HPA activity and neotic and anxiety-like behavior vary among *Peromyscus* species

Lynn B. Martin *, Brian C. Trainor, M. Sima Finy, Randy J. Nelson

Departments of Psychology, Neuroscience, and Evolution, Ecology, and Organismic Biology, The Ohio State University, Columbus, OH 43210, USA

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Abstract

Behaviorally plastic species are more likely to invade and endure in new areas, and behaviorally plastic individuals tend to be attracted to novelty (i.e., neophilic). Furthermore, neophilic behaviors are often influenced by glucocorticoids. Thus in addition to environmental conditions and vicariant events, behavioral plasticity and its endocrinological mediators may influence the extent of vertebrate geographic distributions. Some species of mice in the genus, *Peromyscus*, occupy most of North America whereas others are restricted to small areas. We predicted that one widespread species (*Peromyscus maniculatus*) would interact more with novel objects, more readily explore novel environments, and possess hypo-responsive HPA axes compared to species with small ranges. Our hypothesis was not supported, but given the small number of species in this study and the high anxiety-like behavior in captive *P. maniculatus*, it is premature to reject the hypothesis that behavioral flexibility affects geographic distribution in *Peromyscus*. Indeed, behavioral and HPA axis variation was complementary among species, which is opposite of the pattern typically detected within species, suggesting that future studies of glucocorticoid mediation of neotic and anxiety-like behaviors in *Peromyscus* would be valuable.

Keywords: Anxiety; CRF; Glucocorticoid; Rodent; Stress

1. Introduction

Animals typically exhibit one of two strategies when confronted with novelty (Koolhaas et al., 1999; Wilson et al., 1994). Some are proactive and thus receptive of novel objects, food, or experiences. Others are reactive and avoid novelty in favor of the familiar. These traits are thought to represent equally viable strategies individuals use to cope with adversity (Schjolden et al., 2005). Such behavioral variation also exists among strains of domesticated rodents (Cowan, 1977; Trullas and Skolnick, 1993) and poultry (Minville et al., 2002; Siegel, 1995). Efforts to identify and understand similar variation among wild vertebrates are limited however. One exception involves passerines (Greenberg, 1983, 1990; Lefebvre et al., 1997). Some species tend to be less fearful of novelty than other species (i.e., less neophobic), and are also more behaviorally innovative and flexible. Indeed, behavioral flexibility is oftentimes promoted by reduced neophobia. Behavioral flexibility, including responsiveness to novelty, has been argued to explain different evolutionary diversification rates among taxa (Miller, 1956). If behavioral flexibility can explain diversification in evolutionary time, then behavioral flexibility may also impinge on diversification in ecological time.

We predicted that behavioral flexibility, in particular neophilia, should influence the number and types of habitats species occupy and hence their geographic distributions. As behavioral flexibility helps organisms cope with environmental change and exploit unfamiliar resources (Fragaszy and Mason, 1983; Greenberg, 1983; Lefebvre et al., 1997), increased interest in novel objects and/or locations should promote expansion into and persistence in new habitats (Coleman and Wilson, 1996; Davis et al., 2001; Mayr, 1965; Sol et al., 2002). Although species ranges are

* Corresponding author. Fax: +1 614 688 4733.
E-mail address: lmartin@mail.psych.ohio-state.edu (L.B. Martin).

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limited by many forces (Andrewartha and Birch, 1954), behavioral flexibility also appears important. One of the best predictors of invasion success in birds, a type of range expansion, is behavioral innovation rate (Lefebvre et al., 1997; Sol et al., 2002), or the number of different techniques used to acquire food. As attraction to novelty is typically high in behaviorally flexible species, range expansion would be expected to be more common among species receptive to novel foods, objects, or habitat characteristics. Over time, this increased tendency towards neophilia would promote dispersion across landscapes. Once new areas were invaded, neophilia would presumably also promote persistence in new areas, as neophilic species would be better able than neophobic species to discover and use non-preferred or sub-optimal resources in unfamiliar habitats (Pulliam, 1986). Indeed, habitat generalists tend to be less neophobic, which may explain why they are often more successful than habitat specialists in new habitats (Cassey et al., 2004). Further, more morphologically plastic species are better able to occupy habitats in which competitors are present. For example, hindlimb morphology of blue tits (Parus caeruleus) allows for greater flexibility when foraging, which enables blue tits to feed on substrates that larger (Parus major) and smaller (Parus cristatus) competitors cannot (Moreno et al., 2001). Such increased morphological plasticity has been proposed to explain why blue tits are one of the most cosmopolitan passerines in Europe.

In the present study, we asked whether differences in responsiveness to novelty among four species of Peromyscus mice were complementary of their geographic distributions. Three species are restricted to relatively small areas of the mid-Western US and north-central Mexico (P. aztecus, P. melanophrys, P. eremicus), whereas one (P. maniculatus) occurs across most of continental North America (King, 1968). Three species are also close relatives in the subgenus, Peromyscus, whereas P. eremicus is a member of the subgenus, Haplomyomys. We predicted that geographic distribution would more strongly influence species’ behavior than phylogeny. Specifically, we predicted that P. maniculatus would be quicker to contact/explore novel objects/areas and spend more time handling/exploring them than the other three species. To account for differential anxiousness and response to human handling (i.e., temperament) among species, as these behaviors can confound responsiveness to novelty, anxiety-like behaviors (Carola et al., 2002) and temperament (Wahlsten et al., 2003) were also assessed in all species. Conventional psychobiological assays including the open-field test (Crawley et al., 1997), elevated plus maze (Handley and Mithani, 1984), and light–dark box (Belzung and Lepape, 1994) and a temperament scoring paradigm (Wahlsten et al., 2003) were used to generate these data. Finally, whether behavioral variability was related to variation in components of one important physiological system, the hypothalamic-pituitary-adrenal (HPA) axis, was addressed. In rodents and primates, novelty-seeking individuals usually exhibit low circulating glucocorticoids (Koolhaas et al., 1999) and (in primates) reduced corticotrophin-releasing hormone (CRF) in cerebrospinal fluid and low activation of the right frontal lobe in response to novelty or other anxiety-inducing experiences (Kalin et al., 2004). Here, we measured basal and restraint-induced increases in glucocorticoids and quantified corticotrophin-releasing factor (CRF) in the paraventricular nucleus (PVN) of the hypothalamus after behaviors were assessed.

2. Methods

2.1. Mice

Thirty-five adult virgin male (>55 days old) P. maniculatus (n = 7), P. melanophrys (n = 8), P. eremicus (n = 8), and P. aztecus (n = 8) were acquired from the Peromyscus Genetic Stock Center at the University of South Carolina (Columbia, SC). Each species has been bred in captivity since capture, although duration of time in captivity varies among species: P. maniculatus since 1948, P. melanophrys since 1970 (or 1978), P. aztecus since 1986, and P. eremicus since 1993. We chose to study captive-breeding mice to eliminate confounding effects of experience during development that wild-caught mice may have experienced. This approach promotes characterization of genetic differences in behavior and physiology among species, which is critical to addressing our hypothesis.

Mice were housed in separate polypropylene cages (dimensions: 27.8 × 7.5 × 13 cm) and remained as such throughout the experiments. Ambient temperature and relative humidity were 21 ± 5°C and 50 ± 5%, respectively, for the duration of the study. All mice had unlimited access to food (Harlan Teklad 8640 rodent diet, Indianapolis, Indiana) and filtered tap water. Photoperiod was set to 16 h light (beginning at, 2200 EST) and 8 h dark per day. All mice underwent all behavioral tests, beginning with the elevated plus maze, followed by the open-field test, light–dark box, novel object tests (marble then binder clip), the novel environment test, and finally wildness scoring. Blood samples were later taken for corticosterone (see below). Individuals within species were randomized in terms of order in each test, but all mice experienced all tests in the same sequence. Anxiety tests were performed first in an effort to acclimate all individuals to handling for neophilia behavioral tests. Species were not initially acclimated to novel components of the novel object/place tests to minimize handling and potential subsequent changes in behavior. One mouse died during behavioral tests, but data from this individual were included in all statistical comparisons. All procedures were approved by the Ohio State Institutional Animal Care and Use Committee prior to the study and meet ABS/ASAB guidelines for ethical treatment of animals.

2.2. Novelty responsiveness

2.2.1. Novel objects

Novel object testing occurred 1 h after lights-off; all testing and transport of the mice to the testing room was conducted under dim red light conditions. For each test, one individual was brought into the testing room from the colony, and then a marble (cleaned before each trial with 70% alcohol) was immediately placed in the home cage with the animal. Interactions with the object over the next 5 min were recorded including (i) latency to approach the object, (ii) total contacts with the object, and (iii) total time spent handling the object. The mouse was considered as having approached the object when it began sniffing or touching it. Contact was defined as any part of the body touching the object; handling was defined as holding the object in the mouth or paws. After testing, the marble was removed from the cage and the mouse was returned to the colony. One week later, all individuals were tested in the same manner as above, but this time interest in a metal binder clip was assessed.

2.2.2. Novel environment

During the light cycle, each mouse was removed from its home cage and placed into a plastic box (dimensions: 99 × 52.5 × 12.5 cm). The box
was bisected by a piece of cardboard into which a small hole (10.6 × 8.8 cm) was cut to allow transitions between sides of the box. All mice were initially placed on the same side of the box then given 5 min to explore the two environments. During this period, (i) latency to enter the novel side, (ii) total transitions between sides, and (iii) number of fecal bolus expelled were recorded. After each trial, fecal bolus were removed and the box was sterilized thoroughly with 70% alcohol.

2.3. Anxiety-like behavior

2.3.1. Open field

During the dark period, mice were placed in an acrylic box (40 × 40 cm) lined with rodent bedding, which was housed in a ventilated cabinet (Kim et al., 2002; Pyter and Nelson, 2006). The maze consisted of two closed and two open arms (6 cm wide) in a plus configuration, perpendicular to the periphery, (iii) total beam breaks overall (as an index of activity), and (iv) total rears were recorded. At the conclusion of trials, bedding was removed and the box was sterilized with 70% alcohol.

2.3.2. Elevated plus maze

Elevated plus maze performance was evaluated during the dark period in all mice (Hogg, 1996; Pyter and Nelson, 2006). The maze consisted of two closed and two open arms (6 cm wide) in a plus configuration (~1 m above the floor). The two closed arms were lined by 15 cm tall black plexiglass; all arms were covered with contact paper to prevent mice from falling, and all surfaces were swabbed between trials with 70% alcohol. For each trial, a mouse was released into the center of the maze (where the four arms joined), and each mouse was allowed 5 min to explore the maze. Exploration was videotaped from above and scored later. Mice were considered to have entered arms when all four paws crossed onto an arm. Occasionally, a mouse fell off the maze; small boxes were placed under the maze so mice could quickly be captured and placed back on the maze. If a mouse fell, it was replaced in the maze center and allowed to explore for the remainder of the 5 min trial. Behavior was later scored using The Observer software (Version 5, Exeter Software, Setauket, NY). Several behaviors were quantified including (i) entries into open arms, (ii) entries into closed arms, (iii) entries into the center space, (iv) time in open arms, (v) time in closed arms, and (vi) fecal bolus expelled.

2.3.3. Light–dark box

This experiment closely mirrored that of the novel environment trial above. For these trials, an opaque plastic bag was used to ensure one side of the same box as above was in complete darkness (Kim et al., 2002). During the light period, each mouse was placed on the illuminated side of the box and given 5 min to explore. As above, (i) latency to enter the darkened side, (ii) total transitions between sides, and (iii) number of fecal bolus expelled were recorded. After each trial, fecal bolus were removed and the box was sterilized thoroughly with 70% alcohol.

2.4. Temperament

To characterize how each species responded to capture and handling, each individual was rated on a “wildness” scale developed for domestic mice strains (Wahlsten et al., 2003). To assess capture response, an individual experienced in capturing Peromyscus removed the cage top, and then as quickly as possible attempted to catch the mouse from its cage. All captures were successful within 10–15 s. Simplicity in subduing each individual was scored according to Table 1. Immediately after capture, the mouse was moved to a sitting position on the back of the researcher’s gloved hand while its tail was held lightly between the researcher’s thumb and forefinger. Response to this handling was scored according to Table 1.

### Table 1. Wildness scoring criteria for Peromyscus

<table>
<thead>
<tr>
<th>Score</th>
<th>Capture behavior</th>
<th>Handling behavior</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Minimal resistance</td>
<td>Minor or no struggle</td>
</tr>
<tr>
<td>1</td>
<td>Evades touching by running away</td>
<td>Squeaks or squeals</td>
</tr>
<tr>
<td>2</td>
<td>Jumps onto cage wall</td>
<td>Vigorous struggle (twisting or shaking)</td>
</tr>
<tr>
<td>3</td>
<td>Jumps out of cage</td>
<td>Attempts to bite</td>
</tr>
<tr>
<td>4</td>
<td>Runs from cage</td>
<td>Bites handler</td>
</tr>
<tr>
<td>5</td>
<td>Jumps onto floor</td>
<td>—</td>
</tr>
</tbody>
</table>

2.5. HPA axis activity characterization

2.5.1. Corticosterone and adrenaline measurement

Three weeks after behavioral trials baseline blood samples (~100 μl) were collected from the retro-orbital sinus within 3 min of contacting a cage (Demas and Nelson, 1996). For stress-induced samples, mice were held in polystyrene tubes for 2 h prior to sampling (DeVries et al., 1997; Glasper and DeVries, 2005). Stress-induced samples were collected 2 days after baseline samples to ensure that mice recovered from the initial bleeding sample prior to re-sampling. Post-collection, blood samples were kept on ice until they were centrifuged at 4 k RPM for 20 min. Plasma was then removed and frozen at −80 °C until assays were performed. All samples were assayed in duplicate to calculate intra-assay variation. Baseline and stress-induced plasma corticosterone concentrations were measured using a single 125I radioimmunoassay kit (ICN Biomedicals, Costa Mesa, CA, USA). Cross-reactivity with other hormones in this kit is <0.5%. Because corticosterone in Peromyscus tends to be much greater than Mus and Rattus, for which this kit was developed, plasma was diluted 1:1000 (Glasper and DeVries, 2005). Mean intra-assay variation was 17%, and lower detection limit was 5 ng ml⁻¹. After stress-induced corticosterone samples were collected, mice were anesthetized deeply with isoflurane and rapidly decapitated. Adrenal glands were collected from each individual, and weighed to the nearest 0.1 mg (wet mass). Brains were quickly removed and stored in 5% acrolein in PBS at 4 °C overnight. The following morning, each brain was transferred for 24 h to 30% sucrose in saline, frozen on dry ice, and stored at −80 °C for immunocytochemistry.

2.5.2. CRF immunocytochemistry

Brains were sectioned at 40 μm on a cryostat and alternate free-floating sections were processed for CRF immunocytochemistry. Sections were washed three times in PBS then incubated in 1% sodium borohydride in PBS for 10 min. Sections were then rinsed in 20% normal goat serum and 0.3% hydrogen peroxide in PBS for 20 min. Sections were incubated in primary CRF antibody (1:20000, gift of Wylie Vale, Salk Institute) in 1% normal goat serum in 0.5% Trition-X PBS (PBS + TX) at 4 °C for 48 h. Next, sections were rinsed three times in PBS, and incubated for 90 min with biotinylated goat-anti-rabbit antibody (1:500, Vector Laboratories) in PBS + TX. The sections were then rinsed three times in PBS and then incubated for 30 min in avidin–biotin complex (ABC Elite kit, Vector Laboratories). After three rinses in PBS, the sections were developed in diamnobenzidine for 2 min. Sections were mounted, dehydrated and coverslipped with Permount. Sections containing the paraventricular nucleus (PVN) were identified using a mouse brain atlas (Franklin and Paxinos, 1997). Images were captured at the same time using a Nikon Eclipse microscope. Immunopositive cells were counted with the aid of Neurolucida software (Microbrightfield, Burlington, VT). Control sections in which primary antibodies were not added showed no specific staining.

2.5.3. Statistical analyses

Variance homogeneity and data normality were assessed using Levene’s tests and 1-sample Kolmogrov-Smirnov tests. In most cases, data were normal; some open-field data (i.e., number of beam breaks in each quadrant) were not, so non-parametric tests (Kruskal-Wallis) were used on these data as transformations were unsuccessful. Also, as tempera-
3. Results

3.1. Neophilia

3.1.1. Objects

Species differed in latency to contact \(F_{3,34} = 8.0, P < 0.001\) and time spent handling \(F_{3,34} = 4.4, P = 0.01\) a marble; number of marble contacts \(F_{3,34} = 2.4, P = 0.09\) did not differ among species. Post hoc tests revealed that \(P.\ eremicus\) spent less time interacting with the marble than the other three species (Fig. 1a). Species also varied in latency to contact \(F_{3,33} = 7.3, P = 0.001\) and time spent handling \(F_{3,33} = 8.1, P < 0.001\) a binder clip; number of contacts did not vary among species \(F_{3,33} = 1.3, P = 0.29\). Post hoc tests indicated that \(P.\ aztecus\) spent more time interacting with the binder clip than the other three species (Fig. 1b).

3.2. Environment

Latency to enter the novel chamber \(F_{3,35} = 7.4, P = 0.001\), number of transitions between chambers \(F_{3,35} = 3.0, P = 0.04\), and number of fecal boli \(F_{3,35} = 6.3, P = 0.002\) differed among species. Time spent on initial \(F_{3,33} = 1.3, P = 0.28\) and novel \(F_{3,35} = 1.35, P = 0.28\) sides of the box did not. Post hoc comparisons indicated that \(P.\ eremicus\) explored the novel environment more readily than the other three species (Fig. 1c).

3.2.2. Anxiety

3.2.2.1. Open field. Total activity \(F_{3,35} = 6.2, P = 0.002\) and number of rears \(F_{3,35} = 2.9, P = 0.05\) differed among species. Post hoc tests indicated that \(P.\ aztecus\) was less active than \(P.\ eremicus\) and \(P.\ melanophrys\) (Fig. 2a) and reared less than \(P.\ eremicus\) (Fig. 2b); no other pair-wise species differences were detected. Species also did not differ in number of beam breaks in the box center \(\chi^2 = 0.25, df = 3, P = 0.97\) or on the perimeter \(\chi^2 = 0.25, df = 3, P = 0.97\) once differences among species in terms of activity were taken into account.

3.3. Elevated plus

Number of entries into open arms \(F_{3,34} = 5.8, P = 0.003\) and closed arms \(F_{3,34} = 4.2, P = 0.01\), and number of fecal boli produced \(F_{3,34} = 7.2, P = 0.001\) varied among species. Number of entries into the center space did not \(F_{3,34} = 1.6, P = 0.21\), and no significant pair-wise differences were detected for these measures by post-hoc analyses. Time in open arms \(F_{3,34} = 9.9, P < 0.001\) and closed arms \(F_{3,34} = 10.3, P < 0.001\) differed among species, but

differences among species followed by sequential Mann–Whitney U tests to establish significant pair-wise differences. For the remaining data, univariate ANOVA models were used to compare behavioral and physiological variables among species, followed by Tukey–Kramer post hoc tests to identify significant pair-wise differences. Significance was indicated, where \(P < 0.05\).

3.4. Light–dark box

Latency to enter the dark side of the chamber \(F_{3,34} = 6.6, P = 0.001\), time spent in the darkened chamber \(F_{3,34} = 4.6, P = 0.009\), number of transitions \(F_{3,34} = 7.7, P = 0.001\), and number of fecal boli \(F_{3,34} = 13.8\) varied among species. \(P.\ eremicus\) was most apt of the four species to enter the darkened chamber (Fig. 2d).
3.4.1. Temperament

Both measures of wildness (handling: \( \chi^2 = 14.7, P = 0.002 \); catchability: \( \chi^2 = 21.4, P < 0.001 \)) varied among species (Fig. 3). Pair-wise comparisons indicated that *P. maniculatus* and *P. melanophrys* were the most difficult to catch. *P. maniculatus* was significantly more agitated when handled than the other three species.

3.4.2. HPA axis activity

Adrenal mass (divided by individual body size) varied among species (\( F_{3,34} = 19.3, P < 0.001 \)) with *P. eremicus* having the largest adrenals followed by *P. melanophrys* and *P. aztecus*; *P. maniculatus* had significantly smaller adrenals than all three other species (Fig. 4a). Baseline (\( F_{3,34} = 14.9, P < 0.001 \)) and stress-induced (\( F_{3,34} = 46.6, P < 0.001 \)) corticosterone concentrations differed among species (Fig. 4b); all species showed significant increases in corticosterone post-restraint (\( F_{1,31} = 79.2, P < 0.001 \)). The rate of increase in corticosterone, however, was marginally significantly different among species (\( F_{3,31} = 2.5, P = 0.08 \)). When the absolute change in corticosterone concentrations was compared, however, species exhibited significant variation (\( F_{3,34} = 10.7, P < 0.001 \)), but *P. maniculatus* and *P. aztecus* produced less corticosterone in response to restraint than the other two species (Fig. 4c). Although PVN size varied

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**Fig. 2.** Variation among *Peromyscus* in conventional behavioral tests: (a) open-field beam breaks, which reflects differences in total activity, (b) number of rears in the open field, which reflects differential exploratory activity, and two measures of anxiety, (c) time spent in the closed arms of an elevated plus maze, and (d) latency to enter a darkened chamber in a light–dark box. Bars represent means + 1SEM. Letters represent group membership by Tukey–Kramer post hoc tests.

**Fig. 3.** Variation in temperament in response to capture and handling in *Peromyscus* based on a modified scoring protocol for laboratory strains of mice (Wahlsten et al. 2003). Bars represent means + 1SEM. Letters represent group membership by Tukey–Kramer post hoc tests. Upper case letters depict significant differences in behavior in response to capture attempts; lower case letters depict significant differences in behavior in response to handling by researchers.
among species \((F_{3,27} = 3.0, P = 0.05)\), comparisons of both relative \((F_{3,27} = 19.2, P < 0.001)\) and absolute \((F_{3,27} = 21.4, P < 0.001)\) numbers of CRF-stained cells indicated differences among species. Density of CRF-positive cells in the PVN was fewest in \(P. eremicus\) followed by \(P. maniculatus\), \(P. melanophrys\), and finally \(P. aztecus\) (Fig. 4d).

4. Discussion

\textit{Peromyscus} species differed behaviorally and physiologically, but not in the predicted direction. \(P. maniculatus\), which was expected to be most neophilic, was no more interested in novel objects or a novel environment than the other three species. \(P. maniculatus\) also maintained the lowest basal corticosterone concentrations, produced the least corticosterone in response to restraint stress, and exhibited intermediate CRF positive immunoreactive (ir) staining in the PVN of the hypothalamus. \(P. melanophrys\) and \(P. aztecus\) were comparable in terms of response to novelty (except for the marble, in which \(P. aztecus\) exhibited the most interest of all species). Anxiety-like behaviors were also indistinguishable between these species, even though \(P. aztecus\) was less active than the others. In terms of temperament, \(P. aztecus\) and \(P. eremicus\) were comparably docile whereas \(P. melanophrys\) and \(P. maniculatus\) were agitated when capture was attempted. Among the four species, \(P. eremicus\) was most distinct behaviorally and physiologically. \(P. eremicus\) was most willing of all species to explore the novel arena but least anxious in the open field and elevated plus maze. \(P. eremicus\) also maintained the highest baseline corticosterone and stress-induced corticosterone concentrations and possessed the largest adrenal glands but the lowest CRF-ir cell density in the PVN.

Increased activation of the HPA axis is typically associated with increased avoidance of novel stimuli (Koolhaas et al., 1999). In contrast, we observed that across species, increased HPA activity was associated with increased exploratory behavior. The reduced number of CRH positive cells in the \(P. eremicus\) PVN could reflect reduced CRH expression or increased CRH release into the median eminence. These outcomes could be distinguished by measuring CRH prepropeptide mRNA or direct measurements of CRH in the median eminence. Furthermore, it might be useful to quantify other secretagogues of adrenocorticotrophin (ACTH), such as arginine-vasopressin (AVP). Regardless, the direction of the relationship between aspects of the HPA axis and neotic and anxiety-like behaviors in our study is opposite of what has typically been detected. The direction of the relationships among these species, however, has precedence. High baseline corticosterone concentra-
tions and large corticosterone increases post-stress were coupled with low anxiety-like behavior in prairie voles (*Microtus ochrogaster*), which is often related to high novelty-seeking behavior (Stowe et al., 2005). Meadow voles (*M. pennsylvanicus*), however, were more anxious but maintained lower circulating corticosterone and produced less corticosterone post-stress. The cited inconsistencies between HPA activity and neotic/anxiety behavior in interspecies intra-specific studies highlights the need for further research. Indeed, the paucity of comparative studies of neotic behavior makes generalizations about the hormonal basis (and ecological relevance) of neotic behavior in mammals difficult.

At present, methodological issues may explain as much of the outcome of the present study as stress hormones or geographic distribution. One of the most likely is the amenability of certain behavioral tests to different organisms. In the current study, the species expected to be most interested in novelty exhibited the greatest anxiety-like behavior and was least amenable to handling. Although a lack of support for our motivating hypothesis may be genuine, it may also be driven by the sensitivity of these species to captivity or conditions under which behaviors were assessed. In the wild, the willingness of individuals of *P. maniculatus* to interact with novel objects or explore novel places may be very different than the apparent indifference of novelty detected here. To circumvent this potential limitation, we used multiple tests to characterize behavioral variation, but distinguishing variation among species may yet have been missed. One study in mice, which measured 28 different variables concerning novelty behavior, detected a general continuum of neophilia to neophobia among individuals (Belzung and Lepape, 1994). However, correspondence among behavioral tests was not perfect. In much the same fashion, responsiveness of the HPA axis may be differentially sensitive (among species) to the conditions under which it is measured. HPA activity in our study was characterized in response to restraint stress weeks after all behavioral tests to prevent the stress of blood sampling from compromising later behavioral comparisons. In many previous studies, however, HPA activity was measured in response to novel stimuli. In the future, efforts should be made to account for potential, differential sensitivity to stressors among species when characterizing neotic behavior (Cavigelli and McClintock, 2003). It would also be valuable to consider aversion to predator-related stimuli, as high anxiety-like behaviors may represent differential tendencies to avoid novel predators in unfamiliar habitats.

A second methodological limitation of our study may be our choice of novel objects; more ethologically salient objects may have yielded different outcomes. All *Peromyscus* consume a variety of grains, seeds, fruits, and insects (King, 1968), but variation may exist in the willingness of species to incorporate new foods into their diets (e.g., neophobia), especially because some species live in resource-depauperate habitats where exposure to novel foods is likely to be rare (Mueller and Diamond, 2000). Variation in neophobia may also be influenced by differential diet specialization that may occur among species. Neophobia occurs in rodents; typically immature individuals are more reluctant to consume novel food than adults (Bolivar and Flaherty, 2004). Likewise, neophobia has been found to vary between populations of free-living animals occurring at the edge or center of an expanding range (Martin and Fitzgerald, 2005). Thus, we expect that *P. maniculatus* would be more likely to incorporate unfamiliar items into its diet than the other three species.

Studies in the primate genus, *Macaca*, have suggested that species differences in aggression may also facilitate expansion into or persistence in unfamiliar environments. Across seven species of primates extensive variability exists in aggressive behavior, which is coupled with allelic variation at two genetic loci (serotonin promoter and monoamine oxidase a). More aggressive species generally have greater allelic diversity at both loci whereas less aggressive species tended to be monomorphic (Wendland et al., 2006). It is unlikely that species differences in aggression can account for species differences in geographical distributions in *Peromyscus*, however. *P. californicus* and *P. polionotus* are more aggressive than *P. leucopus* and *P. maniculatus*, respectively (Bester-Merideth et al., 1999; Trainor and Nelson unpublished), yet *P. leucopus* and *P. maniculatus* have broader geographical distributions than *P. californicus* and *P. polionotus*.

In addition to differential neophobia and aggressiveness, study of other aspects of behavioral flexibility, such as problem-solving capacity and behavioral innovation rate, might also be informative. As with the present study, however, these comparisons might be difficult because amenable husbandry practices would have to be developed for each species. In many cases, exotic diets or especially high anxiety may preclude comparisons among important species (e.g., those with very restricted ranges). These limitations are what motivated the use of captive-bred *Peromyscus*, in spite of the limitations of the approach. Indeed, the phylogenetic position of each species (*P. eremicus* being a member of the subgenus, *Haplomyomys*) makes it impossible to determine to what extent complimentarity between HPA physiology and neotic-anxiety behavior is an artifact of evolutionary history. Further, as each of the four species has been maintained in captivity for a different number of generations, differential adjustment to the conditions of captivity may have occurred in such a way as to obscure variation that exists in free-living populations. *P. eremicus* is the most recently captured species, and exhibits the most distinct behavior and physiology. As above for phylogenetic position, this artifact partly obscures the meaning of the patterns detected. It is important to highlight, however, that had exclusively free-living mice been studied, other factors would limit interpretation (e.g., stress of captivity, diet, differential anxiety, experience during ontogeny, and environmental variation among habitats).

Subsequently, we feel that our approach will instigate fur-
ther work to understand the physiological and behavioral mediators of range expansion and maintenance among species. Indeed, a simple second study (i.e., comparisons of the same behavioral and physiological parameters in (i) females of the same species or (ii) males and/or females in single- versus group-housed conditions) would provide extensive insight into our motivating hypothesis (Choubaji et al., 2005).

5. Conclusion

Individuals within populations and populations within species vary in how they respond to novelty. This responsiveness appears orchestrated by the actions of the HPA axis, particularly increased glucocorticoid production. Within species, reduced glucocorticoids promote interest in novelty. Among species, however, the relationship may be more complicated. In other words, the inverse relationship between HPA activity and neophobic/behaviour detected within species in other studies was not observed here; as in some voles, the opposite pattern was found in Peromyscus. This outcome suggests that different species may modify different aspects of their HPA axis (i.e., ACTH secretagogue production, sensitivity of negative feedback processes, tissue-specific receptor distribution and number), which would hinder discovery of simple relationships between HPA activity and neotism and anxiety-like behavior.

In regards to our motivating hypothesis, there was little indication that behavioral or physiological variation complemented geographic distribution. Further study is critical to ascertain whether this outcome is genuine or also a consequence of methodology. Descriptive studies like this one reveal the difficulty of trying to link behavioral to physiological variation in ecologically relevant contexts, especially when multiple species are involved. One circumvention would be to compare populations within species (Martin et al., 2005). We expect that individual P. maniculatus on the edge of the species' range would be more receptive to novelty, more apt to include novel food items into their diets, and more willing to explore novel habitats than more centrally located populations. In light of the preponderance of neotic behavioral variation within species (Greenberg, 1990; Koolhaas et al., 1999) and the influence of behavioral plasticity on rates of evolutionary diversification (Miller, 1956) and invasiveness (Sol et al., 2002), the role of behavioral plasticity in determining species' distributions warrants further study.

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