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Assessing space use in meadow voles: the relationship to reproduction and the stress axis

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Voles are key mammals in understanding how social interactions can affect large-scale population processes. Previous studies have shown that at high population densities, meadow voles (*Microtus pennsylvanicus*) have a lower proportion of breeding animals, higher average corticosterone levels, and can be limited by female territorial spacing. Based on this, we compared corticosterone levels and spatial use between breeding and nonbreeding free-ranging adult meadow voles within populations. We measured intrasexual spatial overlap to examine if breeding females minimize occupying the same areas as other females, and noninvasively assessed corticosterone levels using fecal corticosterone metabolites (FCMs). We found that female meadow voles have much lower intrasexual spatial overlap than males, even though both sexes have similar range sizes, and that females have generally higher FCM levels than males. However, breeding and nonbreeding females did not differ from one another in spatial use or in FCM levels. Conversely, reproductive classes of males differed greatly in all measures: nonbreeding males had FCM levels that were two times higher than those of breeding males, occupied a smaller range, and had lower spatial overlap, indicating they were moving less widely than breeding males. We additionally validated an enzyme immunoassay for noninvasively measuring FCMs in meadow voles. The assay was successful in detecting an increase in corticosterone stimulated by adrenocorticotropic hormone injection; however, dexamethasone did not induce negative feedback. FCMs reflect circulating corticosterone levels approximately 5 h prior. These results highlight differences in FCMs and spacing in meadow voles related to sex and reproductive status, and reflect the respective strategies males and females employ during the breeding season.

Key words: assay validation, glucocorticoids, microtine rodent, sex differences, social stress, space use

Arvicoline rodents (voles and lemmings) are a well-studied clade in ecological research over the past century. Their study has made fundamental contributions to our understanding of population regulation and limitation in mammals, predator–prey interactions, and ecosystem processes (Elton 1942; Tamarin 1985; Krebs 2013; Boonstra et al. 2016). While a variety of factors are known to affect arvicoline population dynamics, such as predation (e.g., Ims and Andreassen 2000; Korpimäki et al. 2002) and food supply (e.g., Huitu et al. 2003; Johnsen et al. 2017), populations can still decline in the absence of changes in these limiting factors (Chitty 1960; Marcstrom et al. 1988; Maron et al. 2010). Thus, intrinsic mechanisms such as social interactions are a factor that can influence population dynamics (reviewed in Boonstra et al. 1998). For this reason, it is imperative to understand social competition in voles and the stress-axis changes it can induce.

A primary factor driving social competition in voles and lemmings is female spacing behavior. The spatial distribution of female animals in the environment is a major determinant of a species' social system and breeding density (Emlen and Oring 1977). This is because the spatial distribution of females can determine how energetically costly it is for males to attempt to monopolize them, and this in turn affects how many males will be in the area per female. In meadow voles (*Microtus pennsylvanicus*), individuals breed in a promiscuous mating system throughout the spring and summer (Boonstra et al. 1993). While meadow voles may nest communally and are socially tolerant during the fall and winter nonbreeding periods, by early May,

females create exclusive territories where they nest solitarily (Madison and McShea 1987). During this time, population density and distribution of meadow voles are determined by female territoriality: females compete for exclusive space, and then density of males is dependent on the number of females in the area (Boonstra and Rodd 1983). Studies using radiotelemetry to track free-living meadow voles have demonstrated that breeding females create discrete territories with minimal overlap with those of other females (Madison 1980; Ostfeld et al. 1988). It is likely that, during reproduction, they need exclusive space for nesting and raising offspring (Boonstra and Rodd 1983), for food resources to meet the high energetic demands of reproduction relative to the low-quality vegetation they consume (Ostfeld 1985), and possibly to prevent infanticide (Wolff and Peterson 1998). Low overlap among territories of females is consequentially associated with better offspring survival in meadow voles and in other polygamous voles (Ostfeld et al. 1988; Jonsson et al. 2002). Males, conversely, have larger ranges that extensively overlap with the ranges of other males (Ostfeld et al. 1988). Male meadow voles do not engage in paternal care (McGuire and Novak 1984), hence it is advantageous for them to attempt to mate with multiple females. Females will also mate with multiple males, their litters can exhibit multiple paternities, and they have rapid postpartum estrus where they will breed again while nursing an old litter (Boonstra et al. 1993). As such, breeding males will overlap most in areas around an estrus female (Madison 1980).

Importantly, social interactions related to spacing behavior can alter the physiology of an animal in terms of measures of hormonal stress (glucocorticoids; cortisol or corticosterone depending on the species). In the majority of territorial species, as population density increases, glucocorticoid levels increase as well (Creel et al. 2013); but, the interaction among individual fitness, changes in stress hormones, and population dynamics is poorly understood. In voles specifically, increases in population density are associated with an increase in blood corticosterone levels at a population level (Boonstra and Boag 1992; Novikov and Moshkin 1998; Charbonnel et al. 2008; Bian et al. 2015). However, it is unknown whether this is due to higher stress levels in breeding females defending territories, higher stress in smaller, nonbreeding adults potentially on the receiving end of such aggression, or general ubiquitous resource competition in the population.

We compared circulating stress hormones and spatial use between breeding and nonbreeding adult meadow voles of each sex. We also tested if breeding females are more successful than nonbreeding females in excluding other females from their territory, and examined how general spatial use and intrasexual territorial overlap changes between breeding and nonbreeding males. We predicted that breeding females should have lower spatial overlap than nonbreeding adult females, as they should be more successful at monopolizing space. Breeding males should have higher spatial overlap than nonbreeding males, as they should invest energy ranging more widely on the grids and surrounding estrus females. Further, we expected that these groups with higher overlap (nonbreeding females, breeding males) should have higher stress hormone levels, as they are likely coming into agonistic contact with congeners more frequently.

In order to use fecal corticosterone metabolites (FCMs) as a noninvasive measure of circulating corticosterone levels, we validated an enzyme immunoassay (EIA) for meadow voles. Circulating corticosterone is metabolized by the liver and excreted in the feces, but the hormone metabolites present and the time it takes to excrete them vary by species (Touma and Palme 2005). Hence, when using a particular EIA for the first time in a species, it is important to verify that the antibody is able to detect an increase in FCMs. To this end, in a second experiment, we used a hormone challenge with adrenocorticotropic hormone (ACTH injection) to stimulate the stress-axis response of voles and dexamethasone (Dex injection) to suppress it via negative feedback, and validate that we can detect this change in the feces. Further, female meadow voles have higher circulating corticosterone levels than males (Boonstra and Boag 1992), and so we expect that they should have higher FCM levels in the feces as well.

Materials and Methods

Validation study.—To validate the assay for FCMs, we captured 12 voles on 13 October 2016 at Koffler Scientific Reserve (KSR) in King City, Ontario, Canada, and brought them into the laboratory at the University of Toronto, Scarborough. Each animal was individually housed in a radiometabolism cage: a 91.5 × 61 × 46 cm polypropylene cage with a metal mesh floor that allows urine and feces to pass through into a collecting tray below. Animals were provided with cotton nesting material, ad lib water, apple slices, oats, and rabbit chow (LabDiet, St. Louis, Missouri; 14.5% protein, 22.6% crude fiber, 2.8% fat). Voles were maintained at a temperature of 15–20°C and a natural photoperiod. Animals ranged from 16 to 29 g and all were nonbreeding (four females, eight males). These animals were not part of the general field study. All validation procedures were approved under University of Toronto animal care protocol #20011748 and followed guidelines approved by the American Society of Mammalogists (Sikes et al. 2016).

For the first 3 days of captivity, animals were allowed to habituate to captive conditions. Fecal samples were collected from the trays under cages using sterile forceps every 4 h from 0600 to 1800 h daily. This provided baseline levels associated with the stress of capture, habituation to the lab, and evidence of circadian patterns in FCM levels. On 17 October, all voles were injected with 44 kBq radiolabeled ^{3}H-corticosterone (1,2,6,7-^{3}H-corticosterone, PerkinElmer Health Sciences Canada, Inc., Woodbridge, Ontario, Canada; specific activity: 2.8–3.9 TBq/mmol) between 0700 and 0730 h. The first fecal sample was collected at 1000 h, and then every 2 h until 1800 h. On the following day, samples were collected every 4 h from 0600 to 1800 h. Aliquots of these fecal sample extracts were analyzed with a scintillation counter to measure radioactivity. One animal excreted very little radioactivity, so it was presumed this injection failed, and the animal was excluded from this portion of the analysis.

On 19 October, six voles (three females, three males) were injected with 0.25 mg/kg of synthetic ACTH (Cortrosyn, Amphastar Pharmaceuticals Inc., Rancho Cucamonga, California) to stimulate corticosterone secretion and examine the lag time between a stressor in the blood and its signature in the feces. On the same date, the remaining six voles (one female, five males) were injected with 250 mg/kg synthetic glucocorticoid Dex (Vétoquinol, Lavaltrie, Quebec, Canada) to induce negative feedback, depress corticosterone secretion, and assess whether this was reflected in the fecal signature. Following the injections (given at 0700 h), fecal samples were collected every 2 h that day until 1800 h, and then every 4 h on the next day, from 0600 to 1800 h.

Field study.—We studied meadow voles living in seminatural field enclosures at KSR over the course of two summers (May–August 2016 and May–August 2017, 8 months total, during the breeding period for this species). Each enclosure was a fenced 25 × 25 m area surrounded by metal hardware cloth extending 0.6 m above and 0.6 m below ground and capped with aluminum to prevent climbing. The outer perimeter was surrounded by additional Vexar plastic fencing to a height of 1.5 m and an electric fence to prevent predators from entering the grids. All enclosures contained a 5 × 5 grid of Longworth live traps spaced 5 m apart (Fig. 1). Enclosures with higher densities ($n = 4$ enclosures, starting density of over 20 adult animals/625 m^2) contained two traps at each grid point (50 traps), whereas enclosures with lower densities ($n = 6$ enclosures, starting density of six or less adult animals/625 m^2) contained one trap at each grid point (25 traps). Trapping took place on a weekly basis from May through August, both years. The general population demography of voles in southern Ontario is currently undocumented, and thus we could not assess the phase of the population cycle from which the founder animals were collected. However, intensive studies in the past have found population cycles in this region (Boonstra 1985). Live traps were baited with oats and contained cotton nesting material. Traps were set at 0400 h and checked at 0800 h. When captured, voles were tagged in the ear with an identifying fingerling fish tag (S. Roestenburg, Riverton, Utah) and the following data were collected: mass (Pesola scales, ± 1 g), breeding condition, and grid location. Breeding condition of males was determined by testes position (scrotal or abdominal) and mass; male *M. pennsylvanicus* produce viable sperm at a median mass of 30 g, when the tubules of the cauda epididymis become defined (Keller and Krebs 1970). Breeding condition of females was determined by vaginal perforation, separation of the pubic symphysis, and lactation. We excluded juvenile animals from analysis, which we considered to be animals < 20 g, a classification similar to that used in prior studies of this species (Boonstra and Rodd 1983).

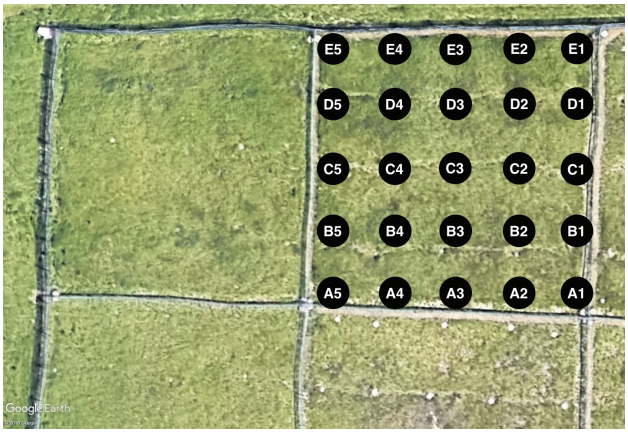

Fig. 1.—Trapping grid layout on a photograph of a study enclosures at Koffler Scientific Reserve, Ontario, Canada.

Fecal samples were collected from the tunnels of live traps six times throughout the summer in 2016 and five times throughout the summer in 2017. On days of fecal sample collection, traps were set at 0600 h and checked at 0800 h to minimize the time voles were confined in traps so that fecal samples would not reflect trapping stress. Fecal samples were collected in 0.5 ml vials and stored temporarily in a cooler with ice packs, then moved to a −20°C freezer at the University of Toronto, Scarborough. All procedures were approved under University of Toronto animal care protocol #20011477 and followed guidelines of the American Society of Mammalogists (Sikes et al. 2016).

Measurement of hormone metabolites.—All fecal samples were freeze-dried using a lyophilizer (Labconco Corp., Kansas City, Missouri) and then homogenized by crushing with a mortar and pestle. Samples were then extracted at a ratio of 0.05 g feces per 1 ml of 80% methanol (Palme et al. 2013). The mixture was vortexed for 30 min at 1,450 rpm and then centrifuged for 15 min at 2,500 g to separate fecal material from the supernatant. Extracts were diluted 1:100 in assay buffer and assayed using a previously described 5α-pregnane-3β,11β,21-triol-20-one EIA (Touma et al. 2004). This EIA has been validated for a variety of rodent species including domestic mice, *Mus musculus* (Touma et al. 2004), laboratory rats, *Rattus norvegicus* (Lepschy et al. 2007), Columbian ground squirrels, *Urocitellus columbianus* (Bosson et al. 2009), North American red squirrels, *Tamiasciurus hudsonicus* (Dantzer et al. 2010), bank voles, *Myodes glareolus* (Sipari et al. 2017), and brown lemmings, *Lemmus trimucronatus* (Fauteux et al. 2017). The inter-assay coefficient of variation (*CV*) for high and low pool sample extracts were 8% and 15%, respectively ($n = 12$ plates) and the average intra-assay *CV* was 13%.

Data analysis.—In the validation study, we examined how treatment (ACTH versus baseline and Dex versus baseline) and time affected FCM levels using linear mixed effect models. Animal ID was included as a random effect, and treatment, time point, and sex were included as fixed effects. All animals were reproductively quiescent, so no reproductive status was included. Sex and time point were not significant in the either of the models and were removed, leaving just animal ID and treatment. We tested if there was a difference in FCM levels by day over the first 3 days of captivity (habituation period) with a linear mixed effects model. Animal ID was included as a random effect, and day was the fixed effect. We also tested the

effect of time of day (circadian rhythm) on FCM levels using a linear mixed effects model where time was the fixed factor and animal ID and date were random factors.

For the field study FCM data, out of the total sample of adult animals where fecal samples were collected ($n = 71$), the majority of animals provided only a single sample throughout the summer. However, a few ($n = 14$) provided multiple fecal samples collected on different dates (e.g., a sample was collected from the same animal once in June and once in August). In this case, one sample from each animal was randomly selected, as the data could not be generally treated as true repeated measures, and averaging repeated samples for an individual would be inappropriate since breeding status or conditions on the same grid could differ between time points. Breeding status was assigned as status on the day the fecal sample was collected.

We calculated two measures of spatial use using the locations where animals were captured on the trapping grids (Fig. 1). Each summer (2016 and 2017), we trapped 2 days per week for 16 weeks. Intrasexual overlap ("overlap score") was calculated among voles, excluding juveniles (< 20 g), of the same sex located within the same grid (i.e., potential competitors). To calculate overlap score, a string of coordinates for each vole was produced, representing a history of where the voles had been captured over the experimental period. Each vole's trapping history was then compared to all others in its same-sex, same-grid cohort and any instances of shared coordinates were added up to form the overlap score. Hence, the overlap was the number of unique locations shared with other unique voles in the cohort. A single vole could score several overlaps with another through sharing multiple different locations; however, two voles that were repeatedly trapped in the same location would only result in a single overlap for each. Furthermore, a single vole could score multiple overlaps from being trapped in a single location if multiple other voles had also been trapped there. The overlap score was cumulative throughout the experimental period, so it was intended to reflect a general measure of an animal's ability to exclude others from its range, as opposed to a weekly measure of territorial spacing. The second spatial measure was total vole range ("range score"), and was simply the number of unique trapping locations an individual vole had been caught at, out of a maximum of 25 trapping locations per grid. Any animals in this sample that had lost eartags or were able to escape to a new grid were omitted ($n = 5$).

Fecal corticosterone metabolite concentrations, range score, and body mass were each compared between sexes and among breeding groups (breeding females, nonbreeding females, breeding males, and nonbreeding males) using a one-way ANOVA. Differences between specific groups were then examined with a post hoc Tukey HSD test. FCM data and range score data were log transformed to meet assumptions of homogeneity of variance and normality. Overlap score data could not be transformed to normality and so a nonparametric Kruskal–Wallis test was used with a post hoc Dunn's test. The relationship between population density in enclosures (minimum number alive per 625 m^2) and overlap score was examined using the Kendall rank correlation test. Data were analyzed in R (version 3.4.3—R Core Team 2017). Linear models were built using the packages nmle (Pinheiro et al. 2018) and lme4 (Bates et al. 2015). Figures were created in R and Prism (V 4.0, 2003; Graphpad Software, La Jolla, California).

Results

Validation study.—At the onset of the validation study, animals were given 3 days to habituate to captivity and feces were collected throughout that time. On the second day of captivity, FCM levels were still slightly higher (0.09 ± 0.052 log ng/g FCM) relative to the first day ($t_{59} = 1.79$, $P = 0.08$), but on the third day of captivity, average FCM levels had dropped (−0.13 ± 0.047 log ng/g FCM, $t_{59} = -2.84$, $P < 0.01$), and thus FCM levels on the third day were considered "baseline" (Fig. 2). We found no evidence of circadian rhythm during the habituation period of the study, as the effect of time was insignificant ($F_{3,60.9} = 0.988$, $P = 0.40$; Fig. 2). When compared with baseline FCM excretion, the ACTH challenge significantly increased FCM levels, with baseline levels being −0.11 ± 0.05 log ng/g FCM lower than ACTH stimulated levels ($t_{46} = -2.43$, $P < 0.05$). Dex had no effect, and FCM levels following Dex suppression were nearly identical to baseline, and the relationship was in the opposite direction than expected, with Dex levels being 0.09 ± 0.05 log ng/g FCM higher than baseline ($t_{42} = 1.74$, $P = 0.09$; Fig. 3). As females have higher corticosterone levels than males, and it is possible that they may be less sensitive to Dex at this concentration than males, we then removed the female ($n = 1$) from the Dex versus baseline comparison. When comparing males only, the Dex treatment still had no effect ($t_{27} = 1.88$, $P = 0.071$) and the relationship was still in the opposite direction than expected (Dex was higher than baseline). For excretion time following ^{3}H-corticosterone injection, animals typically peaked at 5 or 7 h post-injection, with the group mean corticosterone excretion peak at 5 h post-injection (Fig. 4).

Field study.—Fecal corticosterone metabolite concentration differed among sex and reproductive classes ($F_{3,67} = 29.4$, $P < 0.001$; Fig. 5). Breeding females did not differ from nonbreeding females ($P = 0.95$), but had higher FCM levels than both groups of males ($P < 0.001$ for breeding males and $P < 0.01$

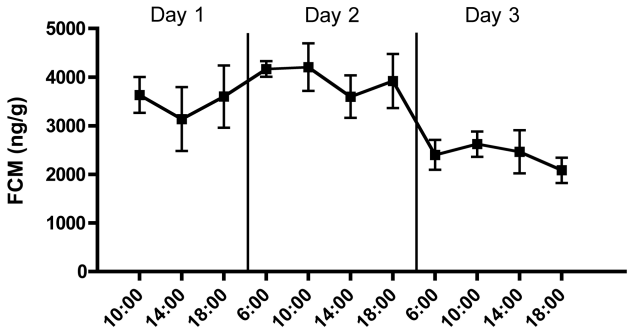

Fig. 2.—Mean fecal corticosterone metabolite (FCM) levels (± *SE*) of all meadow voles (*Microtus pennsylvanicus*) in the validation study during the first 3 days of captivity (the habituation period).

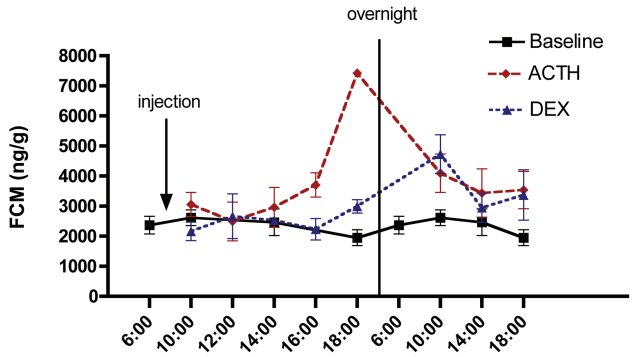

Fig. 3.—Mean fecal corticosterone metabolite (FCM) levels (± *SE*) of meadow voles (*Microtus pennsylvanicus*) after injection with adrenocorticotropic hormone (ACTH) or dexamethasone (Dex) relative to baseline FCM excretion.

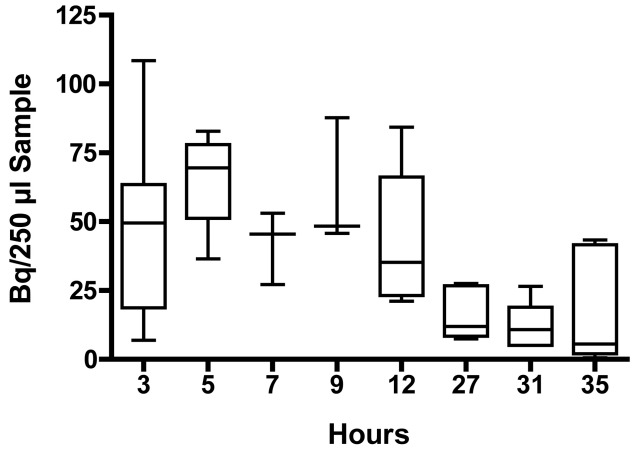

Fig. 4.—Excreted radioactivity in feces of meadow voles (*Microtus pennsylvanicus*) injected with radiolabeled ^{3}H-corticosterone. Times are hours post-injection.

for nonbreeding males). Nonbreeding males had higher FCM levels than breeding males ($P < 0.001$).

There were large differences among groups in spatial use as well. Overlap score differed among groups ($\chi^2_3 = 19.9$, $P < 0.001$; Fig. 5). Breeding males had the highest overlap scores, approximately twice those of nonbreeding males ($P = 0.05$) and approximately six times that of both groups of females ($P < 0.001$). Breeding females, nonbreeding females, and nonbreeding males did not differ from one another. Population density (minimum number alive per 625 m^2) in this study was not correlated with overlap among males ($z = 0.67$, $P = 0.50$) nor with overlap among females ($z = 1.74$, $P = 0.08$). The marginally significant relationship for females was driven by a single observation that was 4.5 *SD*s above the mean. Once that observation was removed, there was no relationship between density and overlap ($z = 1.39$, $P = 0.16$).

Range score also differed among groups ($F_{3,62} = 7.8$, $P < 0.001$; Fig. 5), but only in that range scores of nonbreeding males were less than one-half those of breeding males and breeding females ($P < 0.001$). Breeding females, nonbreeding females, and breeding males were similar in range scores.

Body mass differed among sex and breeding groups ($F_{3,67} = 16.8$, $P < 0.001$; Fig. 5). Post hoc testing showed that breeding males were heavier than nonbreeding males ($P < 0.001$), with an average body mass that was 15 g more than the nonbreeding males. Breeding females had an average body mass that was 8 g heavier than nonbreeding females ($P < 0.01$). Nonbreeding males and nonbreeding females did not differ in mass ($P = 0.11$). Thus, breeding and nonbreeding females only differed from one another in body mass, not FMC levels or space use. However, relative to males, females in general had higher FCM levels, lower intrasexual overlap, and similar ranges.

Discussion

Our results reiterate previous findings of sex differences in spatial use between male and female meadow voles. We additionally found differences in spatial use between breeding and nonbreeding male meadow voles during the breeding season. We found that breeding males ranged more widely on the grids and overlapped with other males more often than nonbreeding males did, which was expected. Breeding and nonbreeding females did not differ in spatial use, which was contrary to our predictions that breeding females should have lower overlap. Further, we compared FCM levels between voles of different reproductive condition and found that breeding males had lower FCM levels than nonbreeders, but that breeding females did not differ from nonbreeding females. We also determined that the 5α-pregnane-3β,11β,21-triol-20-one EIA was capable of detecting stimulated increases in corticosterone levels in meadow voles.

Validation study.—Our stimulation of the stress axis by an ACTH challenge successfully demonstrated that the assay was capable of detecting increases in corticosterone levels. However, Dex injection did not lower corticosterone levels. This is not necessarily a failure of the assay; Dex resistance has been previously found in congener *Microtus ochrogaster* (Taymans et al. 1997). *Microtus pennsylvanicus* and *M. ochrogaster* have higher circulating corticosterone levels than mice and rats (Taymans et al. 1997; Pyter et al. 2005), and so *Microtus* may have tissues that are more resistant to negative feedback with this dosage of Dex. Hence, a higher dose of Dex may be required for future studies in *Microtus*. There may be underlying sex differences in Dex sensitivity that we were not able to assess with the sample size in this study. Interpreting where meadow voles peak in corticosterone excretion following the ACTH challenge is difficult, as sample size was low throughout. Six animals were injected with ACTH; however, at each 2-h collection time point, it was often the case that several of the animals did not defecate enough for a sufficient fecal sample to be collected, particularly later in the day ($n = 1$ at the 1800 h collection point). Thus, in future studies with such small animals and frequent collection of feces, it is advisable to increase sample size.

Excretion of ^{3}H-corticosterone may provide a better indication of how quickly circulating corticosterone is reflected in the feces. Although there was high variation in radioactive

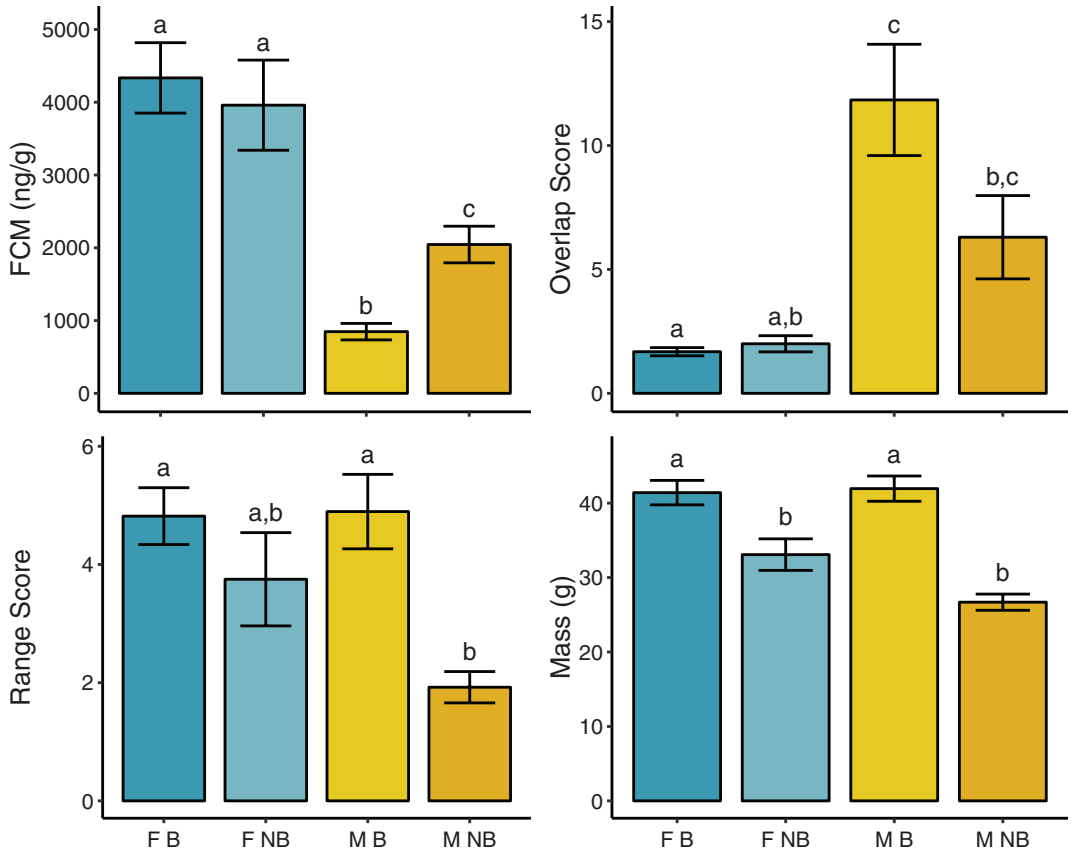

Fig. 5.—Comparisons of fecal corticosterone metabolite (FCM) levels, spacing behavior, and body size among the four groups of meadow voles (*Microtus pennsylvanicus*): breeding females ("F B," $n = 22$), nonbreeding females ("F NB," $n = 13$), breeding males ("M B," $n = 20$), and nonbreeding males ("M NB," $n = 16$). Bars linked by the same letter do not statistically differ from one another.

excretion, and occasionally low sample size ($n = 3$ for 7 and 9 h post-injection), the group mean ^{3}H-corticosterone peak was 5 h post-injection. This is consistent with findings from a highly similar study conducted in bank voles, where the peak excretion of radiolabeled corticosterone in the feces was 6 h post-injection (Sipari et al. 2017). The passage time from circulating corticosterone to peak ^{3}H-corticosterone in the feces for these voles was slightly faster than that of Columbian ground squirrels (7 h—Bosson et al. 2009) and North American red squirrels (10 h—Dantzer et al. 2010) in similar studies. In another arvicoline rodent, the brown lemming, peak FCM levels occurred 4 h after a capture and handling stressor (Fauteux et al. 2017). However, the more rapid excretion of FCMs may be due to the nature of the stressor; lemmings were pursued to their burrows in the wild and hand-captured upon exit, which is likely a more severe stressor than a quick injection in animals habituated to the laboratory.

Spatial use.—Females had much lower intrasexual overlap scores than males. Since range scores did not differ between females and breeding males, this difference was not due to females moving less on the grids, but rather due to sex differences in spacing behavior. The sex differences in our overlap scores are consistent with what is known about meadow vole social organization. The territorial spacing of this species is determined by females, which compete for space for nesting and rearing young, while the males base their movements on the presence of females (Boonstra and Rodd 1983). Radiotelemetry studies have demonstrated that female meadow voles will form discrete, nonoverlapping territories, while male meadow voles will overlap each other extensively, in particular around estrus females (Madison 1980; Ostfeld et al. 1988). Thus, the higher overlap among males was expected, especially in breeding males, which should be most driven to surround females.

Contrary to our predictions, intrasexual overlap score was the same between breeding and nonbreeding females. This was surprising, as increased territorial overlap is associated with poorer offspring survival (Ostfeld et al. 1988; Jonsson et al. 2002), and so it may be advantageous for females that are unable to exclude other females to temporarily suppress reproduction. Yet, the nonbreeding adult females in this study also had low overlap scores. These nonbreeding females were also significantly smaller than breeding females, but even so, were able to engage in the same kind of spacing behavior. Population density did not affect overlap score of males or females. In populations with higher densities (over 20 adult animals/625 m^2), females were still able to maintain low spatial overlap. Even as density increased, females maintained exclusive territories, likely because this behavior is related to the survival of their offspring and thus would be under strong selection. It may be that density in this study was never high enough to force extensive overlap between females; however, population densities in these

enclosures were based on actual densities of free-ranging meadow voles previously studied in southern Ontario (Rodd and Boonstra 1984), so densities higher than these may not be realistic.

Like all estimates with livetrapping data, these overlap and range scores are influenced by an animal's trappability and the number of times an area is trapped. However, when there are multiple trappings, as in this case, using this calculation of territorial overlap appears to be a valid estimate that aligns with results from other methodologies. Yet it may be that with the available data, the overlap score did not reflect subtle differences in spatial use that could be picked up by precise methods such as radiotelemetry and focal follows. With other methods, it is possible that subtler differences in spatial use would emerge between females of different breeding statuses.

Reproductive state and FCM.—There were clear differences in FCM levels between the breeding and nonbreeding males, but it is unclear whether these differences determine whether an animal will become reproductive, or if the animal becomes reproductive and this induces changes in their FCM levels and behavior. In the latter case, it is possible that male meadow voles become reproductive for another reason (e.g., reach a critical mass) and then behavioral and hormonal differences follow. Males that have become reproductive may be driven to move more extensively on the grids and seek out receptive females. Testosterone has widespread physiological effects, and can suppress glucocorticoid production in some cases (Rubinow et al. 2005), so it may be that reduced FCM levels in breeding males are a secondary effect of high testosterone. However, there are many counterexamples in nature where breeding males or socially dominant males have both elevated testosterone and elevated glucocorticoid levels simultaneously (e.g., Boonstra 2005; Czoty et al. 2009; Schoof and Jack 2013; Boonstra et al. 2017), so this relationship is not strongly supported.

In the former case, it is possible that the nonbreeding male meadow voles were social subordinates. They were smaller in size, and had smaller ranges and territorial overlap, indicating that the larger breeding males may have been excluding them from access to estrus females. Reproductive suppression could be induced by the stress of social subordination, as elevated glucocorticoids are known to inhibit reproduction in iteroparous species (Wingfield and Sapolsky 2003; Boonstra 2005). These nonbreeding males may have delayed reproduction until they are large enough to compete with the other males. If the differences in FCMs between groups of males were related to social competition, this would imply that the costs of competition for the breeding males were not particularly severe, as they had the lowest FCM levels of all groups. In social systems where dominance is maintained by frequent physical aggression and challenges, dominant individuals tend to have the highest indices of stress. Conversely, in systems where status is maintained by cues rather than physical combat, subordinates tend to have the highest indices of stress (Sapolsky 2005). Thus, breeding male meadow voles do not appear to be engaging in costly aggressive acts to assert dominance or access females. This is unlike many social systems where dominance or competition between breeding males is costly and results in elevated glucocorticoid levels relative to subordinate or nonbreeding animals (Creel 2001; Boonstra 2005).

Females are more difficult to compare between breeding and nonbreeding adults in terms of FCM levels. Pregnancy naturally raises glucocorticoid levels in the majority of mammals, not necessarily as a result of stress but for developmental and energetic reasons (Edwards and Boonstra 2018). In *M. pennsylvanicus* specifically, pregnancy and lactation raise blood corticosterone levels (Boonstra and Boag 1992). In the breeding females in this study, many were likely pregnant, even if they were not in obvious late pregnancy, as females can breed nearly continuously and exhibit postpartum estrus. That nonbreeding females had FCM levels that were equally high as breeding females here is not necessarily a good indication that the nonbreeding females were not stressed, as the group they were being compared to is not a baseline. In terms of examining differences in social dominance through spatial use, there were no differences between breeding females and the nonbreeding females. This implies that the nonbreeding females were not necessarily social subordinates or unsuccessful at excluding other females from their territory. Further, reproductive status of females did not drive marked changes in spatial use.

When comparing FCM levels and spatial use between breeding and nonbreeding meadow voles of both sexes, males showed distinct differences between reproductive states. It is unclear whether these differences were driven by reproductive state itself, or if there were inherent differences in adult animals that allowed them to become reproductive while others were suppressed until a later time. We found no differences in breeding and nonbreeding female voles in terms of FCM levels, intrasexual overlap, and range, demonstrating that these factors did not seem to differ as a result of breeding status, nor can they predict if an animal is able to be reproductive. However, there were clear differences between females and males in both FCM levels and spatial use, consistent with findings in earlier studies.

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