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Evaluating sex differences in behaviour and glucocorticoids of rodents

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Abstract

In rodents, different selective pressures influence behavioural, physiological and life-history strategies between sexes. Anisogamy and the reproductive cost hypothesis suggests that differences in gamete size and trade-offs in reproduction are driving mechanisms of sex-specific reproductive strategy. However, relationships between behaviour and energetic investment in income-breeding rodents are not fully explored. We investigated behavioural and physiological traits in two rodent species from Algonquin Provincial Park, Canada using two standardized behavioural assays and faecal glucocorticoid metabolites (FGMs) as a proxy for movement and energetic stress. We hypothesized that sex differences in reproductive investment throughout a single breeding season would influence behavioural and physiological traits. We predicted that males would be more explorative and less docile than females due to increased risks associated with mate acquisition. We also predicted that FGMs would be greater in females compared to males due to the increased investment in the development and care of young. In contrast to our hypothesis, we observed some differences in behaviour between sexes in the opposite direction. Male deer mice (*Peromyscus*

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maniculatus) were more docile (mean difference = 0.312, 95% CI = [−0.24; 0.87], $p = 0.27$), and male red-backed voles (*Clethrionomys gapperi*) were less explorative (mean difference =-73.8 s, 95% CI = [−127.5; −19.029], $p = 0.01$) than female counterparts. There was also a high degree of within-individual variation in FGMs in both species. Between-individual variation was only observed in red-backed voles (26.7%), however neither species had a significant relationship between sex and FGMs. Our findings reveal some relationships between behaviour and physiology in income-breeding rodents.

Keywords
anisogamy, corticosterone, docility, exploration, glucocorticoids, income-breeding, reproduction, sex differences.

1. Introduction:

The cost of reproduction hypothesis posits that differences in the energetic investment associated with varied reproductive roles between sexes results in the divergence of life-history strategies (Clutton-Brock & Parker, 1992). Further work on life-history theory has suggested that relationships among life-history traits evolve along a predictable fast-slow continuum (Réale et al., 2010). Fast-paced life history strategies are predicted to have high reproductive output with low investment in parental care, express explorative and bold behaviours, and invest less in self-regulation, including a lower metabolic reactivity (Réale et al., 2010; Hall et al., 2015; Santostefano et al., 2017). Slow-paced life history strategies include traits that reflect the opposite, with a lower reproductive output yet higher investment in young individuals (Réale et al., 2010).

Intra-variation in life-history, behaviour and physiology between individuals is well described; however, sexual differences are less well understood (Tarka et al., 2017). Differences in reproductive roles between sexes can result in sex-specific variation regarding self-maintenance, body composition, organ size, and reproductive strategy (Schulte-Hostedde et al., 2001; Casselman & Schulte-Hostedde, 2004; Hämäläinen et al., 2018; Tarka et al., 2018). Energetic trade-offs between reproductive investment and self-preservation drive natural and sexual selection, resulting in sexually dimorphic traits or sexual differences in behaviour and physiology that influence the position of individuals along the predicted fast-slow continuum (Hedrick & Temeles, 1989; Fairbairn et al., 2007; Shutler, 2010).

Trait expression between sexes often stems from sex-specific reproductive roles influenced by anisogamy and trade offs between self preservation and mate acquisition (Clutton-brock & Parker, 1992; Lehtonen et al., 2016). Anisogamy suggests differences in gamete sizes between sexes influence behavioural and physiological traits, resulting in males that invest more in mate acquisition and females that invest more in self-preservation and the development of offspring. (Schärer et al., 2012; Lehtonen et al., 2016). Males often express behavioural and physiological traits representative of faster life history strategies compared to female counterparts (Tarka et al., 2018). Sex is an important variable when evaluating physiological or behavioural phenotypes. Selective pressures influence physiological traits including immunity (Lee, 2006; Love et al., 2008; Monceau et al., 2017), metabolism (Rønning et al., 2016; Shingleton & Vea, 2023), hormone production (Nelson, 2005), and thermoregulation (Van Jaarsveld et al., 2021) between sexes. Consequently, selective pressures favour alternative behavioural strategies, including in risk-taking behaviours (Holtby & Healey, 1990), foraging patterns (De Pascalis et al., 2020) and aggression (Fresneau et al., 2014). Given the assumptions of anisogamy, males express behavioural and physiological traits differently than females, including higher exploration and risk-taking and reduced physiological reactivity to environmental stimuli.

Stress is the energetic change and physiological response by an organism to environmental stimuli (Hobfoll, 1988; Romero, 2004; Costantini, 2008). Faecal glucocorticoid metabolites (FGMs) are a common non-invasive proxy of metabolic stress in animals (Palme, 2012, 2019; but see MacDougall-Shackleton et al., 2019 for an overview of why glucocorticoids are not "stress" hormones specifically). Glucocorticoids, such as corticosterone, are a group of steroid hormones released from the hypothalamic-pituitary-adrenal axis (HPA-axis) that assist in regulating metabolic function in response to external stimuli (Toufexis et al., 2014; Palme, 2019). Faster strategies are associated with increased exploration and risk-taking behaviour (more proactive strategies) to accommodate mate acquisition (Réale et al., 2010). Consequently, increased exploration-activity, and risk-taking benefit from a lower reactivity to environmental stimuli — thus fast paced individuals express a lower fluctuation of FGMs (Boyce & Ellis, 2005). In contrast, slow-paced strategies prioritize self-preservation (more reactive behaviours) and are more responsive to novel stimuli. Therefore, in maternal-caring rodents males are predicted to express more proactive strategies compared to female counterparts due to their lower investment in young.

Anisogamy and the reproductive cost hypothesis is well described in rodents (Dewsbury, 1982; Roldan et al., 1992; Ramm et al., 2005), where males and females often express alternative behavioural strategies and physiology relating to energetic demands during reproduction (Eccard & Herde, 2013; Immonen et al., 2018). A greater investment in offspring development for females (pregnancy and lactate production) is metabolically expensive and requires increased energetic investment during breeding (Reeder & Kramer, 2005). Thus, we expect differences in behaviours associated with energy gain between sexes.

Despite the importance of sex-specific selective pressures driving life history traits, comparatively few studies investigate sex-differences between physiological and behaviour characteristics (Hämäläinen et al., 2018). In this study, we examined differences in behavioural and physiological strategies between sexes, and within-individuals to determine potential correlations between strategy and reproductive role. We quantified sexual differences in exploration and docility as a proxy of movement and risk-taking behaviour and measured physiological stress through FGMs (Palme et al., 2019), between sexes in two species of rodent, the deer mouse (*Peromyscus maniculatus*), and red-backed vole (*Clethrionomys gapperi*). Both deer mice and red-backed voles are polygynous, with only maternal care of offspring. Both species can have multiple litters in a single reproductive season, with deermice having 4-6 pups on average, and red-backed voles 4–5 pups (Maser et al., 1981; Merrit et al., 1981). Deer mice and red-backed voles both breed year-round in favourable conditions; however, in more northern populations individuals typically invest in reproduction from May through September (Wolff & Sherman, 2008).

We hypothesized that differences in reproductive roles will lead to an alternative expression of traits between sexes. Thus, we predicted that males will express more proactive behavioural phenotypes, including being more exploratory and less docile than females within the same species. Likewise, we predicted that actively breeding males would consistently display lower concentrations of FGMs compared to female conspecifics, but higher FGMs compared to non-reproductive (non-scrotal) males.

2. Methods

2.1. Capture and handling of animals

Deer mice and red-backed voles were surveyed in Algonquin Provincial Park, Ontario, Canada (45°54′N, 78°26′W), from May through September 2022 across 17 historic, and 3 newly established traplines. Each trapline consisted of 20 Sherman traps (H.B. Sherman Traps, Tallahassee, FL, USA) baited with water-soaked sunflower seeds. Traplines were composed of 100-m transects with two traps placed every 10 m, covering an array of forest habitats (see Fryxell et al., 1998 for description of methods and habitat). Historic traplines were baited at dusk and checked the following morning for 3 consecutive nights every other week on a staggered schedule. The newly established traplines were set using the same methods but were only baited every other week during August and September since they were part of another study. Individuals were marked with metal ear tags containing unique alphanumeric codes (National Band and Tag, Newport, KY, USA) for identification. For each individual, we recorded the sex (male or female), age class (juvenile, sub-adult, or adult) based on body mass and hair colour (Schmidt et al., 2019), body mass using a 0.1 g Pesola scale, and reproductive status measured as scrotal or non-scrotal (absence or presence of testes) for males, and non-reproductive (defined as no visible signs of reproduction including an enlarged abdomen, or visibly enlarged mammary glands), pregnant, perforate, or lactating for females.

2.2. Behavioural analyses

Deer mice and red-backed voles were tested in either an open-field test or a handling bag test during each capture day. Since all tests were performed in the wild, and only one test was performed each day, we could not control the order individuals were exposed to tests. All tests were recorded using a portable camera (Sony HDR-CX405) and were later analysed using the behavioural tracking software CowLog 3.0 (Pastell, 2016). For the handling bag tests, individuals were transferred directly from the Sherman trap to a clear, plastic holding bag and left to move freely for 60 s. The handling bag tests is a commonly used behavioural assay for measuring docility, recorded as an individual’s tendency to respond to potential predators (Martin & Réale, 2008). During the handling bag test, bags were held at arms length from the observer < 1 meter off the ground, thus we consider that individuals reacted accordingly to a perceived human-predator. Docility was then measured as the total time an individual spent immobile during the test-period. The open field test consisted of a black plastic arena (51 $\times$ 41 $\times$ 74 cm) with an 8.89 cm PVC opening and a mesh barrier overtop to allow recording of the individual during the test. All open-field tests occurred directly in the field; thus, individuals were transported from the trap immediately to a clear, plastic holding bag immediately after handling. Individuals were then introduced to the arena through the opening and were left to freely explore the arena for a total of 5 min. The open field test was used to measure exploration-activity, as the total amount of time an individual moves freely through a novel, non-risky environment (Carter et al., 2013). To ensure animals were reacting to the arena and not the researcher, once the individual was transferred to the arena, the researcher left the animals field of view. Between each trial, the arena was cleaned using an 80% vinegar solution and then rinsed with water.

2.3. Faecal collection, extraction, and immunoassay analyses

All materials used for the immunoassays were purchased from Avantor Sciences (Avantor, Radnor, PA, USA) unless otherwise specified. Faecal samples were used as a non-invasive measure of glucocorticoid levels in both species. All materials used for the collection of faecal samples were purchased from Fisher Scientific (Thermo Fisher Scientific, Pittsburgh, PA, USA). The total number of faecal samples retrieved with a corresponding behavioural test for each age class, reproductive condition and sex can be found in Table 1. Small mammals often defecate in response to handling; therefore, faecal samples were collected immediately after defecation during handling and placed in an Eppendorf tube containing 1 ml of 80% methanol. Where not possible to collect faecal samples during handling due to insufficient sample volume or lack of defecation, faecal samples were collected from the Sherman trap, no later than 19 h after defecation (Veitch et al., 2021). To control for individual mass, only 1–2 pellets were collected from each individual, with attention to avoid samples potentially contaminated with urine. Eppendorf tubes were then placed on ice in a cooler and were later transferred to a −20°C freezer for temporary storage during our four-month collection period. All samples were later transferred to a −80°C freezer in September, where they were stored until hormone analyses were performed. Traplines were checked in the same order each week to measure faecal sample decay. The maximum 19-h collection period does not influence FGM concentrations based on trap order within this project (Veitch et al., 2021).

Table 1.
Total faecal samples include all samples taken, and unique samples include only the first faecal sample taken for each individual separated by age-class (adult, sub-adult, juvenile), sex and reproductive condition (non scrotal or scrotal for males, non-reproductive, pregnant, or lactating for females).

Category	Faecal samples	Total bag tests	Total open-field test
Deer mice			
Total samples	413	104	45
Unique samples	189	78	41
Males	109	23	10
Females	80	31	10
Adult	98	28	9
Sub-adult	54	11	1
Juvenile	37	15	10
Scrotal	50	17	7
Non-Scrotal	59	15	4
Pregnant	4	1	0
Lactating	8	2	2
Non-reproductive	68	20	9
Red-backed voles			
Total samples	231	52	27
Unique samples	115	36	21
Males	77	15	6
Females	38	7	3
Adult	99	21	8
Sub-adult	7	0	1
Juvenile	9	1	0
Scrotal	42	5	3
Non-Scrotal	34	10	3
Pregnant	9	3	1
Lactating	10	1	0
Non-reproductive	20	3	2

The total bag tests or total open-field tests columns represent the number of unique behavioural tests performed for each category, with individuals that have a faecal sample corresponding to that trap day.

Enzyme immunoassays (EIAs) for measuring FGMs have been validated for red-backed voles and deer mice (Eleftheriou et al., 2020; Harper & Austad, 2000). Glucocorticoid metabolites were extracted using methods detailed by Veitch et al. (2021) with some modifications. Each faecal sample along with any associated methanol from the collection was transferred to a 7 ml glass vial. An additional 1 ml of 100% methanol was used to rinse out the collection tube and was then added to the same glass vial. Samples were left under a fume hood at ambient temperature for 1-5 days to completely evaporate the alcohol to obtain a faecal sample mass. Freshly prepared 80% methanol was added to the samples using a ratio of 0.05 g/ml before vortexing samples for 10 s. Vortexed samples were placed on an orbital shaker at 100 rpm for approx. 20 h. The following day, the vials were centrifuged for 10 min at 2400 g and the supernatants were transferred into fresh glass vials and placed at −20°C until analysis.

Since all faecal samples were placed in 1 ml of methanol directly in the field, the hormone extraction process would start immediately, prohibiting the opportunity to subset samples to control for variation in faecal mass (Palme et al., 2013). Evaporating methanol from faecal samples collected in the field prior to hormone analysis has been previously published (Lynch et al., 2003). Faecal volume ranged from 0.0068–0.3168 g in deer mice, and 0.0073–0.3167 g in red-backed voles. To ensure that as much hormone was extracted as possible, faecal samples were re-extracted with additional 80% methanol in the same vial at a prescribed ratio of 0.05 g faeces/ml methanol, following the evaporation of the original methanol added in the field. Previous studies have shown that a ratio of 30:1 (v/w; 80% methanol: faeces) does not substantially increase the recovery of hormone when compared to a ratio of 10:1 (Palme et al., 2013). In this study the second phase of extraction (0.05 g/ml) was a ratio of 20:1 (Veitch et al., 2021).

FGMs were quantified using methods described by Baxter-Gilbert et al., (2014) and Stewart et al., (2020). Extracts were diluted in EIA buffer (0.1 mM sodium phosphate buffer, pH 7.0, containing 9 g of NaCl and 1 g of bovine serum albumin per litre) at 1:30 for deer mice (Veitch et al., 2021). For red-backed voles, methanol extracts were evaporated (20 μl) and reconstituted in EIA buffer (400 μl) to avoid possible alcohol interference with the immunoassay at 1:20 dilution. Antibody and horseradish peroxidase conjugate were diluted 1:300 000 and 1:1 000 000 in EIA buffer, respectively. Absorbance was measured using a spectrophotometer (Epoch 2 microplate reader, BioTek, Winooski, VT, USA). Intra-assay CV was 7.5%, and inter-assay CVs were 6.6 and 5.9% (45% binding) for deer mouse and red-backed vole respectively, and 8.2 and 12.4% (70% binding) for deer mice and red-backed voles.

Species-specific differences in faecal hormone metabolite profiles are typically assessed using biochemical, physiological and biological validation techniques that ensure appropriate selection of EIAs (Palme, 2019). Parallel displacement between the standard curve and serial dilutions of faecal extract was used as an indirect measure of assay specificity and a biochemical validation of the selected EIAs. Pooled reconstituted faecal extracts were serially diluted two-fold in assay buffer and compared to the respective standard curve. These data were plotted as log (relative dose) vs. percent antibody bound. The slopes of the lines within the linear portion of the curves were determined using linear regression analysis and compared (Soper, 2021) where $p > 0.05$ indicates that the slopes are not significantly different and thus interpreted as parallel. Serial dilutions of pooled faecal extract showed parallel displacement with the corticosterone standard curve for deer mice ($t = 0.71$, df $= 9$, $p = 0.50$, Figure A1 in the Appendix), and red-backed voles ($t = 0.03$, df $= 9$, $p = 0.98$, Figure A1 in the Appendix). Samples were assayed at the dilution factor that corresponded to 50% binding of the serially diluted faecal pool for each assay. Cross reactivities for corticosterone antibody (CJM006) are reported in Metrione & Harder (2011). The recoveries of known concentrations of corticosterone from faecal extracts were $92.0 \pm 2.4\%$ and $109.4 \pm 5.1\%$ for deer mice and red-backed voles respectively. The measured hormone concentrations in the spiked samples correlated with the expected concentrations (deer mice: $r = 0.99$, $p < 0.001$; red-backed voles: $r = 0.99$, $p < 0.001$; Figures A2 and A3 in the Appendix).

2.4. *Statistical analysis*

All statistical analyses were conducted using the statistical software program R version 4.2.3 (R Core Team, 2023). FGMs outside the high and low cut-off values are considered inaccurate and were thus removed as outliers (red-backed voles >6200 or <60 ng/g, deer mice >9500 or <90 ng/g). Low cut-offs were determined as the limit of quantitation (LOQ) using the blank determination method described in Shrivastava & Gulpa (2011); high cut-off values were determined through visual assessment of where values exceeded the standard curve of the parallelism (Figure A1 in the Appendix). A $\log_{10}$-transformation was applied to the total FGM concentrations variable for all models to normalize distribution of data. Samples were collected in the field with no way to control external stimulus overnight (such as inclement weather or a predator interacting with the trap), therefore individuals that had multiple faecal samples from the same trap week (and thus same reproductive period) were averaged to evaluate FGM concentrations during that period. Fluctuations in FGMs are observable after approx. 6–12 h in deer mice and red-backed vole, and longer trap confinement is a more important factor than subsequent capture events for determining FGMs (Harper and Austad, 2000, 2001). However, previous work on this population has shown longer trap confinement within our 12-h timeframe does not significantly impact FGMs (Veitch et al., 2021). Likewise, trap confinement does not select for specific behavioural syndromes (Brehm and Mortelliti, 2018). We then coupled behavioural tests during the same week of collection to analyse the relationship between behaviour and FGMs. Although there are weak correlations between recapture occurrence and behaviour in this population reported in Hughes (2023); we did not see a significant difference in either exploration or docile behaviour for subsequent capture events (Hughes et al., 2024).

Linear mixed effects models were performed using the lme4 package in R (Bates et al., 2015), p-values and likelihood ratio tests (LRT) to compare models were obtained using the package lmertest (Kuznetsova et al., 2017). Intra-class coefficients to determine within and between-individual variation in FGM concentrations were obtained using the package "performance" (Lüdecke et al., 2021). To test fixed effects, we used an LRT using transformed FGMs as a dependent variable first as a null model, then with individual ID included as a random effect, and then with both individual ID and sample date as random effects (Table A1 in the Appendix). To examine how behavioural differences are affected by hormone concentrations we used linear mixed effects models with docility (log transformed for normality) or exploration as a response variable, age, sex, reproductive condition and $\log_{10}$-transformed FGMs as fixed effects, and individual ID as a random effect, for deer mice and red-backed voles separately. For each model we also included an interaction effect between sex and FGMs to determine sexual differences in hormone concentrations. For these analyses, only faecal samples collected during or directly prior the associated bag-test or open-field test were used. Since age was more significant than sex for deer mice, we used a one-way ANOVA followed by an ad-hoc Tukey test to compare differences between age-class, behaviour and FGMs post-analysis.

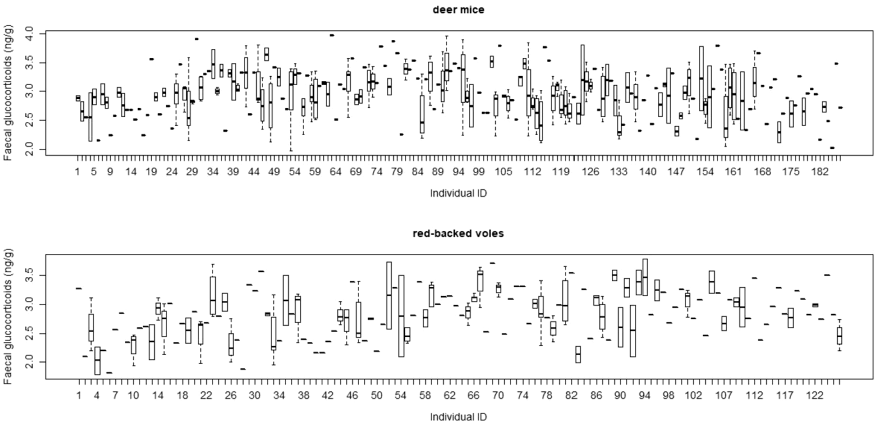

Figure 1. Individual variation in $\log_{10}$-transformed FGM for each observed species including 413 faecal samples of 197 deer mice and 231 faecal samples of 129 red-backed voles. Individuals are represented numerically. Shown are the median (black line) interquartile range (box) and minimum/maximum values (bars) of FGMs, represented by $\log_{10}$-transformed FGMs, for both deer mice and red-backed voles.

3. Results

3.1. *Within-individual variation in FGMs*

For both species, there was a high degree of within-individual variation in FGMs (Figure 1). In deer mice, 9.3% of variation in FGM concentrations was attributable to differences between individuals (ICC = 0.093) and the inclusion of individual ID as a random effect was not significant (LRT = 2.501, df = 1; $p = 0.11$). In red-backed voles, between-individual differences accounted for 26.7% of variation in FGMs and the inclusion of individual ID as a random effect was significant (LRT = 6.0008; df = 1, $p = 0.0014$). There were no significant differences in mean FGMs between or within-sex during different reproductive stages (Figure 2).

3.2. *Relationships between docility and FGMs*

Between species, the pregnant and non-reproductive reproductive conditions were our only significant fixed effects for docility (Table 2). Ad hoc Tukey tests revealed male deer mice were 36% more docile than females (mean difference = 0.312, 95% CI = [−0.24; 0.87], p = 0.27). meanwhile male red-backed voles were 5% less docile than females (mean difference =–0.56, 95% CI = [−0.34; 0.23], $p = 0.69$). However, these effects were not signif-

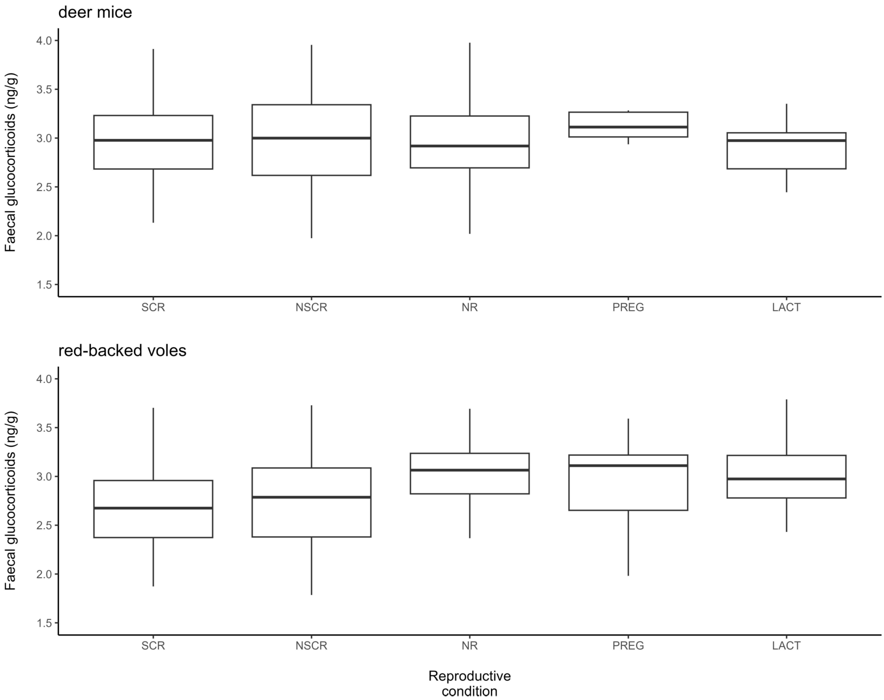

Figure 2. The $\log_{10}$-transformed faecal glucocorticoid metabolite concentrations (FGMs; ng/g) in deer mice (A) and red-backed voles (B). Each column represents reproductive status (NSCR, non-scrotal; SCR, scrotal for males; and PREG, pregnant; LACT, lactating; PERF, perforate, or NR, non-reproductive for females). Dark lines represent the mean value of FGMs (ng/g) while whiskers represent the minimum and maximum values of inter-quartile range.

icant for either species, and the interaction between sex and FGMs revealed no significant differences between sex and hormone concentration (Table 2).

3.3. Relationships between exploration behaviour and FGMs

In deer mice, none of the examined fixed effects were significant for exploration (Table 3). Males were 13 s more explorative than females (mean difference = 13.7 s, 95% CI = [−22.9; 50.4], $p = 0.45$); however, the interaction effect between sex and FGMs showed no significant differences in hormone concentrations between sexes (Table 3). An ad-hoc Tukey test revealed some significant trends in exploration behaviour between sub-adults and juveniles. Sub-adult deer mice were ≈63 s more explorative than juveniles (mean difference = 62.7 s, 95% CI = [2.27; 123.32], $p = 0.045$]. Sub-adult deer mice were also ≈45 s more explorative (mean difference = 44.8, 95% CI =

Table 2.
Summary of fixed effects for linear mixed effects models evaluating the relationship between docility and hormone concentrations in deer mice ($n = 45$) and red-backed voles ($n = 52$).

Fixed effect	Estimate	SE	df	t	p
Deer mice					
Intercept	1.068	1.71	88	0.62	0.54
Log_{10}cort	−0.21	0.58	86	−0.36	0.72
Sex_{M}	1.14	2.21	84	0.51	0.61
Age_{J}	0.26	0.39	89	0.65	0.51
Age_{SA}	0.18	0.35	87	0.51	0.61
Repro_{NR}	1.79	0.85	90	2.11	0.038*
$\text{Repro}_{\text{NSCR}}$	1.32	1.27	84	1.04	0.21
$\text{Repro}_{\text{PREG}}$	3.19	1.53	65	2.09	0.04*
$\text{Repro}_{\text{SCR}}$	1.27	1.31	87	0.97	0.33
$\text{Log}_{10}\text{cort:Sex}_{\text{M}}$	−0.16	0.77	87	−0.204	0.84
Red-backed voles					
Intercept	3.2	0.79	42	4.17	0.00015*
Log_{10}cort	0.03	0.27	42	0.12	0.91
Sex_{M}	−1.19	1.16	42	−1.02	0.31
Age_{J}	0.41	0.51	42	0.83	0.41
Age_{SA}	−0.04	0.37	42	−1.09	0.91
Repro_{NR}	0.47	0.28	42	1.65	0.11
$\text{Repro}_{\text{NSCR}}$	0.39	0.59	42	0.56	0.51
$\text{Repro}_{\text{PREG}}$	0.56	0.29	42	1.93	0.06
$\text{Repro}_{\text{SCR}}$	0.49	0.56	42	0.89	0.38
$\text{Log}_{10}\text{cort:Sex}_{\text{M}}$	0.41	0.33	42	1.24	0.22

All models include age, sex, reproductive condition and FGM concentration (log_{10}cort) as fixed effects, and an interaction between sex and FGMs. Individual ID is also included as a random effect. Age class is represented by J (juvenile) and sub-adult (SA), reproductive condition is represented by non-reproductive (NR), non-scrotal (NSCR), scrotal (SCR) and Pregnant (PREG).

*Significant ($p < 0.05$).

[−8.64; 98.45], $p = 0.11$) than adults; however, this effect was not statistically significant (Figure 3).

For red-backed voles, sex and the pregnant reproductive condition were significant fixed effects, however the interaction effect between sex and FGMs revealed no significant difference in hormone concentrations between sexes (Table 3). Unlike deer mice, which had no significant difference in exploration between sexes, ad-hoc Tukey tests revealed that male red-backed vales were ≈73 s less explorative than females (mean difference =–73.8 s, 95% CI = [−127.5; −19.029], $p = 0.01$; Figure 4). Sub-adult red-backed

Table 3.
Summary of fixed effects influencing exploration behaviour in deer mice ($n = 41$) and red-backed voles ($n = 26$).

Fixed effect	Estimate	SE	df	t	p
Deer mice					
Intercept	183	110	37	1.68	0.102
$Log_{10}cort$	1.99	32.4	37	0.062	0.95
Sex_M	−110	110	37	−0.99	0.33
Age_J	−11.4	18.9	37	−0.602	0.55
Age_{SA}	19.2	18.9	37	1.02	0.32
$Repro_{NR}$	21.5	24.4	37	0.88	0.38
$Repro_{NSCR}$	−1.43	18.9	37	−0.07	0.94
$Log10cort{:}Sex_M$	49.3	32.4	37	1.52	0.14
Red-backed voles					
Intercept	512	136	19	3.75	0.0014
$Log_{10}cort$	−97.9	48.4	19	−2.03	0.057
Sex_M	−378	176	19	−2.14	0.045*
Age_{SA}	55.2	44.0	19	1.26	0.22
$Repro_{NR}$	−92.9	47.5	19	−1.96	0.065
$Repro_{NSCR}$	−25.0	26.0	19	−0.96	0.35
$Repro_{PREG}$	−122	44.0	19	−2.78	0.012*
$Log_{10}cort{:}Sex_M$	85.6	62.3	19	1.37	0.19

Each model includes exploration as a response variable with log_{10}-transformed FGMs, age, sex, and reproductive condition as fixed effects, and individual ID as a random effect. Each model also includes an interaction effect between sex and log_{10}-transformed FGMs. Age class is represented by J (juvenile) and sub-adult (SA), reproductive condition is represented by non-reproductive (NR), non-scrotal (NSCR), scrotal (SCR) and Pregnant (PREG).

*Significant ($p < 0.05$).

voles were ≈45 s more explorative than adults (mean difference = 49.3 s; 95% CI = [−48.3; 146.9] $p = 0.31$); however, this effect was not significant.

4. Discussion

Here we evaluated some differences in behaviour between sexes in deer mice and red-backed voles and found differences to be opposite to our original hypothesis. Male deer mice were more docile although this effect was not statistically significant, and male red-backed voles were less explorative than female conspecifics. However, there were no significant differences in exploratory behaviour between sexes in deer mice, or docile behaviour in red-backed voles. We also found that the non-reproductive and pregnant

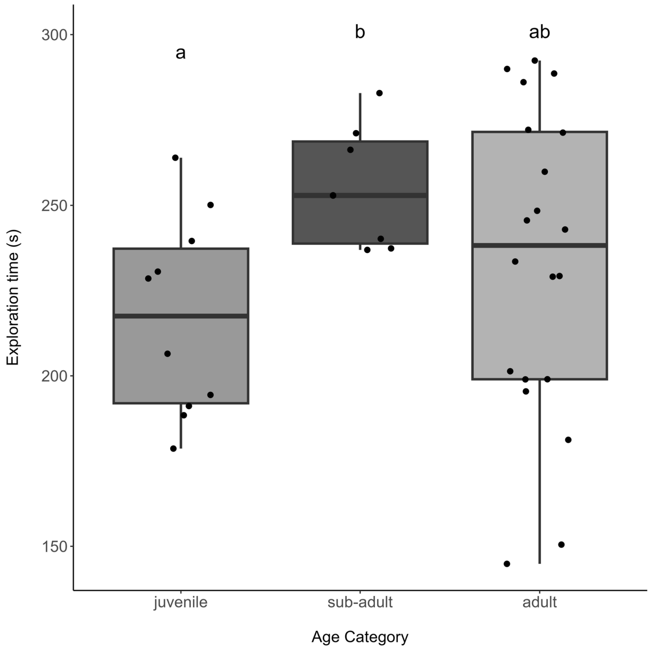

Figure 3. The mean exploration behaviour expressed in deer mice during the first open field test an individual was tested. Sub-adult deer mice were ≈63 s more explorative than juveniles (mean difference = 62.7 s, 95% CI = [2.27;123.32], $p = 0.045$] and ≈45 s more explorative (mean difference = 44.8, 95% CI = [−8.64;98.45], $p = 0.11$) than adults. Individuals are separated by age class, determined using body mass and fur colour. Unique individuals are represented by jitters, dark lines represent the mean exploration time in seconds, boxes represent the interquartile range, and whiskers represent the maximum and minimum distribution. Significance is represented by unique letters where groups that are not significantly different are represented by the same letter above each bar.

reproductive stages were significant fixed effects for deer mice docility, but not exploration. For red-backed voles, none of our fixed effects revealed a significant influence on docility. However, sex and the pregnant reproductive condition were significant effects for exploration. Our ad hoc analyses also revealed some trends in behaviour related to individual age. Sub-adult deer mice were more exploratory than adult and juvenile deer mice, although only the relationship between juvenile and sub-adults was statistically significant. Likewise, sub-adult red-backed voles were more explorative than adults, but these results were not significant. We also report significant within-individual variation in FGMs for both species, and some support for between-individual

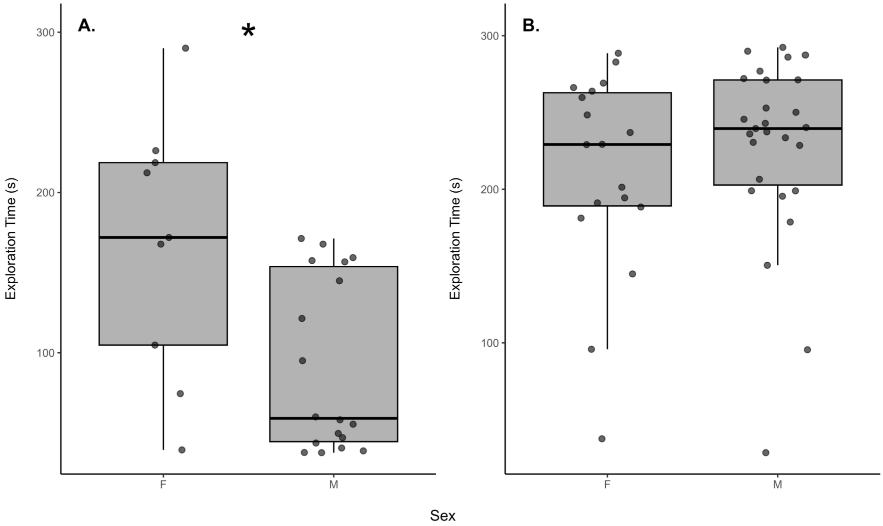

Figure 4. (A) The mean exploration behaviour between male and female red-backed voles during the open field test. Male red-backed voles were ≈73 s less explorative than females (mean difference =–73.8 s, 95% CI = [−127.5;–19.029], $p = 0.01$. (B) By contrast, male deer mice were more explorative than females, however these results were not statistically significant (mean difference = 13.7 s, 95% CI = [−22.9;50.4], $p = 0.45$). An asterisk denotes the significance between the two groups. Black lines represent the mean exploration time for the sample population, while jitters represent each tested individual. Whiskers represent maximum and minimum distribution while boxes represent the interquartile range.

variation in red-backed voles. However, neither species showed a significant relationship in FGMs between sex.

Since only some behaviours were different between sexes, and these differences were opposite to our hypothesis that males would be less docile and more explorative than females, we propose that investment in sex-specific strategies related to exploration and docility may result from a different directionality of selective pressures. Males may incur an increased energetic demand influenced by mate acquisition, while female conspecifics experience similar demand to accommodate the care and development of young (Hämäläinen et al., 2018). In sexually reproducing organisms, anisogamy suggests that the costs of gamete production influences investment in reproduction. However, gamete production is not the only energetic cost for sexually reproducing individuals and our non-significant results in certain behaviours may reflect other factors influencing reproductive costs. These findings coincide with previous literature that suggest the emergence of convergent syndromes between sexes (Hämäläinen et al., 2018; Tarka et al., 2018) and emphasize the importance of investigating multiple seasons to further understand the relationships between sex-specific traits in rodents.

Differences in reproductive strategy between sexes are well described in rodents (Speakman et al., 2007). Throughout the literature, there is conflicting evidence to support sexual differences in behaviour. It is not uncommon to observe non-significant, or negligible differences in individual behavioural traits despite consistent variation across individuals (Vošlajerová Bímová et al., 2016; Strijker et al., 2023). In contrast, body mass and sex can be strong predictors of exploration behaviour (Bednarz & Zwolak, 2022). There is also evidence that male rodents are less active, explorative and respond to external stimuli by freezing or defecating much more frequently than female counterparts (Archer, 1975; Tropp & Marcus, 2001). Such differences in behaviour are possibly linked to differences in risk-perception of external stimuli (Tropp & Marcus, 2001). However, these differences may also be species and study system specific, and there are also examples where male rodents are more explorative, and less docile than females (Eccard et al., 2023). External pressures such as photoperiod, sociality, and resource availability can influence reproductive investment (Pinho et al., 2019; Dantas et al., 2021). For example, shorter day length and restricted food availability can reduce investment in gonadal state for male deer mice (Nelson et al., 1997). Thus, the conflicting evidence to support between sex differences in behaviours may be further masked by system-specific environmental factors.

Sub-adult rodents are generally the primary disperses within some rodent species (King, 1968; Gliwicz, 1992). Thus, sub-adults may express greater exploratory and less docile behaviour than juvenile or adult counterparts (Rohrer & Ferkin, 2020). In some circumstances adult individuals increase behaviour related to movement and mate acquisition as they age to increase reproductive success (Ferkin, 2018). However, such differences in age-related effects may be related to exposure to novel stimuli during early development (Rödel & Meyer, 2011). For example, juveniles are presumably less experienced and therefore less bold and explorative than adult counterparts but increased social play among littermates and maternal influence can affect exploration (Marks et al., 2017; Modlinska et al., 2018).

When evaluating hormone concentrations, our study did not reveal any significant relationship between FGMs and any of the fixed effects we used including sex, age class, or reproductive condition at time of faecal collection. Corticosterone and cortisol are analogous hormones, and corticosterone is the dominant hormone in most rodents (Romero et al., 2008). Although deer mice and red-backed voles are considered corticosterone dominant, recent studies have demonstrated that non-dominant glucocorticoids can also spike during acute stressors (Botia et al., 2023). Thus, to better understand the complete relationship between behavioural traits and glucocorticoids, future research may benefit from the inclusion of both hormones, which was not tested in this study.

Given our results, we suggest that deer mice and red-backed voles may experience similar energetic costs from environmental pressures. Many small mammals including the deer mice and red-backed voles surveyed in this study are income breeders, with females fuelling reproduction by increasing ingestion, rather than burning fat-stores. The increased strategy to compensate energetic demand during reproduction masks potential reproductive costs (Koivula et al., 2003; Williams et al., 2017). For example, experimentally increasing litter sizes of bank voles (*Clethrionomys glareolus*) has been shown to have negligible effect on breeding success (Koivula et al., 2003). Thus, energetic costs associated with reproduction is maintained between some males and females through increased consumption (Williams et al., 2017). For example, North American red squirrels (*Tamiasciurus hudsonicus*) delay reproduction until seasonal food sources become available, and breed earlier following periods of large cone crop production (Fletcher et al., 2013).

Further investigation into seasonal variation in behaviour across winter and summer months, where energetic costs differ, may highlight a more significant relationship since reproduction is linked to photoperiod and seasonal resource availability (Moffatt et al., 1993; Eccard and Herde, 2013; Hämäläinen et al., 2021). Likewise, FGM concentrations increase from spring to summer in most rodents, due to increased energetic demand for reproduction (Romera, 2002; Stewart et al., 2014). However, seasonal energetic costs may be worth investigating to understand the trade-offs in metabolic stress and behaviour. For example, Stead et al., (2024) found that flying squirrels (*Glaucomys* spp.) experience a peak in faecal cortisol metabolites during autumn, a time that coincides with peak energetic demand associated with food caching rather than reproduction. Differences in behavioural strategy may also explain the lack of sexual differences observed within this study. While females actively investing in the care or development of young may express an increased level of energetic stress (Künkele, 2000) similar increased cost may be incurred by males experiencing the associated costs of territory defence, dispersal, spatial movement, or hormone investment (Millar, 1975; Romero, 2002). Understanding the relationships between behaviour and physiology during reproduction is relevant to understanding seasonal ecological processes and energetic strategies. We suggest future work on sex differences in rodents focus on seasonal variation and resource availability for income-breeding rodents.

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Appendix

Table A.1.
Comparison of linear mixed effects models using $\log_{10}$-transformed faecal concentrations (ng/g) of deer mice and red-backed voles.

Model	npar	LogLik	df	χ^2	*p*
Deer mice					
null	3	−222.40			
Intercept ∼ID	6	−221.46	3	1.88	0.59
Intercept ∼ID + date	7	−209.27	1	24.38	<0.001*
Red-backed voles					
Null	3	−129.71			
Intercept ∼ID	6	−121.87	3	15.67	0.0013*
Intercept ∼ID + date	7	−115.88	1	11.96	<0.001*

Age and sex were included as a fixed effect for all models, with either individual ID or individual ID + date of collection as random effects. *Significant, $p < 0.05$.

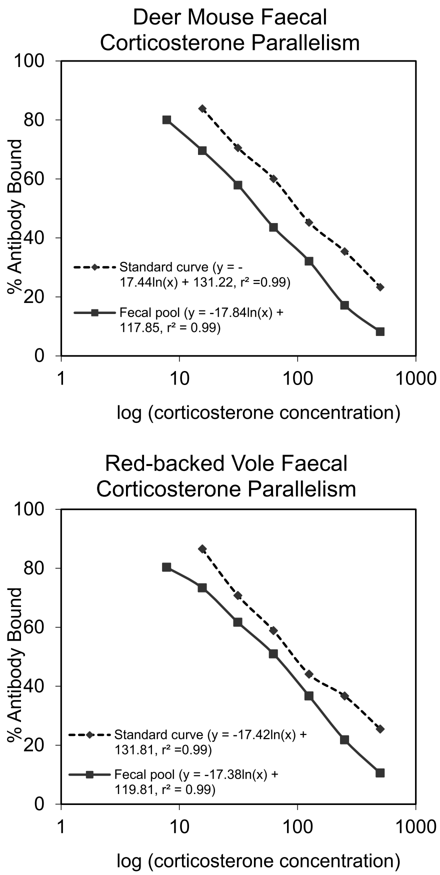

Figure A1. The relationships between total faecal glucocorticoid metabolites (FGMs) and % antibody binding compared to a standard curve generated using Soper (2021). Shown is the relationship for deer mice and red-backed voles.

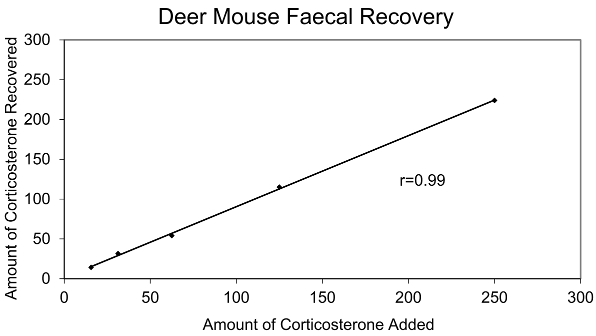

Figure A2. Faecal corticosterone % recovery for the deer mouse ($p < 0.001$); recovery = 92.0 ± 2.4% (mean ± SE).

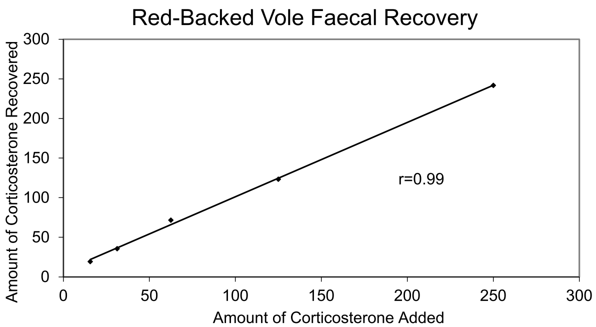

Figure A3. Faecal corticosterone % recovery for the red-backed vole ($p < 0.001$); recovery = 109.4 ± 5.1% (mean ± SE).