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Effects of population density on corticosterone levels of prairie voles in the field

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ABSTRACT

High population density is often associated with increased levels of stress-related hormones, such as corticosterone (CORT). Prairie voles (*Microtus ochrogaster*) are a socially monogamous species known for their large population density fluctuations in the wild. Although CORT influences the social behavior of prairie voles in the lab, the effect of population density on CORT has not previously been quantified in this species in the field. We validated a non-invasive hormone assay for measuring CORT metabolites in prairie vole feces. We then used semi-natural enclosures to experimentally manipulate population density, and measured density effects on male space use and fecal CORT levels. Our enclosures generated patterns of space use and social interaction that were consistent with previous prairie vole field studies. Contrary to the positive relationship between CORT and density typical of other taxa, we found that lower population densities (80 animals/ha) produced higher fecal CORT than higher densities (240/ha). Combined with prior work in the lab and field, the data suggest that high prairie vole population densities indicate favorable environments, perhaps through reduced predation risk. Lastly, we found that field animals had lower fecal CORT levels than laboratory-living animals. The data emphasize the usefulness of prairie voles as models for integrating ecological, evolutionary, and mechanistic questions in social behavior.

1. Introduction

Glucocorticoids function to mobilize resources and channel them to meet environmental demands, ranging from daily activity rhythms to responses to threats and stressors (Sapolsky, 2002). Many natural stressors are profoundly influenced by social factors: conspecifics may compete for territories, commit infanticide, or fuel the growth of predator populations; they may also facilitate food finding, share parental care, or satiate predators with their abundance (Adkins-Regan, 2005; Becker et al., 2002; Solomon, 1949; Wolff and Sherman, 2007). Hence, the social environment has dynamic and complex consequences for individual fitness. It seems fitting that much current attention is focused on the intersection of stress steroids, environmental variability, and social behavior (Creel et al., 2013). In the current study, we test the hypothesis that high population densities promote increases in agonistic encounters and elevate glucocorticoids in the socially monogamous prairie vole, *Microtus ochrogaster*.

Population density cycles are a well-investigated dimension of environmental variation. With respect to glucocorticoids, historical perspectives emphasize how density-associated predation or resource depletion can promote stress and inhibit reproduction, leading to a reduction in population growth (Christian, 1950; Wolff and Sherman, 2007). More recently, researchers have examined how predator abundance or other density-related stressors influence the developing phenotypes of young, causing cycles in stress reactivity that can alter ecological processes as offspring become adults (Breuner, 2008; Love et al., 2013). While population cycles have been examined extensively, their interactions with stress and social behavior are still not fully understood (Creel et al., 2013), and the subject remains a remarkably fruitful area of work.

Because glucocorticoids mobilize resources to deal with unfavorable conditions and limit reproduction, the effects of population density on glucocorticoids often mirror how density contributes to population growth (Christian, 1950; Creel et al., 2013). In many

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taxa, high population density depletes resources, drives agonistic interactions, and/or promotes predation, a set of stressors that also promote glucocorticoid secretion and impair reproduction (Boonstra and Boag, 1992; Christian, 1950; Creel et al., 2013). Perhaps not surprisingly, high density is often associated with high glucocorticoid secretion (Boonstra and Boag, 1992; Christian, 1961; Creel et al., 2013; Wang et al., 2009). Alternatively, environments may actually improve with density (Allee, 1931). For example, higher densities can lower the per-capita risk of predation (Parrish and Edelstein-Keshet, 1999), and facilitate mate-finding (Crowley et al., 1991; Kokko and Rankin, 2006). When the social system includes complex social groups with dominance hierarchies, the relationship between glucocorticoids and population density becomes still more complicated (Creel et al., 2013; Sapolsky, 2005; Wingfield and Sapolsky, 2003), with glucocorticoids affecting individuals unequally depending on dominance status and the stability of social hierarchies.

To investigate the relationship between population density and glucocorticoids, we study the socially monogamous prairie vole. In the wild, most male and female prairie voles form enduring pair-bonds and participate in the rearing of young (Getz and Carter, 1996). Studies spanning decades reveal that their population density undergoes wide annual fluctuations (Getz et al., 1993, 2006, 1990), with heavy spring-summer mortality and low densities (as low as 11 animals/hectare; Getz et al., 1993), contrasted with autumn–winter population spikes (as high as 600 animals/hectare; Getz et al., 1993). The time from conception to adulthood is approximately 9 weeks (Mateo et al., 1994; Stehn and Richmond, 1975); depending on the time of year young are born, offspring may grow to live in similar densities or wildly different densities, providing an interesting challenge to the development of the young. The combination of this well-characterized natural history and the extensive use of prairie voles as laboratory subjects in social neuroscience (Carter et al., 1995; Winslow et al., 1993; Young et al., 1999; Young and Wang, 2004) make them an especially valuable model for the integrative study of stress, sociality, and ecology.

The principal glucocorticoid secreted by prairie voles is corticosterone (CORT). In the lab, acute stressors and exogenous CORT facilitate social bonding in males (Blondel Thesis 2013; DeVries et al., 1996), but does not influence bonding in females (DeVries et al., 1996). Interestingly, stressed males also exhibit increased levels of paternal care (Bales et al., 2006). In several species, parental care or developmental CORT shapes the stress reactivity and social behavior of offspring as adults (Meaney, 2001). Among prairie voles, developmental CORT exposure can alter subsequent parental care and affiliation (Roberts et al., 1996).

CORT levels in the lab are traditionally quantified by invasive procedures such as retro-orbital bleeding (DeVries et al., 1995; Taymans et al., 1997), which are less appropriate for measuring CORT in the field than non-invasive fecal hormone assays (Good et al., 2003). Fecal hormone glucocorticoid assays have been validated successfully in many other mammalian taxa (Crino et al., 2010; Goymann et al., 1999; Harper and Austad, 2000; Mateo and Cavigelli, 2005; Monfort et al., 1997; Wasser et al., 1995), and are currently considered the most reliable, the most practical, and the least invasive method of measuring chronic stress (Dantzer et al., 2014; Sheriff et al., 2011). The fecal hormone assay method has considerable advantages over traditional bleeding methods (all reviewed in Dantzer et al., 2014; Goymann, 2005; Harper and Austad, 2000; Palme et al., 2005; Sheriff et al., 2011): beyond its non-invasive nature and more straightforward collection procedure, each sample also contains an averaged hormone level covering the previous several hours. Hence, measures are more representative of an individual's general hormone exposure than the point sampling of bleeding methods.

In the current study, we begin by validating a fecal hormone assay for CORT in prairie voles, demonstrating that our assay is precise and replicable, and that it can detect acute rises in CORT induced by an arbitrary stressor. We next manipulate population density in semi-natural enclosures, to ask whether density causes changes in social interaction and CORT titers in the field. We focus on males because CORT levels are known to influence both social bonding and parental care of males in the lab, and because limited resources prevented us from looking at both sexes. By using semi-natural enclosures, we allowed social interactions while controlling for factors such as predation, food and water resources (Creel et al., 2013).

2. Materials and methods

2.1. Experimental design summary

We first validated a fecal CORT metabolite hormone assay using a test for assay linearity, followed by a swim challenge to confirm that the assay can detect fecal CORT responses to a standardized acute stressor. Having validated our fecal CORT assay, we conducted a field experiment using a different set of animals. We measured fecal CORT metabolites for these animals in the lab, then placed them in semi-natural enclosures in the field for 19–24 days at one of two densities: low-density (LD) = 80 animals/ha, $n = 12$ males and 12 females per trial, and high-density (HD) = 240 animals/ha, $n = 12$ males and 12 females per trial. In these enclosures we measured individual space use, male–female pairing patterns, and CORT metabolite levels over two separate replicate trials, a "summer trial" and a "fall trial". Animals were briefly trapped and feces were collected during the trial and again at the end of the trial; thus, we collected feces at three time points: pre-field (lab), midway (days 8–13), and at the end of the field trial (days 19–24).

2.2. Subject animals

Study animals were descendants of wild-caught voles from Illinois and Tennessee. Subjects ranged from F6 to F10 generations from the original wild-caught voles. All animals were lab born and raised, and were weaned at 21 days. Upon weaning, voles were placed in same-sex sibling groups. Housing conditions have been known to affect CORT levels, with solo-housed prairie voles exhibiting higher levels of CORT (Ruscio et al., 2007); therefore, all animals used in this study were socially housed in same-sex sib groups of 2–5. Prairie voles reach sexual maturity at 45 days (Mateo et al., 1994); only animals 45 days or older were used as subjects. All procedures were reviewed and approved by the University of Florida Institutional Animal Care and Use Committee (IACUC) in accordance with local, state, and federal regulations to minimize pain and discomfort. Our IACUC protocol number was D289.

2.3. Fecal CORT metabolite extraction and radioimmunoassays

Our sample collection, extraction, and validation protocols included methods established by Harper and Austad (2000), Mateo and Cavigelli (2005) and Crino et al. (2010). For all fecal collection, we collected up to a maximum of five pellets per animal produced during a 15-min defecation period; any feces contaminated with urine were excluded (after Cavigelli et al., 2005). Pellets were stored in a −20 °C freezer.

Feces were freeze-dried and weighed. To minimize variation in weight prior to extraction and radioimmunoassay (RIA), we combined dry pellets, collected at the same time and from the same individual, to a weight of 15–20 mg. If all combined pellets from a given defecation period were less than 15 mg, we still used them;

if there were still remaining pellets after we reached 20 mg, we left those extra pellets out of the analysis and stored them separately. The total feces produced by an individual during a defecation period and used for analysis is hereby referred to as a "sample," and a sample can thus consist of one or multiple pellets.

Samples were then placed in individual glass culture tubes with 1 mL 90% methanol solution, homogenized with a spatula, and then agitated at room temperature for 24 h using a Thermo Scientific Labquake rotator. They were then centrifuged at 2000 rpm at 4 °C for 5 min. We collected ~500 μl of the supernatant from each sample, and stored the supernatant at −20 °C until assayed.

Most of what is present in feces is not CORT itself, but rather CORT metabolites (Palme et al., 2005; Touma and Palme, 2005). In our study, references to fecal CORT will imply fecal CORT metabolites. For fecal glucocorticoid assays, it is standard practice to use antibodies for the original hormone (Goymann, 2005), and thus we used commercial 125I RIA kits (MP Biomedicals, Solon, OH; Catalog Nos. 07120102 & 07120103; minimum detectable dose of 7.7 ng CORT/mL) that have been previously validated for plasma CORT in prairie voles (DeVries et al., 1995; Grippo et al., 2007; Taymans et al., 1997). However, when doing this, it is important to first demonstrate that the antibody for the original hormone can detect the metabolites of the hormone (Goymann, 2005), which was the purpose of our fecal hormone assay validation (Section 2.4). With the exception of the test for assay linearity (Section 2.4.1), which requires a serial dilution, we achieved our best results (i.e., consistently readable from the standard curve) using undiluted extracts for the assays. To account for differences in fecal sample mass, we divided the total CORT metabolites detected (ng/mL) by fecal mass (mg/mL).

2.4. Fecal hormone assay validation

2.4.1. Test for assay linearity

Antibodies targeting CORT can be used to measure CORT metabolites if dilutions of fecal CORT produce parallel changes in binding to the RIA-antibody (Goymann, 2005). This criterion is referred to as the test for parallelism (Beehner and McCann, 2008; Crino et al., 2010; Harper and Austad, 2000; Heilmann et al., 2011; Mateo and Cavigelli, 2005; Sheriff et al., 2011; Vasconcellos et al., 2011). Using serial dilutions of a pooled fecal sample, we checked that assay values varied linearly with hormone concentration within the standard curve boundaries (Buchanan and Goldsmith, 2004; Heilmann et al., 2011) and identified appropriate sample dilutions.

We collected fecal samples from 16 animals and extracted fecal CORT as described in Section 2.3, and then pooled the extracted samples together. Using the pooled extracts, we performed three separate serial dilutions, each consisting of 10 dilutions ranging from 1:2 to 1:2000, using the steroid diluent provided with the RIA kit (MP Biomedicals Lot Number: RCBK1010). All dilution samples were run in duplicate in a single RIA; the intra-assay coefficient of variation (CV) was 4.35%.

We quantified CORT at each dilution level for each of the three replicates, log–logit transformed the antibody binding curves, and used an analysis of covariance to compare this with the slope of the log–logit-transformed antibody binding curves from the standards generated from stock solutions that were supplied with the RIA kits (Harper and Austad, 2000; Mateo and Cavigelli, 2005). Note that this test for assay linearity shows a dose–response relationship and is not sufficient for full demonstration of specificity, as some cross-reacting compounds could result in acceptable results (Möstl et al., 2005); therefore, we also had to demonstrate that the fecal assay could detect an acute stressor, which we did with a swim challenge.

2.4.2. Swim challenge

2.4.2.1. Swim challenge experimental design. We tested the biological validity of our assay (Touma and Palme, 2005) by demonstrating that it could detect the change in CORT associated with an acute stressor (Crino et al., 2010; Harper and Austad, 2000; Mateo and Cavigelli, 2005; Touma and Palme, 2005), a swim challenge. Prairie voles swim instinctively when placed in water; swim challenges are commonly used as standard stressors (Bosch et al., 2009; DeVries et al., 1996; Grippo et al., 2012), and are known to elevate prairie vole CORT levels (DeVries et al., 1995; Taymans et al., 1997).

Sixteen animals (eight male, eight female) were used. Four males and four females were selected at random and assigned to the control group; the remaining four males and four females were swim-treatment animals. Control and swim-treatment animals were run simultaneously to control for any effects of circadian rhythms. 48 h prior to testing, all animals were removed from their sibling-housed cage and individually housed in new cages with clean bedding and *ad libitum* food and water. Although solo-housed voles can sometimes exhibit higher CORT levels than group-housed voles (Ruscio et al., 2007), individual housing was necessary to facilitate collection of feces. Nonetheless, because both control and treatment individuals were housed singly, any treatment-specific differences in CORT levels can be attributed to the swim challenge stressor. A full 48 h was allowed for acclimation prior to the experiment so that any temporary CORT surge related to the cage transfer would have dissipated; we know from prior pilot studies that this time window is sufficient. We collected baseline feces from both control and swim-treatment animals 24 h prior to the swim challenge. On the third day of individual housing, forced swim tests began between 1500 h and 1600 h; additional baseline feces were collected 1.25 h prior to the swim challenge.

To evoke a stress response, each swim-treatment animal was placed in a ten-gallon aquarium filled with 15 cm of 22 °C water for 5 min. This was repeated every 30 min for each treatment animal twice, for a total of three 5-min swim challenges spaced over 75 min. Control animals were unhandled during this period; handling of the treatment animals during the swim challenge was considered part of the overall stressor. Between swimming sessions and after the swim challenge, all treatment animals were dried and returned to their cages, where they quickly resumed normal behaviors.

For 20 h after the swim test (excluding times between 300 h and 500 h), sampling was binned into 2-h intervals in which water bowls were removed from the cage to simplify pellet collection, interspersed with 1-h intervals in which water was present and no feces collected. During collection periods, we monitored both swim-treatment and control animals every 15 min, collecting pellets from each animal once per 2-h interval as soon as we observed them defecating. Not all animals produced feces in every time interval, so actual sample sizes within an interval varied from 5 to 10 subjects per treatment. Times are given "post-stressor" to refer to the time since an individual began its first 5-min swim challenge.

To increase our sampling of the time immediately following the stressor, we performed a second swim challenge with a subset of the animals 72 h after the beginning of the first swim challenge (a time span which our previous pilot studies had shown was well beyond the time required for CORT levels to return to baseline). The second challenge was identical to the prior challenge, but this time we used four of the previous control animals (two male, two female) as treatment animals, and four of the previous treatment animals (two male, two female) as control animals. In this replicate, we observed the animals continuously and collected pellets from all animals as soon as they were produced and hence, had a smaller sample size; fecal pellet collection lasted 4.75 h

post-stressor. We combined the second challenge data with our first challenge data, to improve our overall statistical power. However, we also present a second statistical analysis that omits these additional samples, which avoids entirely any potential confounds of repeated testing on some, but not all, animals.

2.4.2.2. Swim challenge CORT quantification and data analysis. Swim-treatment and control samples were run in duplicate over two RIAs (Lot Numbers RCBK1106A, RCBK114) due to the large number of samples. To control for variation between kits and assays, we ran a subset of 9 of the 169 samples in both assays to allow for direct comparison of CORT values. Of the 169 samples, 4 were excluded because they either fell outside the range of the standard curve ($n = 2$), or because their measured values were more than 2 SD above the mean ($n = 2$:2.5 and 10.3 SD).

The intra-assay average CV was 1.86%. The inter-assay average CV for nine overlapped samples that were run in both assays was 22.3%; however, the CORT measures from each assay for these nine overlapped samples were significantly positively related (simple linear regression, $p < 0.0001$; $R^2 = 0.9931$). We used the resulting relationship, $y = 0.663x + 15.28$, to correct for inter-assay differences.

Note that the above and subsequent inter-assay average CVs are relatively high (generally ~22%). This was due to a "kit age effect" whereby older RIA kits consistently showed higher CORT values for a given sample than younger kits, despite purportedly identical standards and controls used separately in each kit (Blondel, unpublished data). There also may have been a "batch" effect in cases where kits came from different lot numbers. Since we ran a large number of samples in replicate across assays whenever using two kits (ranging from 9 to 40 overlapped samples), and had high R^2 values in our regression analyses (ranging from 0.93 to 0.99), we used these regressions between assays to correct for assay-specific variation.

Next we compared the fecal CORT levels of swim-treatment animals to those of the control animals. As described above, we had two pre-challenge collection time periods (24 h and 1.5 h prior to the challenge); and six 2-h collection periods spanning the 20 h following the swim challenge. Because CORT is released in a circadian rhythm (Sapolsky, 2002), we compared swim-treatment and control treatment animals within each time interval. The CORT measurements were not normally distributed (Shapiro–Wilk, $W = 0.891$, $p < 0.0001$), thus we used non-parametric Mann–Whitney U tests to compare swim-treatment values with controls for a given time interval. To include the results of the second swim challenge in the overall analysis, we averaged CORT values for any animals in the second challenge that produced feces at more than one time point within a 2-h sampling interval.

2.5. Semi-natural enclosures and density manipulation

In order to quantify the effect of population density on CORT levels, we created two population density treatments using semi-natural enclosures: low-density (LD; 24 animals in 0.3 ha = 80 animals/ha) and high-density (HD; 24 animals in 0.1 ha = 240 animals/ha). These density treatments fall within the range (11–624/ha) reported in wild prairie vole populations (Getz et al., 1993). Our field site was located in Jackson County, Illinois, USA, well within the natural geographic range of prairie voles.

The semi-natural enclosures consist of four abutting quadrants, each measuring 0.1 ha (Fig. 1). Quadrant walls are composed of aluminum flashing that extends 30 cm below-ground and 60 cm above-ground. Further, quadrants were separated by large removable gates, allowing them to be combined, thus our high-density animals were distributed in one quadrant while our low-density animals occupied three interconnected quadrants. In addition, this structure allowed us to vary the quadrant used for the high-density

Fig. 1. Semi-natural enclosure facilities, demonstrating density treatments. High density treatment location changed in different trials.

treatment and reduce any confound between location and density effect. Inside the enclosure were abundant tall grasses and sedges, the preferred habitat of the prairie vole (Getz, 1985). Clover was planted to supplement their mostly herbivorous diet (Batzli, 1985). Although their water requirements are typically met by the water content of vegetation and by dew, we also provided two agricultural poultry water stations in each quadrant, which were kept filled during trials and cleaned between trials. Predation-prevention measures included aviary netting covering the top and sides of the enclosure, a secondary perimeter of flashing ~3 m outside the inner perimeter, snake-guards to deter entry along support posts, and a 2-cable electrified fence directly above the outer perimeter.

We ran two 3-week (19–24-day) field trials; Trial 1 was conducted in August 2011 ("Summer trial") and Trial 2 was conducted in October 2011 ("Fall trial"). Each trial consisted of 12 males and 12 females assigned to high density, and another 12 males and 12 females assigned to low density (Fig. 1). All animals were ear-tagged and toe-clipped for identification and future paternity analysis, and affixed with radio-transmitters (model SOM 2028 HWSC, 2.2 g or less, Wildlife Materials, Murphysboro, Illinois). Because this is a fossorial species, toe-clipping was limited to a single rear toe, in accordance with the American Society of Mammalogists guidelines (Sikes et al., 2011). In the wild, male space-use is strongly influenced by female residency (Getz and McGuire, 1993), with males dispersing until they find and join a female at her nest. Thus, we released females first, and released males two days later, to more closely replicate the natural social environment.

Prairie voles exhibit mostly crepuscular activity patterns, and beyond that also exhibit 2–4 h ultradian activity rhythms (Calhoun, 1945; Madison, 1985). The location of animals was noted via radio-tracking from 1 to 3 times per day every day, with a minimum of 1 h between subsequent fixes per individual. Animals were tracked at varying times of the day in order to avoid timetabling issues (Kenward, 2001), and between one and three night fixes were performed per animal over the course of each trial. Locations were estimated to within 30 cm. Radio-tracking was performed with a Telonics receiver (Telonics Inc., Mesa, Arizona) and a 3-element yagi antenna via the homing method (Kenward, 2001), by walking a grid of 3 m × 3 m cells. A minimum of 30 fixes per animal were collected for each trial.

On days 8–13 of each trial, feces were collected from subject animals. Animals were live-trapped out between dawn and 1030 h so as to minimize effects of circadian rhythms on CORT. 192 Sherman live-traps (model LFAHD, 3 × 3.5 × 9″) were used.

These were placed in appropriate micro-habitats throughout the grid. During trap-outs, traps were checked every 15 min to allow calculation of time-of-capture to within 15 min for use in fecal CORT analysis. Animals were placed in buckets, and fecal pellets were collected as soon as they were produced, as described in Section 2.3. Only feces that were collected within 90 min of live-trap capture were used in fecal CORT analysis, as otherwise the CORT levels present in the sample may reflect the trapping event. Following fecal collection, subjects were re-released back into the enclosures at the exact location where they had been trapped. On days 19–24 of each trial, all animals were again live-trapped out, and additional feces were collected. CORT was extracted and measured from the feces as described in Section 2.3. Unless otherwise specified, "field" fecal CORT refers to the combined data from both mid- and end-trial feces collection; if an animal produced feces in both time periods, their CORT values were averaged.

2.5.1. Density manipulation CORT quantification

Due to their large number, samples from the enclosure experiments ($n = 69$) were run in duplicate over two RIAs (Lot Number RCBK1217 for both). The intra-assay average CV was 1.48%. Forty samples were run in both assays; the inter-assay average CV was 22.1%, and the relationship between the assays was $y = 1.1258x + 36.173$ (simple linear regression, $R^2 = 0.93$, $p < 0.0001$).

Although we calculate CORT per mass of sample (ng/mg), there was still a significant effect of sample mass on CORT concentration (simple linear regression, $R^2 = 0.28$, $p < 0.0001$, Fig. 2A). This is a common pattern across taxa when performing methanol-based fecal CORT extractions on samples less than 0.02 g (Millspaugh and Washburn, 2004; Tempel and Gutierrez, 2004). Due to the small size of prairie voles, most of our samples were below this weight. Although the reason for such a sample mass effect is not known, it has been suggested that the effect might be caused by a higher concentration of methanol per unit mass during the extraction process (Millspaugh and Washburn, 2004). We corrected for this effect of mass by identifying the best fit second order polynomial regression (because the effect was mostly at the smaller sample weights; $p < 0.0001$, $R^2 = 0.34$, Fig. 2A). For each sample, we used the sum of its residuals and the overall mean (from the original uncorrected CORT values) to create a mass-corrected CORT concentration for use in subsequent analyses. There was no effect of sample mass on these new mass-corrected CORT values (simple linear regression, Fig. 2B). All further references to our enclosure study CORT data will imply sample-mass-corrected CORT concentration.

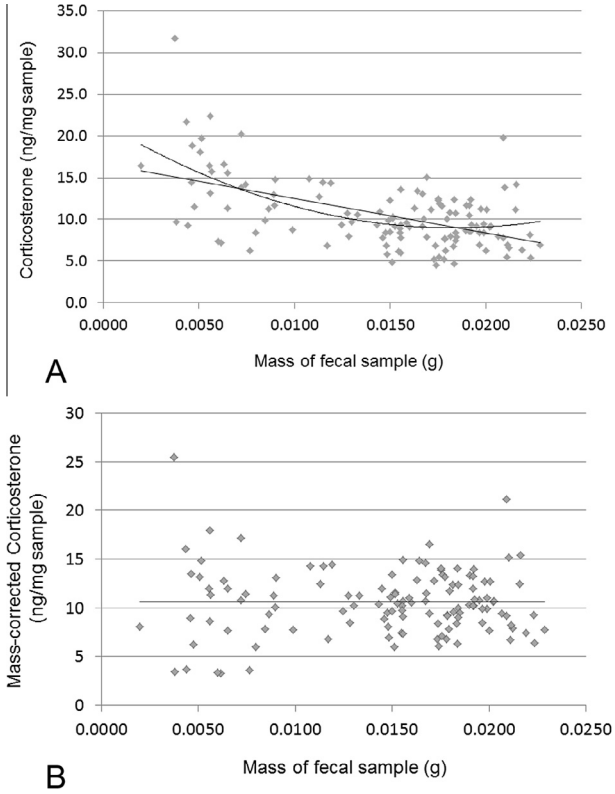

Fig. 2. CORT sample-mass effect. (A) Effect of sample mass on corticosterone concentration, showing both simple linear regression ($p < 0.0001$, $R^2 = 0.28$) and 2nd order polynomial ($p < 0.0001$, $R^2 = 0.34$. (B) Effect of sample mass on mass-corrected corticosterone, simple linear regression (ns).

2.5.2. Density manipulation and space use data analysis

Male prairie voles exhibit two alternative mating/space use phenotypes, resident (paired) males who live in close association with a female and (unpaired) wanderers who have larger home ranges that overlap many individuals (Getz and McGuire, 1993; Ophir et al., 2008; Solomon and Jacquot, 2002). Home ranges of individuals in our study were calculated using the Ranges6 program (http://www.anatrack.com) at the 75% kernel contour, which minimizes the distortion caused by rare foraging events that would be otherwise apparent at higher percent contours. A male and female were considered a pair if their home ranges overlapped one another more than they overlapped all other opposite sex home ranges combined (Ophir et al., 2008). For all but one pair identified in the low density, kernel centers of paired animals were also within 15 feet of one another.

Several variables were calculated for each male resident: number of home range overlaps (the number of animals whose home ranges overlapped that of the focal male, calculated by sex of other animals), percent home range overlapped by mate (the percent of the focal male's home range that was shared with his mate), and percent home range overlapped by other animals of each sex (the cumulative percent of the focal male's home range that was shared with animals of each sex). All of these were calculated with the Ranges6 overlap analysis function. Due to the relatively small number of male wanderers ($n = 6$) and extreme behavioral differences between territorial male residents and non-territorial male wanderers, the above calculations were only performed on resident males.

Enclosure space-use measures and fecal CORT values were analyzed for male residents using ANOVAs that included as factors density, trial, and factorial interactions for all predictive and response variables (i.e., CORT levels, home range size and percent overlap by animals of each sex).

3. Results

3.1. Fecal hormone assay validation

3.1.1. Test for assay linearity

Log–logit transformed curves of serially diluted fecal extracts were compared with log–logit transformed standard curves in a test for parallelism (Fig. 3). The three serial dilutions and the standards had a common slope (dilutions vs standard, test for difference between slopes: DF = 3; VR = 0.6; $p = 0.60$; test for common slope: DF = 1; VR = 2907.6; $p < 0.0001$; standard curve equation $y = -2.08x + 4.68$, $R^2 = 0.996$).

3.1.2. Detecting acute stressors

The combined data from the swim challenges (Fig. 4) showed a CORT surge in the swim-treatment animals relative to time-matched control animals. Specifically there was a significant difference in the second post-stressor collection interval, approximately 4.5–5.5 h post-stressor (one-tailed Mann–Whitney U test; $U = 75$,

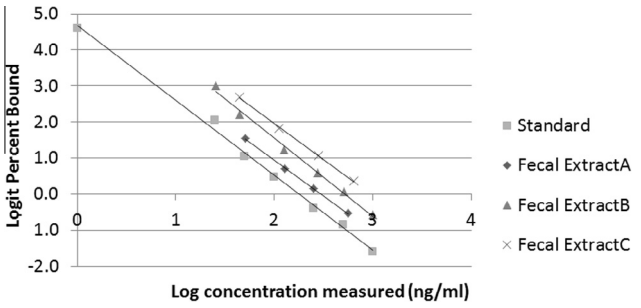

Fig. 3. Log–logit transformed curves of serially diluted fecal extracts compared with log–logit transformed standard curves. Replicates (1:4, 1:10, 1:20, 1:40, 1:100, 1:200) are staggered for ease of viewing, but lie directly on the trendline of standard curve. Standard curve: $y = -2.080x + 4.676$, $R^2 = 0.996$; total fecal extracts: $y = -2.101x + 4.710$, $R^2 = 0.993$.

swim-treatment $n = 11$, control $n = 8$, $p = 0.005$). The differences in swim-treatment and control CORT levels in the first and third post-stressor collection intervals also approached significance (one-tailed Mann–Whitney U test; $U = 50.5$, $p = 0.070$; $U = 24$, $p = 0.058$, respectively). The second swim challenge did not reveal any significant early CORT surge, and was consistent with the time-frame of the surge in the first swim challenge; as such, we combined the data to improve our statistical power. Note that when restricting the data to just the first swim challenge, we still found a significant difference in the second post-stressor collection interval (one-tailed Mann–Whitney U test, $U = 41$, $p = 0.019$).

3.2. Density effects on space use

We identified 27 males as residents ($n = 10$ in summer trial, $n = 17$ in fall trial) and 6 males as wanderers ($n = 2$ in summer trial, $n = 4$ in fall trial) in our density manipulation study (Fig. 5). In the combined high-density (HD) treatments, 83% ($n = 15$) of males were residents, and 17% ($n = 3$) of males were wanderers. In the combined low-density treatments (LD), 80% ($n = 12$) of males were residents, and 20% ($n = 3$) of males were wanderers. Hence, there were no effects of density on the expression of these alternative male tactics (X^2-test, $X^2 = 0.30$, DF = 1, $p = 0.58$). These estimates are consistent with male wanderer estimates from other studies, which range from 4% to 40% of the population (Getz and McGuire, 1993; Ophir et al., 2008; Solomon and Jacquot, 2002). We classified 37 females as residents ($n = 16$ in the summer trial, $n = 21$ in the fall trial), and 7 females as wanderers ($n = 4$ in the summer trial, $n = 3$ in the fall trial). In the combined HD treatments, 94% ($n = 15$) of females were residents, and 6% ($n = 1$) of females were wanderers. In the combined LD treatments, 72% ($n = 15$) of females were residents and 28% ($n = 6$) of females were wanderers. For females, we did see a density effect on alternative tactics, with more females pairing in the HD treatment (X^2-test, $X^2 = 17.15$, DF = 1, $p < 0.001$). As expected (Ophir et al., 2008; Solomon and Jacquot, 2002), male resident home ranges (mean 126 ± 15 SE m^2) were significantly smaller than male wanderer home ranges (mean 194 ± 37 SE m^2; one-tailed t-test, $t_{31} = 1.866$, $p = 0.036$).

Eleven males and eight females died during the summer trial, and one male and two females died during in the fall trial. The summer trial mortality was likely due to the record high temperatures in August 2011; there was no evidence of predation. All mortality occurred within the first few days of the beginning of the trial, and was thus unlikely to have had any significant or confounding effect on the space use data of the surviving animals.

The population density treatments had several significant effects on the space use of male residents. HD home ranges were significantly smaller than LD home ranges (mean HD area = 92 ± 14 SE m^2, mean LD area = 169 ± 23 SE m^2; $n = 15$ HD, $n = 12$ LD; ANOVA with density and trial as effect; $F_{1,25} = 8.311$; $p = 0.008$; Fig. 6A). Density also affected the degree of home range

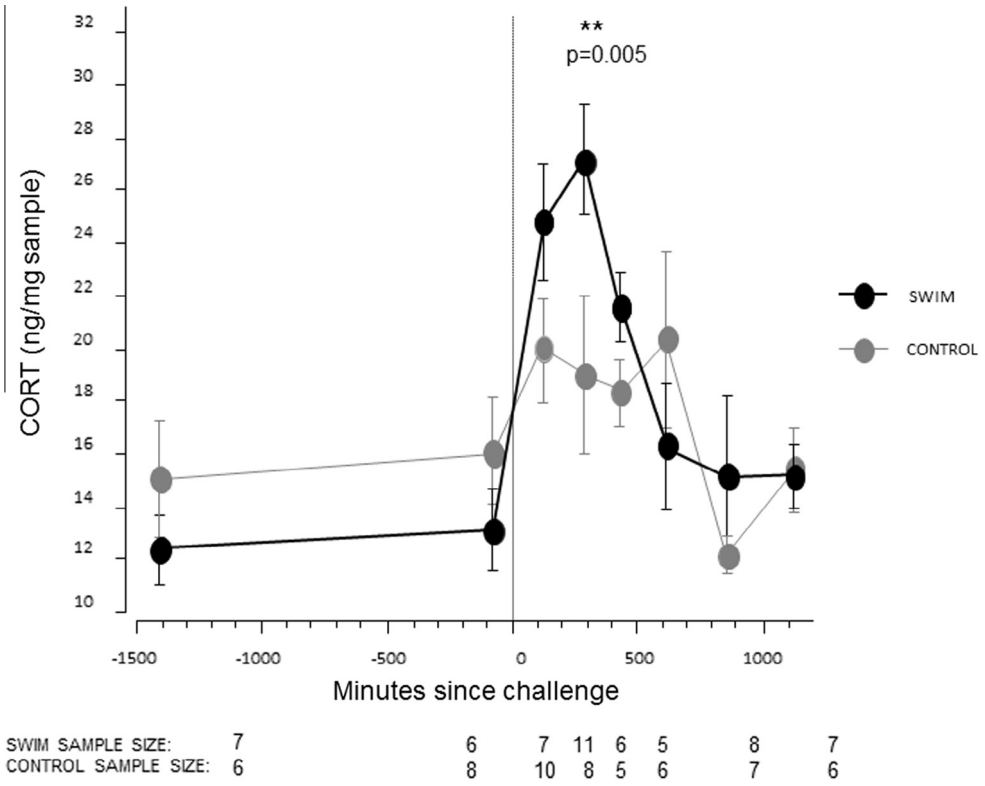

Fig. 4. Effects of swim challenge on fecal CORT metabolites. Mean fecal CORT before and after the swim challenge, with each data point representing the midpoint of each sample collection time interval. Data presented as Mean ± SE. "Time zero" indicates the beginning of the swim challenge. Sample sizes are reported below each time window.

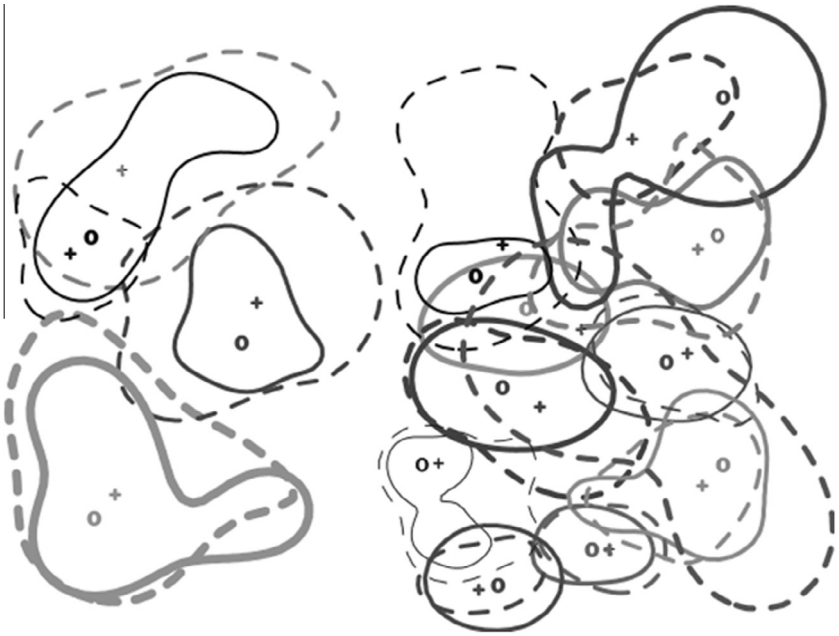

Fig. 5. Home ranges (75% kernel contours) for the high-density quadrant and for one low-density quadrant from Trial 2 dotted lines indicate males, solid lines indicate females. Kernel center (and likely nest site) is indicated by + for males, o for females. Where these would otherwise be overlapping, they have been staggered for visibility. Animals of the same shade have been designated pairs ("residents") and unique shades are unpaired ("wanderers").

overlap male residents experienced. The number of males that overlapped resident male's home ranges was significantly greater in the high-density treatment than in the low-density treatment (mean HD = 2.5 ± 0.3 SE overlaps, mean LD = 1.6 ± 0.3 SE overlaps; n = 15 HD, 12 LD; ANOVA with trial and density as effects; $F_{2,24}$ = 4.796; p = 0.01; Fig. 6B). Similarly, a greater number of females overlapped male resident home ranges in the high-density treatment (mean HD = 3.2 ± 0.3 SE overlaps, mean LD = 2.2 ± 0.4 SE overlaps; n = 15 HD, 12 LD; ANOVA with trial and density as effects; $F_{2,24}$ = 3.671; p = 0.03; Fig. 6C). However, population density did not have any significant effect on cumulative percent overlap by other males, cumulative percent overlap by females excluding the mate, and percent of a resident male's home range overlapped by his mate. Thus, overall, high density animals were more tightly packed in space, but resident males in particular maintained similar degrees of home range exclusivity.

3.3. Density and field effects on fecal CORT metabolites

There was no significant difference between the average field fecal CORT of male residents and wanders (median resident = 9.4 ± 0.4 SE ng/mg, median wanderer = 10.4 ± 1.3 SE ng/mg). However, the density treatment had a significant effect on male resident fecal CORT levels. The average field-collected male resident fecal CORT levels were significantly lower in the HD treatment than in the LD treatment (mean HD = 8.2 ± 0.5 SE ng/mg, mean LD = 10.1 ± 0.6 SE ng/mg; n = 10 HD, 10 LD; ANOVA with trial and density as effects; $F_{1,18}$ = 6.825; p = 0.02; Fig. 6D). Finally, the average pre-field male fecal CORT levels were significantly higher than average field-collected male fecal CORT levels, regardless of density treatment (mean pre-field = 10.8 ± 0.7 SE ng/mg, mean field = 9.2 ± 0.4 SE ng/mg; n = 18 pre-field, 20 field; unpaired t-test, t_{36} = 2.0188, p = 0.05).

4. Discussion

In the current study, we tested the hypothesis that high population densities promoted increases in agonistic encounters and elevated glucocorticoids. In order to test this hypothesis, it was necessary to validate our fecal measures of CORT. Our fecal CORT assay worked successfully, but our results refuted our hypothesis that high population density caused elevations in CORT. We discuss our validation and the results of our population manipulations below.

4.1. Fecal CORT assay validation

Our fecal CORT metabolite assay passed the test for parallelism (Fig. 3), and we demonstrated that the assay can detect changes in CORT levels in response to an acute stressor (Fig. 4). The timing of the CORT surge associated with the swim challenge, an acute stressor, suggests a gut-passage time of around 5 h, possibly slightly shorter (Fig. 4). Gut-passage time has never been formally measured in prairie voles, but prairie voles exhibit ultradian activity rhythms of 2–4 h (Calhoun, 1945; Madison, 1985) and digestion studies have suggested that this is due to a digestive bottleneck limiting food intake, which forces voles into short bouts of rest and feeding (Zynel and Wunder, 2002). Researchers intending to use this fecal CORT assay should ideally collect fecal samples as soon as possible but no later than 2 h after removing a prairie vole from its home cage (in a lab context) or after live-trapping (in a field context), to avoid fecal CORT values more indicative of the handling/trapping experience than baseline values.

4.2. Density effects on social interaction as measured by space use

We found no effects of density on the probability a male would pair. Similarly, the degree of overlap between a male and his partner was similar across densities, demonstrating a stability of affiliative behavior with a mate even in the face of dramatically different population densities. However, high population density seems to provide more opportunities for extra-pair mating, as density nearly doubled the extent of overlap between home ranges of paired males and extra-pair females (Fig. 5). This is consistent with a prairie vole study that showed increased extra-pair paternity at higher densities in wild populations (Streatfeild et al., 2011).

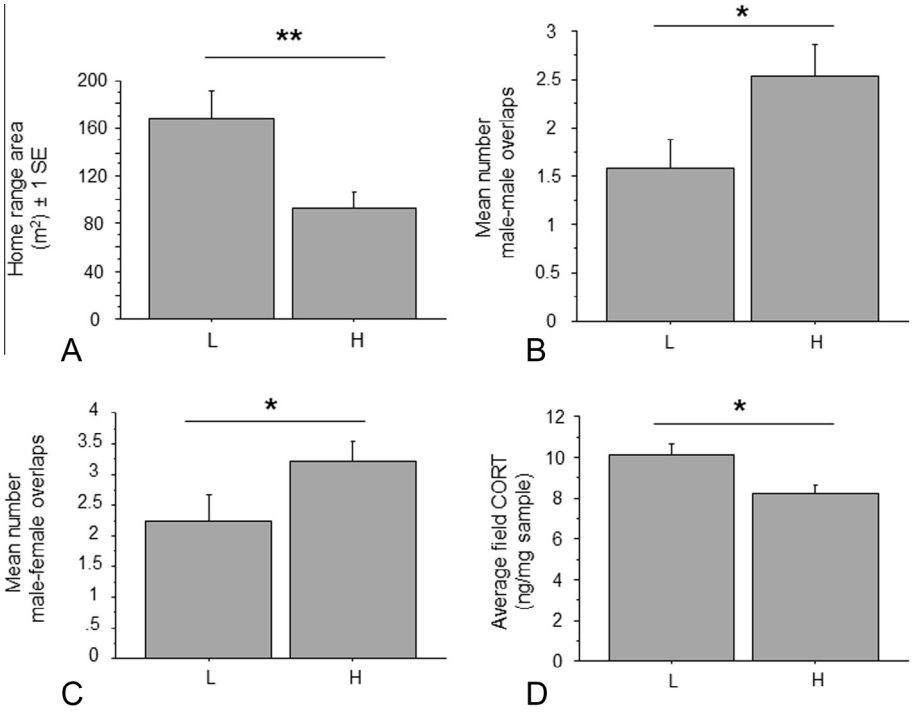

Fig. 6. Effect of density on space use and CORT. (A) Effect of density on 75% kernel home range area, male residents only. (B) Effect of density on number of other males overlapping male resident home ranges. (C) Effect of density on number of females overlapping male resident's home range. (D) Effect of density on average field fecal CORT metabolites. $^{*}p < 0.05$, $^{**}p < 0.01$. Mean ± SE indicated, sample sizes indicated on figure.

Density had mixed effects on male-male interactions: males at high densities had smaller home ranges that overlapped more often with other males (Fig. 6A and B), but the fraction of a male's home range overlapped by other males did not differ with density. Same-sex overlaps occur at the peripheries of home ranges, and may indicate the true boundaries of a defended territory (Maher and Lott, 1995); further, our data are consistent with previous field studies that showed non-residents interacting more at home range peripheries at higher densities (McGuire et al., 1990), yet showing no density-related increase in non-resident nest visits (McGuire and Getz, 1998). Our data suggest that male territories become constricted, but are not necessarily less exclusive at higher densities.

4.3. Density and field effects on CORT

Our density results indicate that, contrary to our predictions, high density animals have significantly lower, not higher CORT levels than low density animals (Fig. 6D). Interestingly, this pattern is the opposite from that seen in most other taxa (Christian, 1961; Nelson, 2000), and would seem to conflict with data indicating increased rates of aggressive behavior among male prairie voles from higher densities (Krebs, 1970). Nevertheless, our results are consistent with some prior findings in prairie voles. Researchers found no differences in actual wounding rates of male prairie voles across densities (Rose and Gaines, 1976; Smith and Batzli, 2006), unlike comparable studies of other voles (Boonstra and Boag, 1992). Perhaps more tellingly, Getz et al. (1997) report that animals have longer life expectancy at higher densities. Moreover, in the lab males have higher CORT when housed in low density rooms than in high density rooms (Nelson et al., 1996). Taken together the CORT and longevity data suggest that high densities might actually be favorable environments, suggesting an Allee/Darling effect of population density on population growth (Allee, 1931; Darling, 1938; Lidicker, 2007).

Creel et al. (2013) reviewed the literature on population density and HPA function, and suggested that departures from the typical positive correlation between glucocorticoid secretion and density could be due to confounding effects of seasonality, recent extreme weather events, reproductive condition, or the effects of trapping and handling stress on glucocorticoids. We avoided most of these confounding effects by running our density treatments concurrently. Moreover, our density differences were concordant across seasons. Both of our trials were during the breeding season when females were in reproductive condition, and male–female pairs remained the major social group type, so we did not see, for example, the potentially confounding effects of winter formation of communal groups (Carter et al., 1995). Finally, due to our non-invasive fecal hormone assay method, trapping and handling are unlikely to have affected or confounded our results.

There are a few possible explanations for the pattern of higher CORT levels at lower densities. These are not mutually exclusive, and can be explored with future research. One explanation is that the larger home ranges observed in lower population densities (Fig. 6A) require more energy expenditure to maintain, and the higher CORT is a means of mobilizing glucose reserves to handle the additional energetic requirements. However, the previous lab density manipulations (Nelson et al., 1996), which showed elevated CORT levels at lower densities of singly housed individuals, makes this an unlikely explanation.

A second hypothesis is that at lower densities, finding a potential mate involves increased searching costs (Kokko and Rankin, 2006); rejecting a potential mate means it may cost more in energy and time to find a suitable alternative mate than it would cost at higher densities. Hence, it may be adaptive to reduce the threshold for pairing to avoid more extensive mate-search costs. We know that in male prairie voles, CORT facilitates social preference formation (Blondel et al., unpublished results; DeVries et al., 1996). We did not find density differences in the fraction of animals paired, and this is consistent with other prairie vole fieldwork (Getz et al., 1993; but see Streatfeild et al., 2011). One possible explanation is that the CORT rise facilitates partner preference and thereby compensates for lower densities. This would predict that

experimentally impairing or clamping CORT function would unmask a density-dependence in pair formation.

Finally, a third explanation is that high population density signals low per capita predation risk. Annual prairie vole population density cycles seem to be driven primarily by predators, with population booms occurring during late autumn and winter months while some predators are inactive (Getz et al., 1990). The combination of low predator abundance and high population density would lead to a much lower per capita predation rate. From this perspective, the elevated CORT present at low population densities might be part of a general increase in vigilance important in high predation environments. This is consistent with the enhanced longevity exhibited at high densities (Getz et al., 1997), as well as lab data demonstrating that high densities promote lower CORT (Nelson et al., 1996). There is precedence for indirect predator cues to elevate CORT levels in prey populations (reviewed in Clinchy et al., 2013), and this is consistent with the sometimes "preparatory" nature of elevated CORT levels (Sapolsky et al., 2000), in anticipation of predictable future stressors.

One reason for studying CORT levels of resident male prairie voles is that acute stressors are known to facilitate paternal care in the lab (Bales et al., 2006). Our data suggest that males might contribute more to care at lower densities, which could in turn influence the stress reactivity of offspring born at different times of the year. While this is an exciting possibility, it is not clear whether the CORT differences we observe will persist during parental care, or whether CORT would have the same effects on paternal care in the more complex field environment.

In addition to population density differences in CORT, we were also surprised to find that lab-housed animals have significantly higher levels of CORT than they do in an outdoor enclosure. This suggests that our subjects generally experienced lab housing as a stressor, despite the fact that animals were housed with same-sex siblings and had been reared in the lab. Moreover, these animals were subject to agonistic interactions and fluctuations in environmental conditions when placed in the field. To our knowledge, this is first time that this has been demonstrated in prairie voles, and is consistent with studies in other taxa that show lower stress levels in field conditions relative to lab housing (Cooperman et al., 2004; Davis and Maerz, 2011). Our results should be kept in mind when interpreting results of laboratory studies, and should be encouraging to researchers who hope to conduct naturalistic enclosure studies.

4.4. Conclusions

We found that high population density increased measures of same-sex and opposite-sex interaction in the field, but lowered the overall level of CORT. Overall, the data suggest that prairie voles may use high population density as a cue to lower predation risk, though several other hypotheses are also plausible. Future experiments are needed to explain why population density has this effect on CORT. Such studies could include sampling wild free-ranging populations at a variety of population densities, across seasons, and while measuring predation risk and fitness correlates such as survivorship and offspring. Regardless of the reason for fluctuations in CORT levels, the fact that density influences CORT levels among prairie voles raises interesting new questions regarding the possible cycling of stress-reactivity as well. By validating an assay for fecal CORT and documenting the effects of density on CORT, we hope to facilitate the future use of prairie voles as integrative models in the study of endocrinology, behavior, and evolution.

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