increased between 10 and 20 minutes of restraint stress (*p < 0.01, F = 13.36).
Figure 3-2. Parallelism of log transformed fecal CORT serial dilutions and logit-transformed percent bound with transformed standard curves. Standard curve (triangles): $y = 4.34 - 1.91x$, $r^2 = 0.87$. Fecal extract curve (circle): $y = 4.97 - 1.43x$, $r^2 = 0.70$. 
CHAPTER 4
DIVERGENT STRESS COPING STYLES IN MALE SHORT-TAILED SINGING MICE IN TWO DISTINCT HABITATS

Introduction

Physiological and behavioral responses to stress vary greatly between individuals in many taxa. These variable responses have been shown to form distinct complexes of correlated traits, known as stress coping styles, which form consistent behavioral patterns over time (Koolhaas et al. 1999). Stress coping styles are commonly described in a proactive/reactive dichotomy (Koolhaas 1999). Animals that exhibit the proactive stress coping style display low behavioral wariness in response to novelty, actively avoid or manipulate stressful stimuli, and have rigid, learned routines (Koolhaas et al. 1999, Koolhaas et al. 2007). Conversely, reactive animals are behaviorally wary in response to novelty, passively respond to stressors, and display flexible learning patterns (Koolhaas et al. 1999, Koolhaas et al. 2007).

In addition to behavioral characteristics, proactive/reactive stress coping styles are characterized by a suite of physiological responses to stress. Proactive animals are characterized by high sympathetic activity and low hypothalamic-pituitary-adrenal (HPA) activity and reactivity (Øverli et al. 2007). Because of their low HPA activity/reactivity, proactive animals have low basal levels of glucocorticoids (the main hormones involved in the stress response) and achieve low levels of stress-induced glucocorticoids (Øverli et al. 2007). Compared to proactive animals, reactive animals show higher parasympathetic reactivity in response to stressors (Øverli et al. 2007). Additionally, reactive animals have higher HPA activity and reactivity, resulting in higher titers of basal and stress-induced glucocorticoids (Øverli et al. 2007).

Divergent stress coping styles are thought to give animals a survival advantage in specific environmental contexts (Marchetti and Drent 2000, de Boer et al. 2003). For example, because of their behavioral wariness, reactive animals may have higher survival in environments that are
densely populated by predators. Conversely, in predator depauperate environments, reactive animals may have lower survival compared to proactive animals because of the substantial morbidity costs associated high levels of glucocorticoids such as reduced immunocompetence and longevity (Cavigelli and McClintock 2003). Additionally, divergent stress coping styles may influence reproduction by creating tradeoffs between the expression of sexually selected traits, such as display vocalizations, and predator avoidance. Therefore, the frequency and fitness of divergent stress coping styles in populations is likely to be highly dependent on specific environmental factors (Sih et al. 2004).

Despite being described in a number of species including laboratory rodents (De Boer et al. 2003), pigs (Ruis et al. 2001), birds (Marchetti and Drent 2000), fish (Dingemanse et al. 2007; Ward et al. 2004), and humans (Tyrka et al. 2006), few studies have examined the prevalence of divergent stress coping styles in wild animals. I examined the prevalence of divergent stress coping styles in short-tailed singing mice from two sites in Boquete, Panama (Chapter 2). I measured behavioral response to novelty (open-field test) and HPA activity and reactivity. Additionally, to examine the interaction between divergent stress coping styles and reproductive behaviors, I measured the call rate of mice and compared this to behavioral and physiological stress responses. Singing is presumed to be important for mate attraction in singing mice (Chapter 2). Examining the interaction between stress responses and singing behavior may provide insight into trade-offs between reproduction and survival generated by divergent stress coping styles.
Methods

Animal Trapping and Site Descriptions

I trapped male singing-mice from May - July 2006 and May – June 2007 using the protocol described in Chapter 1. I housed mice in the laboratory for seven days using a standard protocol (Chapter 1).

Behavioral Stress Response: Open-Field Assay

To measure behavioral stress response I conducted open-field trials the morning following capture (Day 2) between seven AM and noon. The open-field test assays behavioral stress by taking advantage of the natural tendency of rodents to avoid open spaces. 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. I lined the arena with corn cob bedding. I changed the bedding between trials and cleaned the arena with ethanol. Prior to the initiation of the ten minute open-field trial, I placed mice in an opaque covered container in the center of the arena for five minutes. I videotaped trials using a Sony CCD-RTV118 Handycam and used JWatcher (Blumstein et al. 2000) to quantify the following behaviors: immobility (sec), singing, grooming (sec), jumping, posting, defecating, and crossing between the inner and outer circle (described in Table 4-1). I collected the feces produced by the mice during the ten-minute period to quantify fecal CORT titers.

Hypothalamic-Pituitary-Adrenal (HPA) Activity and Reactivity: Fecal and Serum Corticosterone (CORT)

I measured HPA reactivity by exposing mice to a zero (control) or 20 minute restraint stress treatments. For the 20 minute restraint stress treatment, I held animals by the scruff and restrained them on their back. After restraint I immediately collected 100-150 μL of blood from the retro-orbital sinus. For animals in the control treatment, I collected a blood sample from the
retro-orbital sinus in less than two minutes (including capture time) to obtain a measure of basal CORT titers (Romero and Reed 2005). I centrifuged whole blood at 3000 rpm for four minutes and then isolated and stored plasma at -20°C.

Using a standard protocol, I extracted CORT from fecal samples (Mateo and Cavigelli 2005). I weighed feces and placed them in individual test tubes with 1 ml of 90% methanol. Using a spatula, I homogenized the samples and agitated them for 24 hours. I centrifuged the samples at 2000 rpm for five minutes, decanted the extract, and stored it glass test tubes at -20°C until assayed.

**Radioimmunoassays (RIA)**

I measured serum and fecal CORT using $^{125}$I-radioimmunoassay kit (MP Biomedicals, Solon, OH; catalog no. 071200103). Using a combination of validation methods described by Good et al. (2003) and Touma et al. (2004; Chapter 2), I physiologically validated the blood and fecal assay to ensure that it was sensitive enough to detect biological meaningful alternations in hormone alterations.

I diluted fecal samples to 1:63 and plasma samples to 1:255 in assay diluent prior to radioimmunoassay. I 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.

**Adrenocorticotropic Hormone (ACTH) Supplementation and Singing**

I measured spontaneous call rate from seven AM until noon the day following capture (Day 2). I placed mice in opaque acoustically insulated chambers (40cm x 40cm x 40cm) and allowed them to acclimate for 30 minutes prior to determining call rate. To measure call rate, I counted the number of songs in a two hour period.

To examine the direct role that the HPA axis plays in singing behavior, I experimentally manipulated CORT titers and measured the resulting change in singing behavior. In a paired
design, I exposed animals to an ACTH and a saline treatment, alternating the order of treatments to account for treatment order effects. I injected animals with 0.03 IU/ml of ACTH suspended in gelatin or 0.03ml of saline. I allowed animals to acclimate to acoustically insulated chambers for 30 minutes and then counted the number of songs produced in one hour.

**Results**

**Behavioral Stress Assay: Open-Field Behavior**

There were significant site by year interactions for all open-field behaviors. For this reason, I analyzed open-field behavior separately for each year. In 2006, compared to males from the Park, males from the Reserve spent significantly less time immobile after trial initiation \( (p = 0.03, F = 5.03) \), jumped significantly more \( (p = 0.02, F = 5.69) \), crossed from the inner to outer circle significantly more times \( (p = 0.03, F = 5.69) \), and had a non-significant trend to post with paws on the side of the arena more \( (p = 0.08, F = 3.19; \text{Figure 4-1}) \). There were no significant differences between the Park and Reserve in number of defecations \( (p = 0.14, F = 2.13) \) or time spent grooming \( (\text{sec}; p = 0.42, F = 0.66; n = 29 \text{ for Reserve and 14 for Park}) \). Finally, there were significantly more males from the Reserve who sang in the open-field \( (n = 14) \) compared to singing males from the Park \( (n =0; p < 0.00, \chi^2 = 10.02) \).

In 2007, there were no significant differences between males from the Park \( (n =22) \) and the Reserve \( (n = 17) \) in immobility behavior \( (p = 0.37, F = 0.83) \), jumps \( (p = 0.42, F = 0.67) \), number of crosses between the inner and the outer circle \( (p = 0.32, F = 1.02) \), number of posts with paws on arena wall \( (p = 0.22, F = 1.57) \), number of defecations \( (p = 0.92, F = 0.10) \), or time spent grooming \( (\text{sec}; p = 0.98, F = 0.001) \). Though there were no significant differences between sites in any open-field behaviors in 2007, it is interesting to note that many of the trends in the data or the opposite of the trends from 2006 (Figure 4-2).
To determine whether individual differences in open-field behavior represented a cohesive set of behavioral traits, I examined correlations between measures of immobility (latency to move, latency to cross pegs) and measures that could be interpreted to reflect active responses to the stressful environment (jumps, posts, crossing from inner to outer circle), using animals from both sites and years. All pairwise correlations among these variable were significant (Pearson's coefficient, $-0.50 < r < -0.27; \text{all } p<0.02; n=78$)

**HPA Activity and Reactivity: Fecal and Serum CORT**

I combined data from 2006 and 2007 because there was no significant site by year interaction for fecal CORT ($p = 0.74, F = 0.11$) or serum CORT ($p = 0.20, F = 1.70$). Males from the Reserve had significantly higher fecal CORT titers during the open-field trials than males from the Park ($p = 0.03, F = 4.94; \text{Figure 4-3}$). Although the metabolism of corticosterone can vary greatly between rodent species, in the lab mouse peak corticosterone metabolites are recovered from the feces 10 hours after manipulation (Touma et al. 2003). This suggests that stimulation of the HPA-axis during the open-field trials would not influence corticosterone titers in feces collected 10 minutes after trial initiation. In 2006 I measured fecal CORT titers after seven days of captivity in the laboratory and found no significant difference between males from the Reserve and males from the Park ($p = 0.43, F = 0.65, n = 8 \text{ and } 9$ respectively). However, after seven days in captivity, males from the Reserve had significantly lower fecal CORT titers compared to titers measured after two days of housing in the laboratory (Figure 4-4, $p = 0.01, t = -4.81, n = 6$). Similarly, males from the Park had significantly lower fecal CORT titers on day seven compared to day two (Figure 4-4, $p = 0.03, t = -2.69, n = 8$).

Fecal CORT titers were not significantly higher for mice that sang in the spontaneous singing trial (callers) compared to mice that did not sing (noncallers; $p = 0.33, F = 0.96, n = 22$ and 41 respectively). Likewise, among callers, fecal CORT titers did not predict calling rate ($p = 0.33, F = 0.96, n = 22$ and 41 respectively).
There was a significant negative correlation between fecal CORT titers and the number of feces produced during the open-field trial. No other open-field behaviors were significantly related to fecal corticosterone titers.

Examining acute stress reactivity, I found no significant difference between Reserve and Park males in basal (control) serum CORT titers (p = 0.72, F = 0.13, n = 7 and 17 respectively). Conversely, males from the Park achieved significantly higher serum CORT titers following 20 minutes of restraint stress (ANCOVA, p = 0.02, F = 6.68, n = 12 and 10 respectively, Figure 4-5).

**Singing Behavior and Stress Responses**

Combining data from 2006 and 2007, significantly more males from the Reserve (n = 46) called than males from the Park site (n = 35; p < 0.00; \( \chi^2 = 17.01 \); Figure 4-6) in the two hour sampling period. 52% of males from the Reserve (n = 46) sang while only 8% of males from the Park site sang (n = 35). However, calling males from the Reserve (n = 24) did not call significantly more than calling males from the Park (n = 3; MannWhitney U; p = 0.17; U = 18.0). Interestingly, spontaneous call rate is significantly positively correlated with anogenital distance (AGD) of calling males in both populations (n = 24; p = 0.04; Figure 4-7).

In 2006, after seven days of captivity, there was no significant difference between the number of callers and noncallers from the Reserve and the Park (p = 0.40, \( \chi^2 = 0.70 \), n = 21 and 11 respectively). Calling males from the Reserve sang significantly more on the seventh day in captivity compared to the second day (p = 0.1, t = -3.41, n =13). Although not significant, there was a trend for Park males to increase singing rate on the seventh day (p = 0.08, t = -2.24, n = 6). Among seventh day callers from the Park and the Reserve, there was a non-significant positive correlation between fecal CORT titers and calling rate (Spearman correlation, p = 0.07, z = 1.83, n = 11).
Among calling males from both the Park and the Reserve in 2006 and 2007 (n = 24), there was non-significant negative correlation between fecal CORT titers and spontaneous singing rate (p = 0.21, F = 1.68). Additionally, there was no significant relationship between basal serum CORT titers (as measured from the restraint stress experiment) and calling rate in calling males from both sites and both years (p = 0.47, F = 0.55). Looking at differences in open-field data between callers and non-callers, calling males spent significantly less time in the inner circle after trial initiation than non-calling males (p = 0.03, F= 5.10) and posted with their front legs against the wall significantly more than non-calling males (p = 0.001, F = 11.59; Figure 4-8). However, there were no significant difference between calling and non-calling males in other open-field behaviors including the number of jumps against the arena wall (p = 0.56, F = 0.34) or the number of times an animal crossed between the inner and outer circle ( p = 0.25, F = 1.36; Figure 4-8).

**Effect of ACTH on Singing Behavior**

Experimentally manipulating ACTH titers (and thus increasing CORT) did not alter singing rate compared to control treatments (Figure 4-9, Wilcoxon signed rank, p = 0.21).

**Discussion**

The results from this study indicate the existence of differences in the behavioral and physiological stress responses between short-tailed singing mice from two distinct habitats. In 2006, male singing mice from the Reserve display a proactive-like stress phenotype while males from the Park fit the reactive coping style. In the open-field test they spent less time immobile and were more active (jumped and moved between circles more) compared to males from the Park. Proactive animals will often attempt to repeatedly solve a problem in the same way even when it proves ineffective; reactive animals will either attempt a new solution or abstain from further problem solving attempts (Korte et al. 1996). The males from the Reserve jumped
significantly more in the open-field than mice from the Park. If this behavior constituted an attempt to escape the open-field apparatus, this routine formation lends further support to the proactive behavioral nature of Reserve males.

Interestingly, the open-field behavioral trends I observed in 2006 were absent in 2007. In fact, though not significant, many of the trends were the opposite of what I observed in 2006. Because stress responses are highly context dependent, it’s possible that some extrinsic ecological factor influenced open-field behavior between years. Wild-type rats (*Rattus norvegicus*) show no difference in some behavioral response tests such as the 8-arm radial maze and the Morris water maze on first exposure, but reactive animals will behave more anxiously during the second exposure (File et al. 1996, Veenema et al. 2003). This suggests that prior exposure to stressors influences an animal’s behavioral response. The habitat in the Park site is constantly and unpredictably changing as the area is used for agriculture. It’s possible that subtle habitat changes at this site between 2006 and 2007 influenced previous stress exposure and that these alterations were responsible for the shifts in open-field behavioral trends. Although many of the open-field measures varied by site and year, there were strong overall correlations among open-field behaviors across individuals. This supports the interpretation that these reflect a common suite of behaviors that can be interpreted as a coping style.

Unlike the behavioral data, the assays of physiological stress response yielded consistent results across years. Physiologically, the proactive/reactive dichotomy predicts that males from the Park would have higher basal CORT titers than males from the Reserve. Contrary to this prediction, I observed no significant difference between basal serum CORT between Reserve males and Park males. However, Park males achieved higher serum CORT titers following a 20
minute period of restraint stress than Reserve males, indicating a more reactive HPA response as I would predict from a reactive phenotype.

Interestingly, Reserve males had significantly higher fecal CORT titers than males from the Park when assayed on the second day of captivity. Fecal samples integrate circulating hormone titers over time including basal and acute episodic hormone fluctuations. For this reason, measuring hormones from fecal samples is thought to more accurately represent an animal’s hormonal status because it does not capture a single point in time like blood samples (Touma et al. 2004). If this is true, it is interesting and unexpected that males from the Reserve had higher fecal CORT titers than males from the Park. One possible explanation is that, though Park males achieve a higher stress-induced CORT response, they are able to attenuate HPA activity more rapidly or metabolize CORT more rapidly than males from the Reserve. In an environmental context where animals are exposed to chronic stress, the ability to rapidly attenuate HPA activity or metabolize endogenous CORT can reduce morbidity costs associated with elevated levels of glucocorticoids such as suppression of reproduction, muscle wasting, growth suppression, neuronal death, and immune system suppression (Sapolsky et al. 2000, Wingfield et al. 1997). Attenuation of HPA activity in response to chronic stress has been demonstrated in European starlings (Rich and Romero 2005, Cyr and Romero 2007), pigs (Ruis et al. 2001), and rats (Swiergiel et al. 2007). Mice from the Park may chronically be exposed to stress as their environment is unpredictably manipulated for agricultural purposes, which could influence resource availability, conspecific population density, or predator density. If this is true, it would explain why mice from the Park have a higher HPA response to an acute stressor, but lower levels of fecal CORT.
I found a greater prevalence of mice that sang at the Reserve than at the Park. Looking across years and sites, animals that vocalized (callers) were more likely to display some open-field behaviors associated with the proactive stress coping style (jumping and shorter periods of immobility). While singing behavior is associated with behavioral stress coping style, it does not appear to be related to the physiological component of stress coping styles. Singing behavior was not predicted by second day fecal CORT titers or basal serum CORT titers and ACTH supplementation did not decrease singing behavior as would be predicted if propensity to sing was modulated by a CORT mechanism. Although I observed an increase in singing behavior and a concordant decrease in fecal CORT titers from the second to the seventh day in captivity, this could represent an overall attenuation of the stress response to captivity and not a causative mechanism. In addition to differential HPA activity and stress response behaviors, proactive/reactive animals are also characterized by differential levels of conspecific aggression. Serotonin is an important neuromodulator in the regulation of social behavior and aggression and has been shown to modulate display vocalizations associated with dominance in weakly electric fish (*Apteronotus leptorhynchus*, Telgkamp et al. 2007). It’s possible that propensity to sing in singing mice is controlled by a similar mechanism and would explain why ACTH supplementation had no effect on singing behavior.

In conclusion, I describe behavioral and physiological differences in stress responses in 2006 that form phenotypic assemblages that closely mirror the characteristics of proactive and reactive phenotypes described by Koolhaas et al. (1999). However, in 2007, behavioral stress responses in the open-field assay do not conform to the proactive/reactive dichotomy, nor were they consistent with the behavioral trends in 2006. Conversely, differences in physiological stress response between sites are consistent between years and, in part, conform to the
proactive/reactive dichotomy. The anomalous behavioral trends between years and the lack of correlation between behavioral and physiological responses that typify the proactive/reactive stress coping style dichotomy suggest that the proactive/reactive stress coping style dichotomy does not appropriately or consistently describe differences in stress responses between Park and Reserve animals. However, the consistency in the relationship between stress coping style and singing across years and between sites suggests that vocalization behavior can be mapped onto the proactive/reactive dichotomy. Specifically, I suggest that behaviorally proactive animals are more likely to engage in display vocalizations that reactive animals. This suggests that animals may face trade-offs between reproductive success and stress coping style. The unique vocalization behavior of singing mice provides the opportunity to examine these tradeoffs in wild animals, making them an ideal system in which to study the ecological and evolutionary relevance of divergent stress coping styles.
Figure 4-1. Mean ± SEM measures of open-field behavior in 2006 with n = 29 for Reserve and n = 14 for Park. Males from the Reserve (A) had significantly shorter periods of immobility (p = 0.03, F = 5.03), (B) had a non-significant trend to post with paws on the wall more than Park males (p = 0.08, F = 3.19), (C) jumped significantly more against the arena wall (p = 0.02, F = 5.69), and (D) crossed from the inner to the outer circle significantly more than Park males (p = 0.03, F = 5.69). * indicates p < 0.05.
Figure 4-2. Mean ± SEM measures of open-field behavior in 2007 with n = 17 for Reserve and n = 22 for Park. There were no significant differences between (A) period of immobility following trail initiation (p = 0.37, F = 0.83), (B) number of posts with paws on the arena wall (p = 0.22, F = 1.57), (C) number of jumps against the arena wall (p = 0.42, F = 0.67), and (D) number of times individuals crossed from the inner to the outer circle (p = 0.32, F = 1.02).
Figure 4-3. Mean fecal CORT ± SEM for 2006 and 2007 combined. Reserve males had significantly higher titers of fecal CORT than Park males (*p = 0.03, F = 4.94, n = 38 and 27 respectively.)
Figure 4-4. Mean fecal CORT (ng/g) ± SEM on the second and seventh day in captivity. Mice from the Reserve (A) had significantly lower fecal CORT titers on the seventh day in captivity compared to the second day (*p = 0.01, t = -4.81, n = 6). Likewise, mice from the Park showed a significant decrease in fecal CORT titers from the second to seventh day in captivity (**p = 0.03, t = -2.69, n = 8.)
Figure 4-5. Mean ± SEM CORT secretion in basal and stress (20-minute restraint) conditions. Animals from the Park showed higher post-stress levels of CORT compared to animals from the Reserve (n = 12 and 10 respectively, *p = 0.02, F = 6.68 ANCOVA). No site differences were observed for the control (n = 7 and 17, p = 0.72, F = 0.13, ANOVA, Fisher’s PLSD).
Figure 4-6. Percentage of males that sang at least once in a two hour period (callers) and males that did not sing (noncallers). The Reserve had significantly more caller’s than the Park (P < 0.00, $\chi^2 = 17.0$).
Figure 4-7. Singing rate in a two hour period in relation to anogenital distance. Linear regression with 95% confidence bands for mean ($r^2 = 0.14$, $P = 0.04$, $n = 25$).
Figure 4-8. Mean ± SEM measures of open field behavior in 2006 and 2007 with n = 20 for Callers and n = 62 for Noncallers. Compared to Noncallers, Callers (A) spent significantly less time in the inner circle after trial initiation (p = 0.03, F = 5.10) and (B) posted with their front legs against the arena significantly more (p = 0.001, F = 11.59). There were no significant differences between (C) number of jumps against the arena wall (p = 0.56, F = 0.34) or the number of times an animal traversed between the inner and outer circle (p = 0.25, F = 1.36). * indicates p < 0.05.
Figure 4-9. ACTH treatment did not significantly change singing behavior compared to control treatments (Wilcoxon signed rank, $p = 0.21$, $n = 7$ per treatment).
Table 4-1. Description of behaviors and activities measured in the open-field behavior assay.

<table>
<thead>
<tr>
<th>Behavior/Activity</th>
<th>Description</th>
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<tbody>
<tr>
<td>Immobile</td>
<td>Stationary behavior after trial initiation; measured until animal took one step</td>
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<tr>
<td>Vocalization</td>
<td>Stereotyped vocalizations longer than 3 seconds</td>
</tr>
<tr>
<td>Groom</td>
<td>Time spent engaged manipulating fur or tail with paws or mouth</td>
</tr>
<tr>
<td>Jump</td>
<td>Both hind legs leave the ground</td>
</tr>
<tr>
<td>Post</td>
<td>Front paws placed on arena wall, animal standing on hind legs</td>
</tr>
<tr>
<td>Defecate</td>
<td>Number of pellets</td>
</tr>
<tr>
<td>Cross circle</td>
<td>Crossing the circle of push pins designating the inner and outer circles</td>
</tr>
</tbody>
</table>
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Andrea Crino was born in Forest Grove, Oregon, in a cabin in the woods. A childhood spent rampaging through the wilderness, eating questionable plants, and poking unsuspecting animals with sticks instilled Ms. Crino with a deep respect and sense of passion for the natural world. She pursued this interest by earning a Bachelor of Arts degree in biology at Lewis & Clark College in 2002. As an undergraduate, Ms. Crino spent a semester in Costa Rica studying tropical ecology and conservation with the Organization of Tropical Studies. This fantastic experience motivated Ms. Crino to pursue independent research through two Research Experience for Undergraduates fellowships with the National Science Foundation at Baylor University and the Rocky Mountain Biological Station. Ms. Crino was actively involved in community service at her undergraduate institution. She served as a teaching assistant for introductory biology courses, led middle school girls in weekly science activities to increase confidence, and volunteered with the Nature Conservancy.

After graduating Ms. Crino, worked as an intern at Archbold Biological Station and the Smithsonian Tropical Research Institute and as a research technician at Arizona State University before returning to school to pursue a Master of Science at the University of Florida. She completed her degree in August of 2008.

While in graduate school, Ms. Crino completed two half-marathons, two full marathons, and two Olympic-distance triathlons. She is a devoted rock climber, an amateur surfer, consummate booty-shaker, and enjoys a fine Pinot Grigio on warm, summer days in Florida.