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PHYSIOLOGICAL ECOLOGY – ORIGINAL RESEARCH

Is chronic stress a causal mechanism for small mammal population cycles? Reconciling the evidence

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Abstract

Chronic stress has long been hypothesized to play a role in driving population cycles. Christian (1950) hypothesized that high population density results in chronic stress and mass "die-offs" in small mammal populations. Updated variations of this hypothesis propose that chronic stress at high population density may reduce fitness, reproduction, or program aspects of phenotype, driving population declines. We tested the effect of density on the stress axis in meadow voles (*Microtus pennsylvanicus*) by manipulating population density in field enclosures over three years. Using fecal corticosterone metabolites as a non-invasive measure of glucocorticoid (GC) concentrations, we found that density alone was not associated with GC differences. However, we found that the seasonal relationship of GC levels differed by density treatment, with high-density populations having elevated GC levels early in the breeding season and decreasing towards late summer. We additionally tested hippocampal glucocorticoid receptor and mineralocorticoid receptor gene expression in juvenile voles born at different densities, with the hypothesis that high density may reduce receptor expression, altering negative feedback of the stress axis. We found that females had marginally higher glucocorticoid receptor expression at high density, no effect in males, and no detectable effect of density on mineralocorticoid receptor expression in either sex. Hence, we found no evidence that high density directly impairs negative feedback in the hippocampus, but rather female offspring may be better equipped for negative feedback. We compare our findings with prior studies to attempt to disentangle the complicated relationship between density, seasonality, sex, reproduction and the stress axis.

Keywords Field study · Glucocorticoids · Intrinsic regulation · Maternal programming · Maternal effects · Population regulation · Stress axis · Vole cycles

Introduction

Voles and lemmings (arvicoline rodents) have been one of the most intensely studied group of mammals in ecological research over the past century. At least 7,678 articles with vole, lemming, *Microtus*, *Clethrionomys*, or *Myodes*, in their titles have been published to date (Web of Science search, July 2021). Many of these rodents undergo 3–5 year cycles in abundance (reviewed in Krebs and Myers 1974; Boonstra and Krebs 2012; Krebs 2013; Myers 2018; Oli 2019; Andreassen et al. 2021). The cycles are a prominent feature of ecosystem dynamics in the Northern hemisphere (Ims and Fuglei 2005; Boonstra et al. 2016). A key topic has been understanding the mechanisms driving these cycles. Some studies have demonstrated that cyclic population declines can persist even in the absence of food limitation, predation, and other environmental factors, demonstrating that intrinsic vole social dynamics and territorial competition at high density may play a role in population processes (Chitty 1960; Cole and Batzli 1978; Boonstra et al. 1998; Maron et al. 2010). The cycles are additionally associated with phase-dependent changes in animal quality in terms of survival and reproduction. Populations at the peak of the

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cycle, when density is highest, tend to have a lower proportion of breeding individuals in the population and a shorter breeding season. This decrease in reproduction is driven by delayed sexual maturation of offspring born at high density, which persists into the decline (Boonstra 1985; Oli and Dobson 1999; Novikov et al. 2012). Further, there is evidence that juvenile survival is poorer at high density (Krebs and Myers 1974; Boonstra 1985; Norrdahl and Korpimäki 2002). Hence, there has been great interest in elucidating intrinsic mechanisms that could drive these changes in phenotype in these small mammal populations.

It has been hypothesized that chronic stress, acting at high population density, drives population declines. The hypothalamic–pituitary–adrenal (HPA) axis drives responses to both physical and psychosocial stressors in vertebrates. In response to a stressor, corticotrophin-releasing hormone is secreted from the hypothalamus, triggering release of adrenocorticotrophin from the anterior pituitary into the circulation, which causes the adrenals to synthesize glucocorticoids (GCs; primarily corticosterone or cortisol depending on the species). These GCs induce a suite of physiological effects to bring the organism back to homeostasis, which can include catabolizing energy reserves, down-regulating functions that are not immediately necessary, and generally altering the expression of genes throughout the brain and body (Sapolsky et al. 2000). The secretion of GCs in response to a stressor is ultimately reduced via negative feedback, where GCs bind to glucocorticoid receptors in the hippocampus, hypothalamus, pituitary, and adrenals, attenuating the stress response. The HPA axis additionally has many basal (i.e., not in response to a stressor) functions related to regulating energy balance. Basal HPA axis activity appears to be largely regulated by mineralocorticoid receptors, which are co-localized with GRs in the hippocampus but bind to GCs with a higher affinity (de Kloet et al. 1998). Thus when GC levels are low, GCs are primarily bound to mineralocorticoid receptors in the hippocampus, but when GC levels are peak, they begin to saturate the glucocorticoid receptors as well (de Kloet et al. 1998; ter Heegde et al. 2015). Yet, there is evidence that mineralocorticoid receptors also play a role in response to stressors, especially related to stress effects on memory, potentially via lower affinity membrane-bound receptors (ter Heegde et al. 2015).

The origins of the idea for stress-driven small mammal population cycles can be traced to the work by Hans Selye and his proposal of the "General Adaptation Syndrome" (Selye 1946). This work was based on laboratory research in rats, where chronic adrenocortical secretion of corticosterone eventually led to 'exhaustion' of the stress response, resulting in disease, shock, and subsequent death. Inspired by Selye, Christian (1950) proposed that chronic stress was a driver of small mammal population cycles and that increased adrenocortical activity at peak density triggered a population-wide "die-off." Early tests of this hypothesis involved comparing adrenals from voles in different phases of the cycle as a measure of adrenocortical activity (reviewed Krebs and Myers 1974). Following the development and increased availability of immunoassays and their application to small mammals (Bradley et al. 1980), subsequent studies compared corticosterone levels (the primary glucocorticoid in voles and lemmings) from animals sampled in high and low-density populations. These studies have produced somewhat variable results, possibly because many factors can influence corticosterone levels including sex, reproductive status, predation, and other environmental conditions.

Though the general adaptation syndrome has been rejected (Sapolsky 2004; Fink 2010; Boonstra 2013) and Christian's (1950) stress hypothesis has been generally discredited (including by the author himself; Christian 1978) its impact and appeal has caused some to still cite it as evidence that stress effects may be a driver of vole population declines. However, stress-driven population declines in voles and lemmings could occur for other mechanistic reasons. Elevated GCs could drive physiological changes that impact fitness, such as poorer body condition or immune system function. Prior work has shown that elevated GCs in root voles (*Microtus oeconomus*) are associated with inhibited immune function (Du et al. 2016). Elevated GCs may also impact reproduction. By 1978, Christian had updated his original hypothesis to propose that suppressed reproduction was the mechanism by which stress drives population declines (Christian 1978). There are several known mechanisms by which GCs can directly inhibit reproductive function in other species (Bambino and Hsueh 1981; Chandran et al. 1994; Whirledge and Cidlowski 2013; Annie et al. 2019). However, elevated GCs do not always result in a suppressed reproductive axis across species. In some cases, reproductive individuals have higher GC levels than non-reproductive individuals in the population (e.g., Boonstra et al. 2001; Creel 2005; Hunnick et al. 2020), and elevated GCs are a normal part of some aspects of reproductive function (e.g., Fanson and Parrott 2015; Edwards et al. 2018). Therefore, it is unclear if elevated GCs would directly suppress reproduction in voles and/or at what threshold.

Finally, elevated GCs in mothers can alter offspring phenotype. When mothers are exposed to high stress conditions during pregnancy, lactation, or their offspring's early life, it can alter offspring stress axis function and behavior (Love et al. 2013; Moisiadis and Matthews 2014a; McGowan and Matthews 2018; Stead et al. 2021). This can occur by alteration of offspring neural expression of glucocorticoid and mineralocorticoid receptors (along with other mechanisms; reviewed in Moisiadis and Matthews 2014b). Thus, given that glucocorticoid receptor and mineralocorticoid receptor levels are key in GC

regulation, any stressor that alters their expression levels will alter the ability of the organism to cope. For example, in laboratory rats, maternal stress and poor maternal care has been shown to result in decreased hippocampal glucocorticoid receptor expression, suppressing negative feedback of the stress axis, resulting in a higher magnitude response to stressors (Meaney 2001). In laboratory mice, maternal exposure to predator odor during pregnancy results in more cautious offspring with higher hypothalamic mineralocorticoid receptor expression (St-Cyr et al. 2018). In addition to influencing offspring stress response, elevated maternal GCs can shape offspring phenotype by altering their reproductive physiology, behavior, growth/metabolism, and other traits (e.g., Smith and Waddell 2000; Dantzer et al. 2013; Meise et al. 2016; Yao et al. 2021). Thus, elevated GCs may play a role in vole population cycles by two major mechanisms. They may directly influence fitness, or they may alter the phenotype of offspring (Fig. 1).

To examine both GC levels and potential HPA axis programming at different population densities in voles, we carried out a field experiment on *Microtus pennsylvanicus*. We created high and low-density field enclosure populations over three breeding seasons (May–August of 2016–2018) and compared vole fecal corticosterone metabolite (a non-invasive measure of adrenocortical activity; Palme 2019) levels by density. This experimental design allowed us to directly manipulate population density, while other environmental conditions were held constant. We then compared brain glucocorticoid receptor and mineralocorticoid receptor expression levels in the hippocampus of juveniles born in high or low densities, with the prediction that voles born in high-density populations may have lower levels of these receptors (Fig. 1). We discuss the overall evidence pertaining to whether or not elevated glucocorticoid levels are a viable mechanism for driving their population cycles.

Materials and methods

Study site and enclosures

Field enclosures were constructed at Koffler Scientific Reserve (King City, Ontario, Canada; 44°01′48″ N, 79°31′56″ W). 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 out. The outer perimeter was then surrounded by additional Vexar plastic fencing to a height of 1.5 m and an electric fence to prevent terrestrial predators from entering the grids (for pictures of the enclosures and the fence, see Fig. 1, Edwards et al. 2021a). All enclosures contained a 5 × 5 grid of Longworth live-traps spaced 5 m apart. High-density enclosures contained two traps at each grid point (50 traps), whereas low-density enclosures contained one trap at each grid point (25 traps).

In early May of each year, replicate low-density enclosures were established with founding populations of 4–6 voles, and high-density enclosures with founding populations of 20–26 voles. Enclosures had approximately 40–50% males and 50–60% females. These densities and sex ratios are based on naturally occurring conditions in wild meadow voles in Southern Ontario at the onset of the breeding season in spring, which had a minimum density of 96 voles/ha and a maximum density of 549 voles/ha between 1978 and

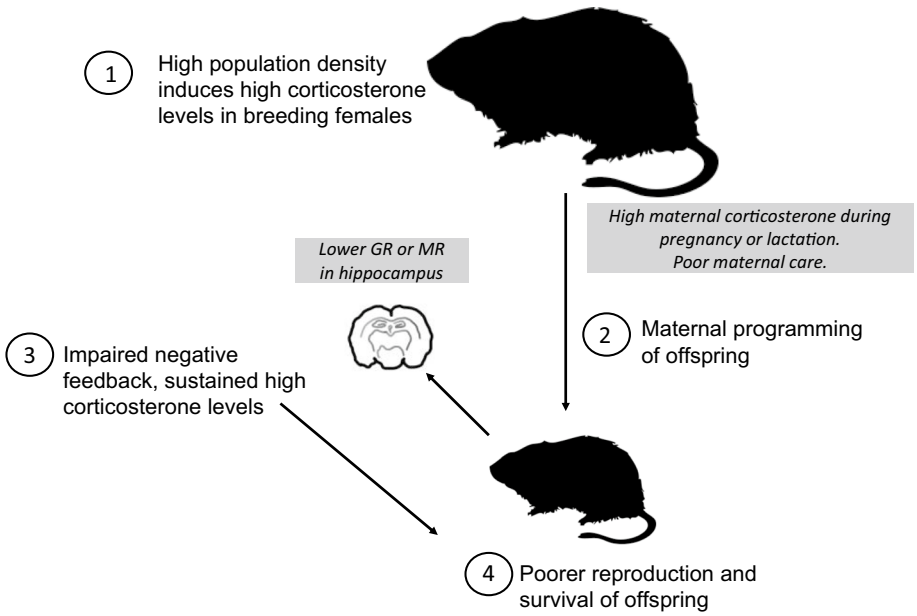

Fig. 1 A schematic of the hypothesis of how high population density could potentially program offspring phenotype in voles. High population density may elevate glucocorticoid (corticosterone) concentrations in adult voles, which could influence offspring during gestation, lactation, or by altered maternal behavior. If elevated maternal glucocorticoids alter offspring expression of glucocorticoid receptors or mineralocorticoid receptors, this could alter offspring negative feedback of the stress axis, resulting in elevated and sustained stress responses. Ultimately, these elevated stress responses could impact offspring fitness by affecting reproduction and survival

1981 (Boonstra and Rodd 1983). A founding density of 4–6 voles per enclosure is equivalent to 64–96 voles/ha and a founding density of 20–26 voles per enclosure is equivalent to 320–416 voles/ha.

To establish the founding enclosure populations, meadow voles were live-trapped in the meadows of the Koffler Scientific Reserve in the spring (late March-early April) of each of the 3 years of the study (2016, 2017, and 2018). At that time, most females were not yet reproductive. The number of voles captured in each of these 3 years was markedly different, with many fewer being caught in 2017, despite with much more effort being made in that year. These voles were then individually housed in the University of Toronto Scarborough wildlife research facility in 91.5 × 61 × 46 cm polypropylene cages. Voles 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) and maintained at a temperature of 15–20 C and a natural photoperiod. The purpose of this month-long holding period was to ensure all founding females released into the enclosures were not pregnant (meadow vole gestation period is 21 days). Thus, all animals born in field enclosures were conceived and gestated at either high or low density.

Live-trapping

In early May of each year, all voles temporarily housed in the wildlife research facility were randomized with respect to meadow of capture, and released into their respective enclosures simultaneously. In spring 2016 (the first year of the study), all enclosures were heavily live-trapped to remove any residents. In the springs of 2017 and 2018, we again live-trapped the enclosures to remove any voles that may have remained after our intense capture sessions at the end of the summers of 2016 and 2017. Low-density enclosures were maintained at low density by cropping them continuously throughout all summers by releasing animals outside of the fences. This cropping was performed during the weekly trapping sessions. Animals released during cropping were juveniles of weaning weight, equal sex ratio, to reduce potential artifacts from removing the original founding animals, which may have social dominance effects on the population. During all years, voles were live-trapped on a weekly basis from early May through August (~ 16 trap weeks a year). Longworth 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 (Ameri-marks, Utah). Body mass was measured using Pesola spring scales (± 1 g), the grid location was recorded, and reproductive condition was assessed. Female voles were considered breeding if they were lactating and/or had vaginal perforation and opening of the pubic symphysis. Male voles were considered breeding if their testes were scrotal and had reached a minimum body mass of 30 g, which is the median body mass males produce viable sperm (Keller and Krebs 1970).

Independent populations were established each spring, as all animals in the enclosures from the previous year were trapped out and released elsewhere (aside from those collected for tissue samples) at the Koffler Reserve at the end of August. When enclosures were trapped out each year, final population counts could be established, and this also ensured that vegetation would not be destroyed over the winter by high-density, resident populations. Treatment grids were switched each year so that high-density populations were in grids that had previously held low-density populations, to counteract any cumulative damage on the vegetation. Weekly population density was calculated as minimum number alive (MNA; Krebs 1966) per enclosure. Treatment differences in average enclosure MNA across the study period were compared to check if any enclosures markedly deviated in density from the treatment they were intended to be (Fig. 2). One enclosure which was initially intended to be high density, grid "F" in 2017, failed to stay at high density. This change appeared to be due to a disappearance early on in the study (May–June) of founding animals. Missing animals were not recovered, and may have either escaped through a weakness in the fence (gap or tunnel) or died, and were never trapped elsewhere. We reassigned this enclosure to low density. Any animals that were found to change grids during the study (by tunneling, etc.) were included in density estimates but were not included in analysis of treatment differences, as their density history was varied. 16 animals were documented as grid changers. The total number of enclosure

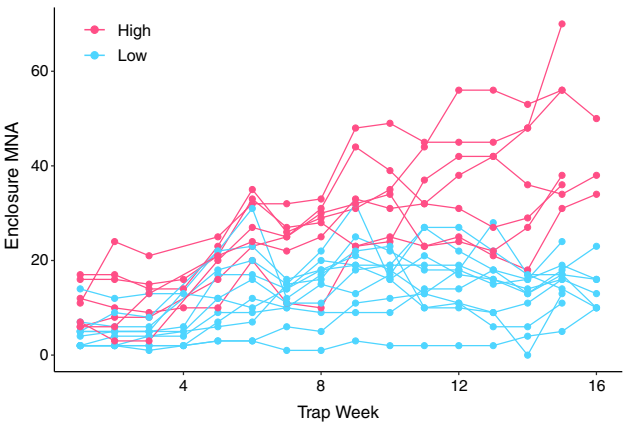

Fig. 2 Population average minimum number alive (MNA) across trap weeks in each 25 × 25 m enclosure during the 3 years of the study. Each line represents a separate enclosure population. Live-trapping began in early May (week 1) and concluded at the end of August (week 16). High-density treatment enclosures are in pink and low-density treatment enclosures are in blue

populations studied across all years was 18 (1 l low density, 7 high density). The total number of enclosures used each year was 6 in 2016, 4 in 2017, and 8 in 2018.

Fecal hormone metabolite analysis

Fecal samples were collected during 4–6 trapping sessions per year. FCMs represent circulating hormone levels approximately 5–7 h prior in meadow voles (Edwards et al. 2019), and therefore samples collected from traps represent baseline FCM levels, not levels reflective of trapping stress. On days of fecal sample collection, traps were set at 06:00 and checked at 08:00. Samples were then collected from the tunnels of the Longworth live-traps. Only a single individual is typically captured per trap, and tunnels were cleaned out between trapping sessions, thus the identity of the individual the sample was associated with was known. Feces contaminated with urine were not collected. Samples were placed into 1.5 mL vials and stored immediately in a cooler on ice packs until transfer to a −20 °C freezer later that day.

Fecal samples were weighed and extracted in a ratio of 0.05 g fecal matter to 1 mL 80% methanol, then extracts were diluted 1:100 in assay buffer. FCMs were measured by a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay (described Touma et al. 2003) that we have previously validated for this species (Edwards et al. 2019). Samples were run in duplicate. The inter-assay coefficient of variation of sample pools run on all plates was 9.6% and the intra-assay coefficient of variation of pools run at the beginning and end of plates was 12.9% ($N = 12$ plates).

Brain tissue collection and receptor expression

Brain samples were collected in late August of each year. Live-traps were set at 0600 h, and checked at 0800 h. Juvenile animals were collected with the criteria that they had not yet reached reproductive maturity (females had no vaginal perforation or lactational tissue and males had testes that had not descended into the scrotum). These animals were likely born in from mid-July to early August, though the exact ages were not known. Voles were transported in the live-traps to the Koffler Reserve laboratory (< 5 min from the field enclosures). Proceeding in a random order, each animal was removed from the trap and rapidly euthanized via isoflurane overdose. The brain was removed and placed on sterile aluminum foil inside a cooler filled with dry ice. After freezing, brains were wrapped in parafilm and transported on dry ice to the University of Toronto Scarborough where they were stored at −80 °C until analysis. Brains were sectioned into 30 µm slices with a Cryostat (Leica CM3050S, Leica Biosystems) and the hippocampus was dissected out based on brain landmarks in the Allen Mouse Brain Atlas (Lein et al. 2007). mRNA was extracted from the tissue using MasterPure Complete DNA and RNA Purification Kit (Epicentre).

To quantify gene expression, mRNA was converted to cDNA (High Capacity cDNA Conversion Kit, Applied Bio-Systems) and the quantity was assessed using a spectrophotometer (Nanodrop ND-2000C, Thermo Scientific). Primers were designed based on congener *M. ochrogaster* sequences (NCBI GenBank assembly accession GCA_000317375.1). Primer sequences for NR3C1 (glucocorticoid receptor) were: *Forward primer*: 5′-CAGAACTGGCAGCGCTTTTA-3′ and *Reverse primer*: 5′-AACGTCTGGAAGCAGTAGGT-3′. Primer sequences for NR3C2 (mineralocorticoid receptor) were: *Forward primer*: 5′-CGAGGCAGCTATGGAAACCA-3′ and *Reverse primer*: 5′-GGTCCTTTCTGCAGGTCCAA-3′. Amplicon lengths were 92 bp and 121 bp, respectively, annealing temperatures were 64 °C and 58 °C, respectively. qPCR reactions were performed using Fast SYBR Green master mix (Applied Biosystems) and 20 ng of cDNA. Purified water was run on each qPCR plate as a no template control. Samples were run in triplicate and analyzed with StepOne Plus real-time PCR software. A melt curve was run as a final cycle, and single product amplification was determined by the presence of a single peak. Glyceraldehyde phosphate dehydrogenase (GAPDH), actin beta (ACTB) and succinate dehydrogenase complex subunit A (SDHA) were chosen as reference genes (see Edwards et al. 2021a). We then tested whether hippocampal expression of these reference genes showed treatment effects; if so, would make them unsuitable as reference genes. ACTB did show an effect of treatment ($P = 0.02$), hence, the normalization factor was calculated for each sample using the geometric mean of GAPDH and SDHA. Treatments, sexes, and years were mixed across qPCR plates ($n = 3$ plates per gene). Relative gene expression of candidate genes for each sample was determined using the delta CT method (Schmittgen and Livak 2008), and the final values analyzed were the $-\Delta CT$ normalized to the series mean of each candidate gene.

Statistical analysis

Fecal corticosterone metabolite levels were analyzed using linear mixed effect models (LMMs) with year as a random effect in all models. While some individuals had up to 5 samples collected, the majority of individuals had one or two feces samples collected throughout the study and therefore repeated measures with individual ID as a random effect were not included. If an individual had multiple samples, only one per individual was kept in the analysis and the others were randomly dropped. The total sample size after dropping repeated individuals was 212 (121 female and 91 males). Among the females, 63 were breeding and 58 non-breeding. Among the males, 47 were breeding and 44 non-breeding. Fixed effects included in the models were

density treatment, sex, reproductive status (breeding or non-breeding), trap week (treated as a continuous variable), and all interaction effects. Since this model was heavily parameterized, we additionally performed stepwise model selection using the AIC values, and also report the results model with the lowest AIC score. This model had all of the effects in the omnibus model except for the status and trap week interaction effect, and the 3-way interaction effects (aside from density treatment, sex, status interaction which was still included). There was only one additional model within 2 AIC of the top model (Δ AIC = 1.62) and this model had the additional effect of the status and trap week interaction. Fecal corticosterone metabolite data was log transformed to so that residuals fit assumptions of normality and homoscedasticity. Figures showing fecal corticosterone metabolite levels also display log transformed data due to the high variation in raw concentrations.

For hippocampal gene expression, 80 juvenile brains (49 females and 31 males) were analyzed. To compare receptor expression by treatment, we used mixed models using year as a random effect and treatment, sex, and the treatment and sex interaction as fixed effects. For this relative receptor expression data, due to the skew of the data, we used generalized linear mixed models (GLMMs) with a Gamma distribution for each of the response variables (glucocorticoid receptor and mineralocorticoid receptor). All analyses were conducted in R version 3.6.3 (R Core Team 2020) and models were built using the package 'nlme' (Pinhiero et al. 2020) and 'lme4' (Bates et al. 2015).

Results

In the main model with all interaction effects (Table 1A), the main effect of density treatment was not statistically significant ($\beta = -0.22 \pm 0.21$, $p = 0.31$) and had a comparatively smaller effect size relative to the main effects of sex ($\beta = -0.32 \pm 0.22$, $p = 0.15$) and reproductive status ($\beta = 0.30 \pm 0.18$, $p = 0.10$). There was a significant negative effect of trap week ($\beta = -0.03 \pm 0.01$, $p = 0.01$), with fecal corticosterone metabolite levels declining as trap weeks progressed from the spring to end of summer.

In the top model as based on AIC score (Table 1B), the main effect of density treatment was again not statistically significant ($\beta = -0.21 \pm 0.14$, $p = 0.15$), but there was a significant density treatment and trap week interaction effect ($\beta = -0.03 \pm 0.01$, $p < 0.01$), indicating that fecal corticosterone metabolite levels within the two treatments did not show the same pattern across trap weeks (Fig. 3). There was a sex and trap week interaction effect ($\beta = 0.03 \pm 0.01$, $p < 0.01$; Fig. 3), but a density, sex, and trap week 3-way interaction effect was not present in the best fit model. There was additionally a significant main effect of sex on fecal corticosterone metabolite levels ($\beta = -0.40 \pm 0.14$, $p < 0.01$), with males having lower fecal corticosterone metabolite concentrations than females, and a marginally significant main effect of reproductive status ($\beta = 0.16 \pm 0.08$, $p = 0.05$), though the effect size of reproductive status was smaller than that of both sex and density treatment, albeit with less variation. Important for this relationship, there was a highly significant sex and reproductive status interaction ($\beta = -0.55 \pm 0.12$, $p < 0.001$), where breeding females had similar fecal corticosterone metabolite levels to non-breeding females (Tukey post hoc $p = 0.35$), but breeding males had lower fecal corticosterone metabolite levels than non-breeding males (post hoc $p = 0.01$).

Finally, there was a marginal treatment, sex, and status interaction effect ($\beta = 0.37 \pm 0.20$, $p < 0.06$; Fig. 4). Post hoc tests indicated breeding females did not differ in fecal corticosterone metabolite by density ($\beta = 0.07 \pm 0.09$, $p = 0.99$), nor did non-breeding females differ by density ($\beta = -0.03 \pm 0.09$, $p = 0.99$). Similarly, neither breeding males ($\beta = -0.26 \pm 0.11$, $p = 0.20$), nor non-breeding males ($\beta = 0.00 \pm 0.12$, $p = 0.99$) differed by density. However, breeding males at high density had lower fecal corticosterone metabolite levels than non-breeding males at high density ($\beta = 0.39 \pm 0.09$, $p < 0.001$). Breeding males at low density had comparable fecal corticosterone metabolite to non-breeding males at low density ($\beta = 0.13 \pm 0.14$, $p < 0.98$; Fig. 4).

Hippocampal mRNA expressions of glucocorticoid and mineralocorticoid receptors in juvenile voles are shown in Fig. 5. There was a significant effect of density treatment on glucocorticoid receptor expression levels ($\beta = 0.47 \pm 0.20$, $p = 0.02$), no significant main effect of sex ($\beta = 0.02 \pm 0.14$, $p = 0.90$), but a significant density and sex interaction effect ($\beta = -0.70 \pm 0.24$, $p < 0.01$). Post hoc testing indicated that females born at high density had marginally higher glucocorticoid receptor expression than females born at low density ($\beta = -0.48 \pm 0.20$, $p = 0.07$), but male glucocorticoid receptor expression did not differ by density ($\beta = 0.22 \pm 0.14$, $p = 0.38$). In contrast, there was no effect of density on mineralocorticoid receptor expression levels ($\beta = 0.15 \pm 0.13$, $p = 0.23$), nor of sex ($\beta = 0.27 \pm 0.20$, $p = 0.17$), though the effect size of sex for mineralocorticoid receptor expression was considerably higher than the effect size of sex for glucocorticoid receptor expression. There was an interaction effect of density and sex on mineralocorticoid receptor expression levels ($\beta = -0.60 \pm 0.26$, $p = 0.02$), but post hoc tests reported no particular between sex and density treatment comparisons were statistically significant (all $p > 0.20$).

Table 1 Summary of the mixed effect models where fecal corticosterone metabolite concentrations (log ng/g) were fit to density treatment (high or low; high as the intercept), sex (female or male; female as the intercept), reproductive status (breeding or non-breeding; non-breeding as the intercept), trap week (continuous) and their interaction effects

	Parameter	Estimate ± SE	*df*	*T*	*p*
(A)	**Intercept**	**3.52 ± 0.13**	**194**	**27.43**	**0.00**
	Density treatment	−0.22 ± 0.21	194	−1.02	0.31
	Sex	−0.32 ± 0.23	194	−1.43	0.15
	Reproductive status	0.30 ± 0.18	194	1.64	0.10
	Trap week	**−0.03 ± 0.01**	**194**	**−2.55**	**0.01**
	Treatment × sex	0.00 ± 0.45	194	0.01	0.99
	Treatment × status	−0.13 ± 0.29	194	−0.44	0.66
	Sex × status	**−0.73 ± 0.28**	**194**	**−2.60**	**0.01**
	Treatment × trap week	0.03 ± 0.02	194	1.43	0.15
	Sex × trap week	0.03 ± 0.02	194	1.49	0.14
	Status × trap week	−0.02 ± 0.02	194	−0.84	0.40
	Treatment × sex × status	0.38 ± 0.52	194	0.73	0.47
	Treatment × sex × trap week	−0.00 ± 0.04	194	−0.10	0.92
	Treatment × status × trap week	0.00 ± 0.03	194	0.04	0.97
	Sex × status × trap week	0.02 ± 0.03	194	0.75	0.45
(B)	**Intercept**	**3.57 ± 0.11**	**199**	**33.62**	**0.00**
	Density treatment	−0.21 ± 0.14	199	−1.45	0.15
	Sex	**−0.40 ± 0.14**	**199**	**−2.77**	**< 0.01**
	Reproductive status	**0.16 ± 0.08**	**199**	**2.00**	**0.05**
	Trap week	**−0.03 ± 0.01**	**199**	**−3.93**	**< 0.01**
	Treatment × sex	−0.03 ± 0.15	199	−0.23	0.82
	Treatment × status	−0.10 ± 0.13	199	−0.82	0.41
	Sex × status	**−0.55 ± 0.12**	**199**	**−4.59**	**< 0.001**
	Treatment × trap week	**0.03 ± 0.01**	**199**	**2.33**	**0.02**
	Sex × trap week	**0.03 ± 0.01**	**199**	**3.18**	**< 0.01**
	Treatment × sex × status	0.37 ± 0.20	199	1.86	0.06

Samples were collected from 63 breeding females, 58 non-breeding females, 47 breeding males, and 44 non-breeding males. Bold font indicates $P < 0.05$. (A) The omnibus model with all factors and their interaction effects. AIC = 150.73. The random effect of year was included in the model ($\sigma = 0.06$). The $r^2_{(m)} = 0.34$ and the $r^2_{(c)} = 0.37$. (B) The top model based on stepwise model selection using AIC values. AIC = 141.95. This model did not include the status and trap week interaction effect, nor any of the 3-way interaction effects except for treatment × sex × status. The random effect of year was included in the model ($\sigma = 0.06$). The $r^2_{(m)} = 0.35$ and the $r^2_{(c)} = 0.38$

Discussion

Our experiment resulted in several key pieces of evidence in understanding the relationship between population density and stress in vole cycles. First, we found that while density alone had no detectable direct influence on fecal corticosteroid metabolites levels, density treatment interacted with trap week to impact fecal corticosteroid metabolites levels, declining as trap weeks progressed at high density, but not low density (Fig. 3). In high-density populations, fecal corticosteroid metabolites levels were peak in spring in the early breeding season, and lower towards the end of the summer. The opposite pattern was seen at low density. While the impact of trap week on fecal corticosteroid metabolites levels was influenced by sex (Fig. 3), surprisingly, no 3-way interaction effect between density, trap week, and sex was present in the top model. However, it is clear that the negative relationship of fecal corticosteroid metabolites levels across trap weeks at high density is primarily driven by the females, as males at high density do not show this pattern (Fig. 3). The seasonal findings in our study align with meadow vole reproductive biology, where the early breeding season is a competitive time for breeding females acquiring territories. Females are socially tolerant during the fall and winter, when most individuals are not breeding, but by early May establish exclusive territories at the onset of reproduction (Bujalska 1973; Madison 1980; Ostfeld et al. 1988). This spring change in female social behavior is known to be driven by reproductive hormone levels and photoperiod (Beery et al. 2008), and acts to increase offspring survival through securing resources and potentially preventing infanticide by unrelated females (Boonstra 1980; Ostfeld et al. 1988; Wolff and Peterson 1998; Jonsson et al. 2002). Male meadow voles do not undergo this process; they have highly

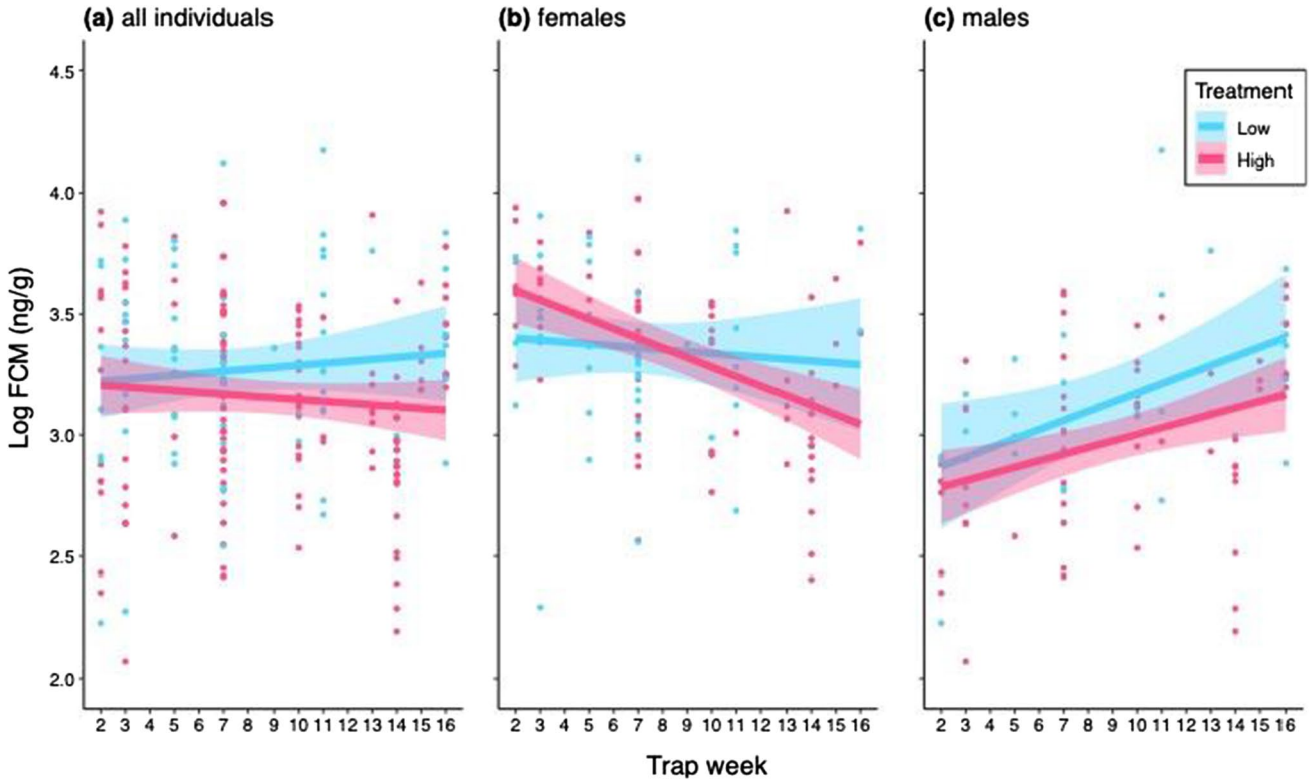

Fig. 3 Fecal corticosterone metabolite concentrations (log FCM ng/g) by density treatment, trap week, and sex in: **a** all meadow voles, **b** female voles only, and **c** male voles only. Live-trapping began in early May (week 1) and concluded at the end of August (week 16). High-density treatment enclosures are in pink and low-density treatment enclosures are in blue. There was a significant density and trap week interaction effect on fecal corticosterone metabolite levels ($p = 0.02$), as well as a sex and trap week interaction effect ($p < 0.01$), but no 3-way interaction was present in the best fit model, nor was it significant in the omnibus model

overlapping territory and their spatial distribution is driven instead by the presences of reproductive females in the area (Madison 1980; Boonstra and Rodd 1983; Edwards et al. 2019). Hence, higher fecal corticosteroid metabolites levels in females in the early breeding season, particularly at high density, could potentially be associated with establishing this spacing behavior.

We additionally detected a density treatment, sex, and reproductive status interaction effect. At high density, breeding males had significantly lower fecal corticosteroid metabolites levels than non-breeding males, but this difference was not seen at low density. This indicates that the overall reproductive status effect within males in the dataset is particularly driven by the high-density males. Among females, post hoc tests indicated that there were no differences in fecal corticosteroid metabolites levels at high and low density. It is important to note that we have found evidence of reduced reproduction at high density in these same populations of meadow voles. In high-density enclosures, there were a lower proportion of sexually mature animals, and within the adult (> 20 g) females, in high-density enclosures there were a lower proportion of lactating individuals, particularly later in the summer (Edwards et al. 2021a). Therefore, the high-density treatment was sufficient to induce reduced reproductivity in young born on the grid and in mature females in summer. However, we did not detect clear fecal corticosteroid metabolites effects by density in these same populations, indicating that these reproductive effects may not be driven directly by the stress axis.

We found little evidence of inherent changes in the stress axis of juveniles born at high or low density. We predicted glucocorticoid receptors would be lower at high densities, as studies in some other species have shown that maternal or early environmental stress reduced these receptor levels, decreasing negative feedback of the stress axis (Meaney 2001). However, males showed no detectable differences in glucocorticoid receptor expression in the hippocampus (Fig. 5). Female juveniles had higher glucocorticoid receptor levels at high density, but this relationship was contrary to our predictions of reduced capacity for negative feedback at high density. This does not support the hypothesis that high density results in stress axis dysregulation in juveniles, which may have served as a proximate explanation for the poorer juvenile survival observed in decline populations. Females may possibly instead be better equipped for stress axis negative feedback

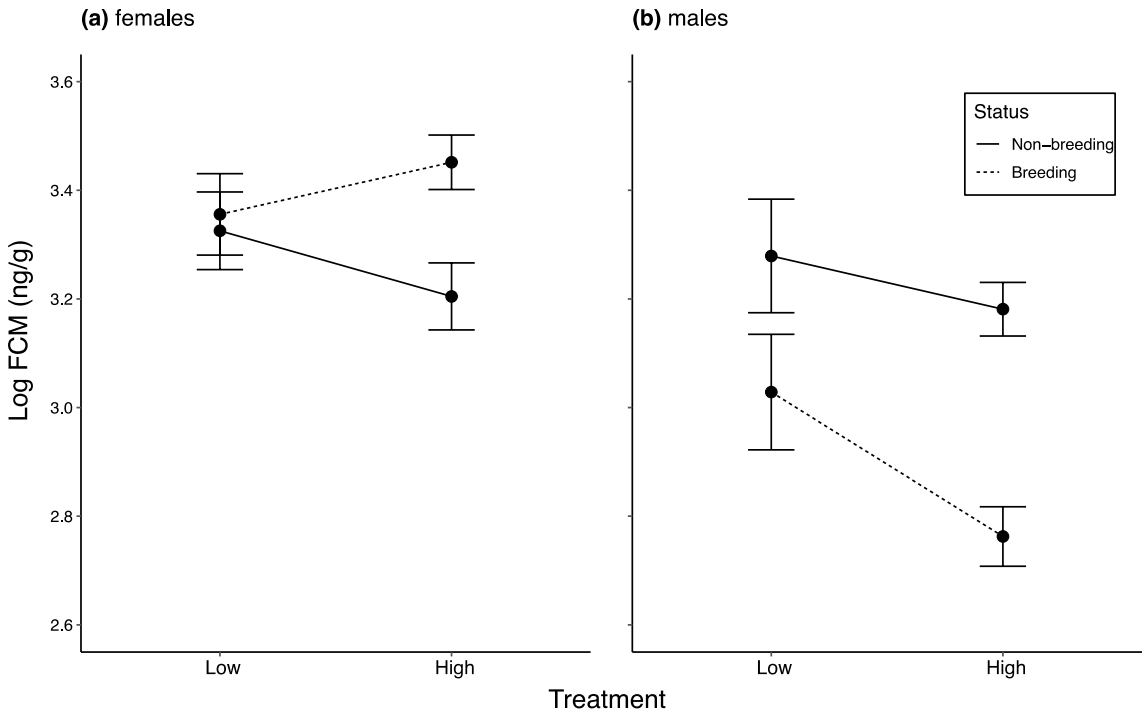

Fig. 4 The interaction effect between density treatment and reproductive status for observed data on fecal corticosterone metabolite concentrations (log FCM ng/g) in: **a** female meadow voles, and **b** male meadow voles. Error bars represent standard error. Breeding individuals are marked with a dashed line, and non-breeding individuals are marked with a straight line. Post hoc tests indicated that, at high density, breeding males have lower fecal corticosterone metabolite concentrations than non-breeding males ($p < 0.001$)

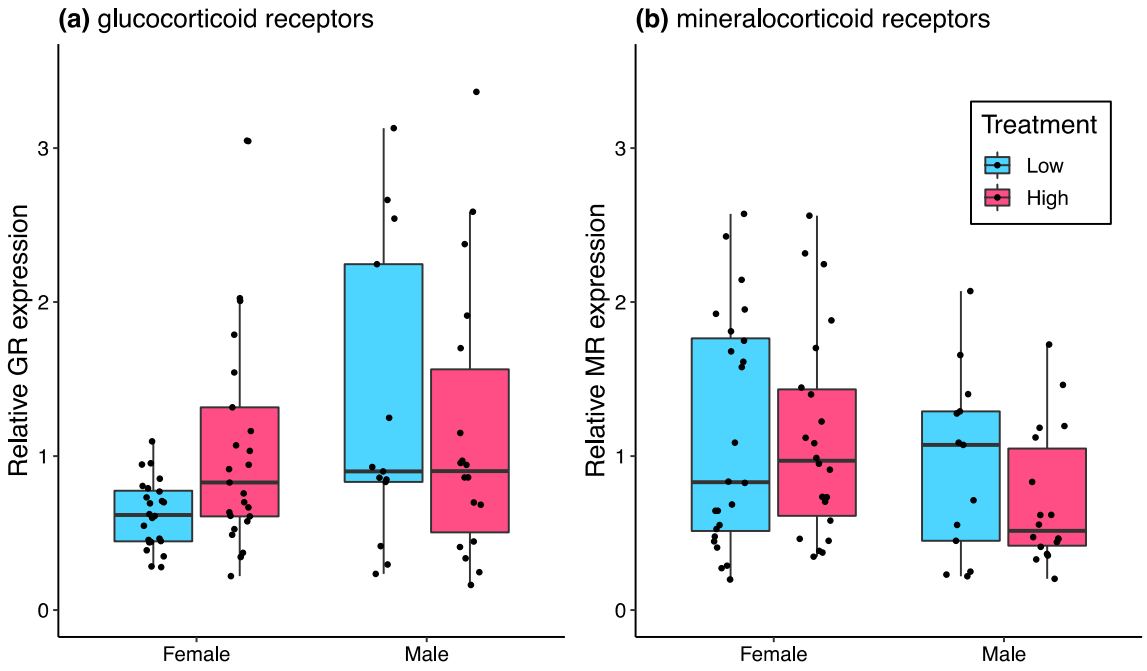

Fig. 5 The relationship between density treatment and **a** glucocorticoid receptor, and **b** mineralocorticoid receptor expression in hippocampal tissue collected from juvenile voles. Juvenile females ($N = 49$) born at high density had marginally higher glucocorticoid receptor expression than those born at low density ($p = 0.07$). There were no detectable effects of density on male ($N = 31$) glucocorticoid receptor expression ($p = 0.38$), or mineralocorticoid receptor expression overall ($p = 0.23$)

when they are born in high population densities. Neither sex showed density-related differences in mineralocorticoid receptor expression.

These GC results can be compared with those of other vole studies that examined corticosterone level differences in high and low-density populations, either monitored naturally over population cycles or manipulated in field enclosures (Table 2). We did not include studies that compared singly and group housed voles in the laboratory (e.g., Kravchenko et al. 2016) nor studies that compared laboratory rooms with either many or few cages of singly housed voles to simulate high and low density (e.g., Nelson et al. 1996). Though these laboratory studies have value for understanding other aspects of vole biology, these conditions have little ecological relevance to natural density conditions where individuals compete for space, resources, and reproductive access. Several vole studies have found a positive association between GCs and population density; however, this effect was not found in all studies (Charbonnel et al. 2008; Blondel et al. 2016, our study).

Understanding the confounding factors involved in the density and corticosterone relationship is critical in reconciling these results. In our study, there was a seasonal effect, where fecal corticosterone metabolites were higher early in the breeding season and declined as the summer progressed. Population density within the enclosures tended to increase across the breeding season as new litters were born (Fig. 2; though low-density enclosures were cropped), the overall trajectory of fecal corticosterone metabolites over the breeding season was in the opposite direction of potential density-driven stress: they were higher earlier in the breeding season when density was lower. Therefore, we do not think this main effect of trap week is driven by increasing density, but is instead an effect of spacing behavior early in the breeding season as females establish territories. Differences in fecal corticosterone metabolite levels across the breeding season raise the possibility that density and GC studies conducted at different parts of the breeding season could have different results. For example, a study found that in fossorial water voles (*Arvicola scherman*) in France, GC levels were lower at peak density than they were in the following decline year (Charbonnel et al. 2008). This study was conducted at the end of their reproductive period (September), which could be a time of generally lower GC levels at high density, if they are similar to our meadow vole populations in this respect. Alternately, this could indicate that GC levels are not responding to immediate population density in this species, and may be instead driven by delayed density-dependent effects. In southern red-backed voles (*Myodes gapperi*), late breeding season populations displayed a marginal increase in GC levels at high density, which may potentially have been more pronounced had earlier breeding season animals been sampled (Harper and Austad 2004).

Another set of contradictory data is the relationship between population density and corticosterone levels in male voles. We found that breeding male meadow voles had generally low fecal corticosterone metabolite levels compared to other groups, but this effect was less pronounced at low density, where breeding males had higher levels that were more comparable to non-breeding males (Fig. 4). This is somewhat similar to findings in Blondel et al. (2016) where adult male prairie voles had higher GC at low density. A possible explanation for this effect in males may be that, at low density, breeding males are expending more energy seeking the few estrous females in the area. Interestingly, both our study and Blondel et al. (2016) used enclosure populations, so it is possible that we created a stressor for breeding males at low density by restricting their ability to seek receptive females elsewhere. All of the studies that found the opposite density effect in males were in free-ranging populations (c.f. Bian et al. 2015). It may be that the primary concern for female voles during the breeding season is acquiring space and excluding competitors, whereas the primary concern for male voles is finding mates throughout the breeding season. Hence, females may be more physiologically responsive to changes in the social environment (clearer seasonal pattern in fecal corticosterone metabolites, increased glucocorticoid receptor levels at high density).

We conclude that in our populations, there does not appear to be density-related programming of the stress axis (at least at the level of the hippocampus) nor markedly elevated maternal GCs. It may be that differences in offspring phenotype at high and low density still occur through maternal programming, but are programmed by mechanisms other than GCs. Maternal programming can occur by many other non-GC physiological mechanisms including androgen levels and quality/bioactive compounds in milk, among others (reviewed Edwards et al. 2021b). It is clear that voles are highly susceptible to maternal programming. Van Cann et al (2019a, b) created early growth conditions in the laboratory (repeated social confrontation and protein diet manipulation during pregnancy and nursing) that then affected both male and female offspring phenotype and survival in the field, though the proximate mechanism was unknown. However, we stress that to understand the basis of vole population cycles, one needs to replicate as close as possible the conditions that the voles experience in nature. Our evidence in these meadow vole populations indicate that juveniles born at different population densities have differential expression of gonadotropin releasing hormone mRNA in the brain, though we do not know if this is driven by maternal programming or another early-life mechanism (Edwards et al. 2021a). Aside from maternal effects, conditions in the early-life environment at high density may act to influence reproduction and other aspects of phenotype, such

Table 2 Studies examining population density or population cycle phase and corticosterone levels in wild voles

Study	Species	Population	Factors in analysis	Sample type	Collection	Corticosterone levels
Boonstra and Boag (1992)	Meadow vole *Microtus pennsylvanicus*	Early breeding season (March–May)	Split by sex	Plasma	Following live-trapping, trap-stress elevated	Density: high density ↑ (both sexes)
		Southern Ontario, Canada	Female reproductive status: non-reproductive, lactating, pregnant			Sex: females ↑ Reproductive: pregnant and lactating females ↑
Novikov and Moshkin (1998), Novikov et al. (2012)	Northern red-backed vole *Myodes rutilus*	Breeding season (June–August)	Split by sex	Plasma	Live-trapped voles habituated to the laboratory for 3–5 days and sampled in the laboratory	Density: high density ↑ (all except overwintered females)
		Sampled from cycling populations over 5 years	Age: overwintered or young of the year			Sex: females ↑ Reproductive: breeding males ↓
		Lake Teletskoye valley, Russia.[a]	Reproductive status: sexually mature, immature, pregnant females			No difference among females, except pregnant females ↑
Harper and Austad (2004)	Southern red-backed vole *Myodes gapperi*	Late breeding season (July–November)	Sex	Plasma and feces	Voles taken from live-traps to the laboratory for 2 months prior to blood sample collection	Density: high density marginally ↑ (both sexes)
			Age: juvenile or adult			Sex: no difference
					Feces collected from live-traps	Age: no difference
			Reproductive status: sexually mature or immature			Reproductive: no difference
Charbonnel et al. (2008)	Fossorial water vole	Late breeding season (September)	Sex	Feces	Collected from live-traps	Density high density ↓ than decline (both sexes)
	Arvicola scherman	Sampled from a high density year and a subsequent decline year	Age: months			Sex: females marginally ↑
			Reproductive status: sexually mature or immature			Reproductive: breeding animals ↓ (both sexes)
		Jura Mountains, France	Body condition			
			Study site			
Navarro-Castilla et al. (2014)	Common vole *Microtus arvalis*	Summer populations (July)	Sex	Feces[b]	Collected from live-traps	Density: high density ↑ (both sexes)
			Body mass. Reproductive status: breeding or non-breeding			Sex: females ↑ Reproductive: breeding females ↑
			Habitat type: crops or field			no difference in males (but breeding male $n = 3$)

Table 2 (continued)

Study	Species	Population	Factors in analysis	Sample type	Collection	Corticosterone levels
Bian et al. (2015)	Root vole *Microtus oeconomus*	Fall and spring populations	Trapping session	Feces	Collected from live-traps	Density: high density ↑ (both sexes)
		Density-manipulated enclosure populations				Sex: no difference a priori
						Reproductive: N/A
						Trapping session: later in the year ↑ (both sexes)
Blondel et al. (2016)	Prairie vole *Microtus ochrogaster*	Two sets of late breeding season 3-week trials (August and October)	N/A: adult males only sampled	Feces	Collected within 90 min of live-trapping	Density: high density ↓ (males only)
		Density-manipulated enclosure populations				Sex: N/A
						Reproductive: N/A
Current study: best fit model	Meadow vole *Microtus pennsylvanicus*	Breeding season (May–August)	Sex	Feces	Collected from live-traps	Density: no main effect
		Density-manipulated enclosure populations	Reproductive status: breeding or non-breeding			Sex: females ↑
						Reproductive: breeding males ↓
		Southern Ontario, Canada	Trap week			Trap week: ↑ in earlier trap weeks (main effect and at high density)

[a]Novosibirsk Scientific Center population was also reported in the 2012 study, but never reached cyclic highs in density, and so comparisons are not reported here

[b]No published validation of the FCM methods used in this study, to our knowledge

as resource availability, diet and plant defenses (Massey et al. 2008; Reynolds et al. 2012), pheromone exposure (Massey and Vandenbergh 1980; Kruzczek et al. 1989), and social interactions with conspecifics. Thus, there are many other potential mechanisms that may shape the vole decline phenotype aside from GCs alone.

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Author contribution statement PE and RB designed the research, collected the data, and wrote the manuscript; RP developed and provided the fecal metabolite assays; all the authors contributed to revising the manuscript and gave final approval for publication.

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Availability of data and materials The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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