



The glucocorticoid response to environmental change is not specific to agents of natural selection in wild red squirrels

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ABSTRACT

Evolutionary endocrinology aims to understand how natural selection shapes endocrine systems and the degree to which endocrine systems themselves can induce phenotypic responses to environmental changes. Such responses may be specialized in that they reflect past selection for responsiveness only to those ecological factors that ultimately influence natural selection. Alternatively, endocrine responses may be broad and generalized, allowing organisms to cope with a variety of environmental changes simultaneously. Here, we empirically tested whether the endocrine response of female North American red squirrels (*Tamiasciurus hudsonicus*) was specialized or generalized. We first quantified the direction and magnitude of natural selection acting on three female life history traits (parturition date, litter size, offspring postnatal growth rate) during 32 years of fluctuations in four potential ecological agents of selection (food availability, conspecific density, predator abundance, and temperature). Only three of the four variables (food, density, and predators) affected patterns of natural selection on female life history traits. We then quantified fecal glucocorticoid metabolites (FGMs) across 7 years and found that all four environmental variables, regardless of their effects on patterns of selection, were associated with glucocorticoid production. Our results provide support for a generalized, rather than specific, glucocorticoid response to environmental change that can integrate across multiple co-occurring environmental stressors.

1. Introduction

Endocrine systems shape the evolutionary trajectories of species through a variety of pathways (Cox et al., 2016; Lema, 2014; Zera, 2007), including coordination of phenotypic responses to changes in the environment that promote adaptive phenotypic integration (Dantzer and Swanson, 2012; Flatt et al., 2005; Ketterson et al., 2009; Lema and Kitano, 2013; Sinervo and Svensson, 1998; Zera, 2007). When environments shift, individuals can experience changes in the direction and/or magnitude of natural selection acting upon phenotypic targets. The complexities of endocrine systems and their associated receptors, carrier and binding proteins, clearance mechanisms, and hormones allow for pleiotropic effects on multiple phenotypic targets of selection (life

history traits, behavior, morphology). Shifts in hormone production in response to environmental changes can thus coordinate adaptive phenotypic plasticity (Denver, 1997a, 1997b; Dufty et al., 2002) and mediate multidimensional phenotypic adjustments in response to these changes (Ketterson et al., 2009). Yet, whether endocrine responses reflect generalized sensitivities to the environment broadly, or specific sensitivities to only those environmental changes that impact the direction or magnitude of selection on phenotypes, remains largely untested.

Steroid hormones in particular respond to a wide variety of environmental changes like predator abundance, climate, intraspecific competition, and food availability (Cox, 2020). For example, individuals experiencing heightened predation risk often increase production of

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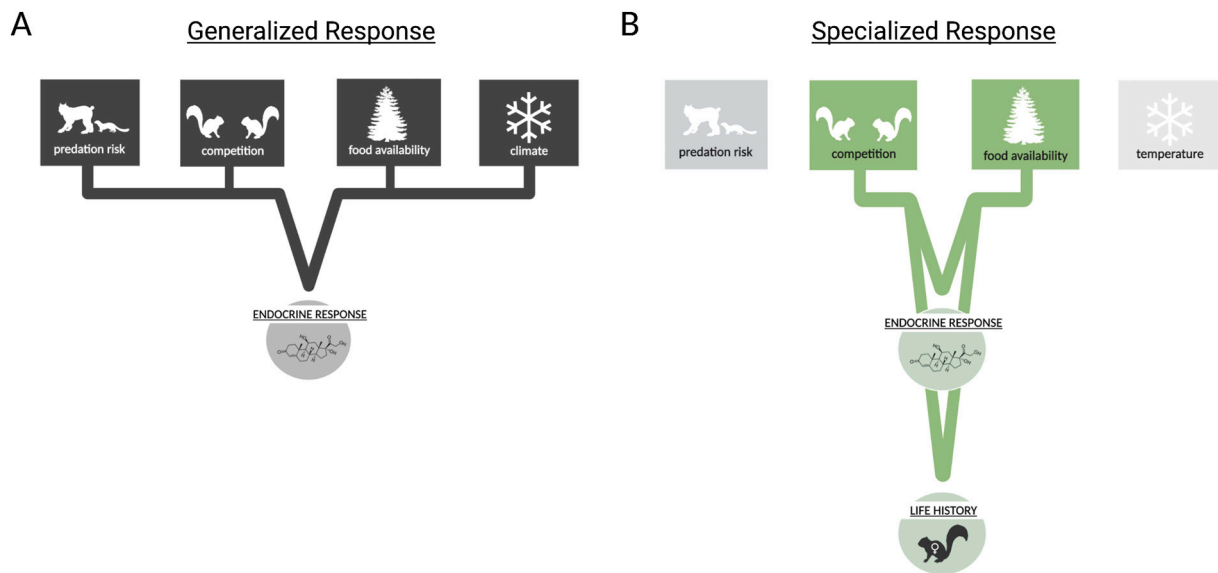


Fig. 1. Generalized versus specialized endocrine responses to environmental changes. Conceptual figure illustrating (A) a generalized endocrine response to environmental fluctuations in which the endocrine system responds to each factor and (B) a specialized endocrine response to only those environmental variables that ultimately affect patterns of natural selection on life history traits.

glucocorticoids (GCs), end-products of the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Sapolsky et al., 2000). Increased GCs in turn reduce foraging behavior or induce morphological adjustments that optimize predator avoidance (Denver, 2009; Denver, 1997a, 1997b; Fraker et al., 2021; Middlemis Maher et al., 2013). In this example, predation acts as the ecological agent of selection (i.e., an ecological factor that affects natural selection/the relationship between a trait and fitness; sensu Wade and Kalisz, 1990) and the hormonal response mediates changes in the phenotypic targets of selection (behavior and morphology). This hormonal response may reflect a specialized sensitivity to a specific predator cue because that cue induces adaptive phenotypic plasticity in environments with high predation risk (Fig. 1B), or it may instead reflect a generalized physiological pathway that integrates not only predator cues but also other environmental changes regardless of their effects on patterns of natural selection (Cohen et al., 2012; Potticary and Duckworth, 2020, Fig. 1A).

Distinguishing between generalized and specialized hormonal responses to environmental change is challenging yet crucial to understanding how natural selection shapes the endocrine system over evolutionary timescales. Foundational early work by Selye (1946) suggested that the physiological stress response, including the HPA axis, is generalized or nonspecific such that it responds uniformly across stimuli regardless of the positive or negative effects of those stimuli on the organism. Though Seyle's idea of a nonspecific HPA axis has since been disproved, the co-opting of a generalized response pathway may be an inexpensive and parsimonious way for animals exposed to multiple environmental stressors over the course of their lives to respond to harsh environments. Such generalized hormonal responses may, however, also be more likely to produce maladaptive phenotypes. Although such bet-hedging may be expected in heterogeneous environments in which ecological changes are unpredictable (Donaldson-Matasci et al., 2013; Sæther and Engen, 2015; Seger, 1987), it may come with a cost: repeated or chronic HPA axis activation can have damaging effects on somatic cells, (e.g., telomeres or oxidative damage to proteins or lipids, Costantini et al., 2011; Haussmann and Heidinger, 2015; Monaghan, 2014), accelerate senescence (Gassen et al., 2017), and decrease lifespan (Campos et al., 2021; Rakotoniaina et al., 2017).

By contrast, in predictable heterogeneous environments, adaptive phenotypic plasticity is expected to evolve (Lande, 2009; Sæther and Engen, 2015; Tufto, 2000) and thus specific, rather than general,

hormonal responses to environmental changes may be favored. For example, an organism's ability to produce a differentiated response to a signal such as an ecological agent of selection rather than to noise (i.e., ecological factors that do influence the relationship between a trait and fitness) may be expected in highly fluctuating and multidimensional environments (Wiley, 2006). In the same vein, sensory biology predicts that organisms inhabiting complex acoustic environments are faced with a “cocktail party problem”, in which they need to discern only those sounds that are relevant to their fitness (McDermott, 2009). The specialization of the HPA axis response in this way may circumvent some of the expected consequences of repeated or chronic HPA axis activation and reflect broader patterns of endocrine system evolution over time. Empirical tests of this hypothesis require detailed data on multiple environmental stressors, patterns of natural selection acting on the phenotypic responses to these stressors, and hormonal responses to these stressors (Crespi et al., 2013) across larger time scales than used in Selye's (1946) work.

Here, we use detailed physiological, life history, and fitness (reproductive success) data collected as part of a longitudinal study of free-ranging North American red squirrels (*Tamiasciurus hudsonicus*) in the Yukon, Canada to test whether the glucocorticoid response to environmental change is generalized or specialized to ecological agents of natural selection. Red squirrels are highly territorial and experience extreme fluctuations in four environmental variables. First, squirrels must cope with dramatic fluctuations in their primary food source, seeds from the cones of masting white spruce (*Picea glauca*, Fletcher et al., 2013), in which a superabundance of cones are produced every 4–7 years during mast events with little to no cones produced in the years in between (Lamontagne and Boutin, 2007). Second, as a result of these masting cycles, squirrels experience pronounced changes in intraspecific competition due to the swelling and shrinking of squirrel population densities (Dantzer et al., 2020). Third, squirrels must cope with changes in predation risk due to oscillations in the densities of squirrel predators and their alternate prey sources. For example, Canada lynx (*Lynx canadensis*) prey-switch to squirrels when the numbers of their preferred prey, snowshoe hares (*Lepus americanus*), crash (Krebs et al., 2001b; O'Donoghue et al., 1998). Finally, squirrels must manage annual changes in overwinter temperatures, which are increasing due to human-caused climate change in the region (Krebs et al., 2019; Lavergne et al., 2021).

Table 1

Predicted patterns of selection on female red squirrel life history traits as a result of environmental fluctuations. Table shows expected phenotypic responses in three life history traits (parturition date, litter size, offspring growth rate) of female red squirrels to changes in each of the four potential ecological agents of selection (conspecific density, food availability (mast year), overwinter temperature, and predation risk) based on prior work in our study population.

	Prediction	Reference
Increased conspecific density		
parturition date	Negative selection (earlier parturition date)	Dantzer et al., 2013; McAdam et al., 2019; Réale et al., 2003
litter size	Positive selection (larger litters)	Dantzer et al., 2013
growth rate	Positive selection (faster growth)	Dantzer et al., 2013; Fisher et al., 2017
Mast year (y/n)		
parturition date	Positive selection (later parturition date)	McAdam et al., 2019
litter size	Positive selection (larger litters)	Boutin et al., 2006; McAdam et al., 2019
growth rate	No effect	McAdam et al., 2019
Colder overwinter temperature		
parturition date	No effect	
litter size	No effect	
growth rate	No effect	
Increased predation risk		
parturition date	No effect	
litter size	No effect	
growth rate	No effect	

We first coupled data on these four environmental fluctuations with data on reproductive success (number of surviving offspring) and three female life history traits (parturition dates, litter size, offspring growth rate) from individual female squirrels to quantify directional selection. Consistent with prior work in this population, we predicted that variation in these life history traits would depend on spruce cone production and conspecific densities, but not on predators or temperature (Table 1). We then quantified the association between all four environmental stressors on HPA axis responses by measuring fecal glucocorticoid metabolites (FGMs) as an index of circulating glucocorticoids in red squirrels (Dantzer et al., 2010; Van Kesteren et al., 2019). Although we have previously shown that FGM production increases in response to elevated squirrel densities (Dantzer et al., 2013; Guindre-Parker et al., 2019), whether FGMs respond to mast years, predator abundance, and/or overwinter temperature in our study population was unknown. If the glucocorticoid response to changes in these variables is generalized, we expected to find an association between FGMs and all four variables regardless of their effect on patterns of selection on life history traits. However, we predicted that rather than a generalized stress response, the glucocorticoid response would be specialized and thus respond only to those environmental changes that we predicted to impact selection on female life history traits (i.e., food availability and conspecific density).

2. Methods

2.1. Study species and ecosystem

This study took place as part of the ongoing long-term Kluane Red Squirrel Project (KRSP), which monitors North American red squirrels (*Tamiasciurus hudsonicus*) living in the Yukon, Canada (61°N, 138°W). Here, we have been collecting life history and fitness data from individual red squirrels since 1987 and hormone data since 2006. We follow individual squirrels throughout the entire course of their lives: they are tagged using a unique combination of colored wire or pipe cleaners as well as numbered ear tags (Monel #1; 5 digits). We also perform a census of all squirrels living in our study area twice a year (May and August), with a yearly probability of detection that does not differ from 1.0 (Descamps et al., 2009). The present study used data collected from two separate study grids (Kloo or KL, Sulphur or SU) of approximately ~40 ha each from the years 1989–2021.

2.2. Data collection

2.2.1. Temperature data

We obtained daily temperature records from the Haines Junction weather station (Climate ID 2100630, 60.77°N, 137.57°W), located ~35 km SE of our study area. In line with prior studies showing an effect of mean overwinter temperature as a proxy of thermoregulatory costs (Hendrix et al., 2019) and litter failure (Studd et al., 2015), we expected that annual mean overwinter temperatures would better capture climatic pressures on red squirrel physiology and life history compared to precipitation or extreme weather events (Hendrix et al., 2019). Thus, we calculated the annual average overwinter temperature as the mean temperature across the months of October to the following March.

2.2.2. Predator data

We used population monitoring data on Canada lynx (*Lynx canadensis*) from the Kluane Boreal Forest Ecosystem Project (1987 through 1996) and the Community Ecological Monitoring Program (from 1996 onward, Krebs et al., 2001b). Red squirrels are susceptible to predation by both terrestrial and avian predators: squirrels have historically comprised part of the diets of owls and raptors in the region (Krebs et al., 2001b), though data collection on avian predation has since ceased. In terms of terrestrial predators, we have witnessed terrestrial predation of squirrels by lynx at our field site and routinely collect data on lynx abundance, thus we chose to focus on lynx in this study. Lynx snowtrack counts collected across transects during winter were used to calculate a species-specific predator abundance index for each year (average snow track counts / 100 km transects). Coyotes (*Canis latrans*) are also present in this region and may consume squirrels, but coyote abundance is largely collinear with lynx and coyotes appear to consume fewer squirrels than lynx (O'Donoghue et al., 1998) so we only included lynx abundance in our statistical analyses. We also considered the abundance of the primary non-squirrel prey of Canada lynx: snowshoe hares (*Lepus americanus*). Lynx primarily consume hares, which exhibit dramatic population cycles (Krebs et al., 1995). Hare density estimates were generated using mark-recapture (see Krebs et al., 2001a for more details). When hare densities are low, lynx exhibit prey switching to red squirrels (O'Donoghue et al., 1998), and have been observed killing squirrels on our study grids (Boutin, Dantzer, Lane, McAdam: personal observations).

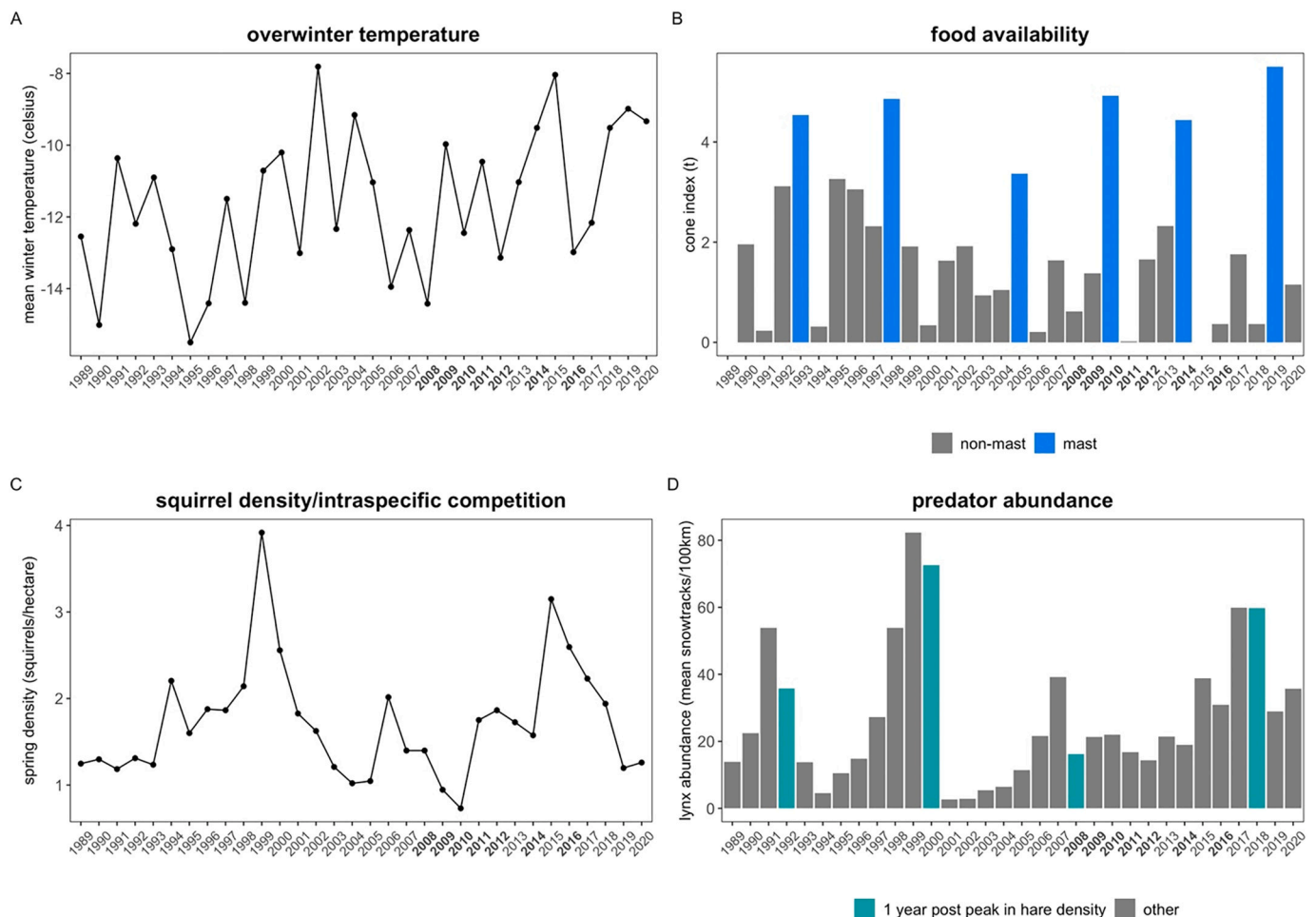


Fig. 2. Red squirrels experienced pronounced environmental fluctuations over the course of the 34-year study period. Fluctuations in (A) mean overwinter temperature measured as degrees Celsius for the preceding year's October through the current year's March, (B) food availability measured as current year's spruce cone index (mean $\ln(\text{cone count} + 1)$), with mast years (in which a superabundance of cones are produced) indicated in blue, (C) spring squirrel density (i.e., intraspecific competition), and (D) predation risk as a function of the lynx-hare cycle, indicating years in which prey switching to squirrels (i.e., 1 year following the peak in snowshoe hare density, when lynx abundance remains high but hares are low) is expected. Years in which fecal glucocorticoid metabolite (FGM) data were collected are denoted in bold on the x-axis.

2.2.3. Conspecific population densities

We calculated adult squirrel population densities as the annual number of adults present on each of our study grids expressed as squirrels per hectare. Because adult dispersal is low and site fidelity is high (Berteaux and Boutin, 2000; Cooper et al., 2017), and all individuals are uniquely tagged, we are able to completely enumerate the number of adult squirrels living on our study grids each year using live-trapping and behavioral observations (Dantzer et al., 2020).

2.2.4. Spruce cone abundance

Red squirrels in our study areas primarily consume seeds from white spruce trees (Fletcher et al., 2013; Ren et al., 2017), which they cache underground in a midden on their territory (Fletcher et al., 2010). We quantified white spruce cone production by calculating the number of spruce cones visible on one side of each tree as an annual index of cones produced on a consistent subset of trees (between 159 and 254 trees) on each of our two study areas each year (LaMontagne et al., 2005). Transformed counts ($\ln x + 1$ transformation) were averaged across all trees within a year for each study area to create an annual cone index, which has been calibrated to the actual number of cones produced per tree (Boutin et al., 2006; LaMontagne et al., 2005).

2.2.5. Life history data

We collected data on three life history traits from female squirrels: parturition date, litter size, and offspring growth rate. These traits have previously been found to associate with fitness and be affected by spruce cone abundance and conspecific density (see Table 1). We determined when females were reproductively active by fitting them with radio-collars (model PD-2C, 4 g; Holohil Systems Limited), which allows us to subsequently locate their nests shortly after parturition. Litter sizes (first litters only) were quantified as the number of pups produced at this first nest entry at ~ 0 d following birth. To calculate offspring growth rates, we temporarily removed pups from their nests for weighing at both the first nest entry (day of birth) and again at ~ 25 d old. This 25-day period represents a linear period of offspring growth during which pups rely solely on maternal milk (McAdam and Boutin, 2003). Growth rates were then calculated as the gain in mass between the two nest entries (g/day).

2.2.6. Fitness data

We calculated short-term fitness as maternal annual reproductive success, which we measured as the number of pups surviving to 200 d of age (Berteaux and Boutin, 2000; Larsen and Boutin, 1994; McAdam and Boutin, 2003). This is a critical survival bottleneck in this population as it hinges on juvenile squirrels acquiring their own territories prior to

winter, which is necessary to recruit successfully into the breeding population (Larsen and Boutin, 1994) and greatly reduces overwinter mortality (Hendrix et al., 2019). Here, we focused on annual reproductive success (number of surviving offspring) rather than adult survival because offspring survival is more important to lifetime fitness in our population (McAdam et al., 2007).

2.2.7. Hormone data and measurement of fecal glucocorticoid metabolites

We collected fecal samples from adult female red squirrels (mean age = 2.5 y, range = 1–7 y) during routine live-trapping over 7 separate years (2008–2012, 2014, 2016) from March through August during which the four ecological factors of interest (food availability in current year, spring squirrel density, spring predator abundance, and prior overwinter temperature) varied (Fig. 2). We trapped squirrels using Tomahawk live traps (Tomahawk Live Trap Co.) baited with a small amount of peanut butter on each individual's territory. Whole fecal samples were collected from beneath the traps, frozen on ice, and transferred to a -80°C freezer.

To determine responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis, we measured fecal glucocorticoid metabolites (FGMs) from frozen whole fecal samples. FGMs provide an index of circulating glucocorticoids (primarily cortisol) in red squirrels (Dantzer et al., 2010; Van Kesteren et al., 2019). Our live-trapping procedures do not affect FGM levels (Dantzer et al., 2010), thus our FGM measures are expected to be an accurate reflection of HPA axis activity prior to handling. To show that our FGM measures were repeatable across assays, we ran pooled samples repeatedly on different plates ($n = 115$) and found that the estimates of optical density were highly repeatable ($R = 0.851$, 95 % CI 0.543–0.925). We then used a linear mixed-effects model to partition the variance in the optical density recorded from these pooled samples and found that the sample itself explained the majority of the variance (85.1 %), with little explained by intra-assay variation (4.9 %) or inter-assay variation (9.9 %). Additionally, fecal samples were analyzed in two different labs ($N = 396$ at University of Toronto, Scarborough, and $N = 902$ at University of Michigan) using the exact same protocol. To confirm that our FGM measures were repeatable across both labs, we ran a separate group of fecal samples ($N = 128$ samples) in both labs and found that the optical densities of these samples were closely correlated (Pearson Correlation = 0.876). All samples were run in duplicate and only samples with intra-assay CVs $<15\%$ were included in this study.

2.3. Statistical analysis

We conducted all analyses in R version 4.0.2. We used the package lme4 (version 1.1.23) to conduct generalized linear mixed models and constructed forest plots using the sjPlot package (Lüdtke, 2018). In all models, continuous predictor variables were scaled to zero mean and unit variance. We verified selection model fit and assumptions by visually assessing residuals using the package DHARMA (Hartig, 2022). All figures were created in R with the exception of Fig. 1, which was created in BioRender (www.biorender.com).

2.3.1. Selection models

To identify ecological agents of selection on life history traits, we built a generalized linear mixed-effect model with the number of pups that recruited into the population (# of pups survived to 200 d of age, i. e., annual reproductive success, ARS; sensu Berteaux and Boutin, 2000; Larsen and Boutin, 1994; McAdam and Boutin, 2003) as the dependent variable (Poisson distribution, log link). Life-history traits were scaled to zero mean and unit variance within each grid and year (Fig. S1). We focused this analysis on only the first litter for each female each year. The number of females producing two ($n = 171$) and three ($n = 17$) litters per year is substantially less than the number of females producing one litter ($n = 1124$), and females often only attempt second or third litters if the first litter did not survive to weaning (McAdam et al., 2007).

The dataset for this model contained 1104 litters from 623 females across 32 years. We used maternal identity and year as random effects to account for multiple observations of females across years and any annual variation in dependent traits not captured by our fixed effects. To construct the fixed effects, we built two-way interactions between each life history trait (parturition date), litter size (number of pups), mean pup growth rate (g/d) with each potential ecological agent of selection (squirrel density, whether it was a mast year or not, cone crop from the preceding year (cones $t-1$), year in the lynx-hare cycle, and mean winter temperature (Fig. S2). Typically, high hare abundance facilitates high lynx production such that both species have peak numbers in the same years. When the hare population crashes, the lynx population follows but can remain relatively high for 1–2 years. These years following a hare crash represent periods when lynx are likely to prey-switch to red squirrels. We thus binned years into a four-level categorical variable depending on the stage within the lynx-hare cycle: peak, 1-yr post peak, 2-years post peak, or other. We also included study area (KL vs SU) and linear and quadratic age terms as covariates. While we included spruce cone abundance in the previous year as a continuous variable (cone index $t-1$), we included a categorical variable of mast year (yes/no) rather than spruce cone abundance in the current year (cone index t) as a continuous variable in our models. Mast years (1993, 1998, 2005, 2010, 2014, 2019) were easily qualitatively determined because they result in a superabundance of cone crops that is strikingly different from non-mast years (Lamontagne and Boutin, 2007). Female red squirrels exhibit anticipatory reproductive plasticity in response to ecological cues of future cone abundance (Boutin et al., 2006). Thus, we expected that an upcoming masting event would elicit physiological and phenotypic responses more strongly in that year than continuous changes in cone production. We then used backward stepwise elimination to remove nonsignificant ($p < 0.05$) interactions and construct the final model. We used Type III Wald chi-square tests to determine the significance of independent terms.

2.3.2. Hormone model

We built a generalized linear mixed-effects model in which fecal glucocorticoid metabolites (FGMs) were set as the dependent variable and log-transformed to achieve residual normality. We included fixed factors for each potential ecological agent of selection: mast year (y/n), mean overwinter temperature, squirrel density, and predator abundance, controlling for the previous year's cone index (cone index $t-1$). For predator abundance, we used lynx density estimates (average snowtrack counts / 100 km transect) as a continuous variable rather than binning by year of the lynx-hare cycle. This was to minimize collinearity in this model as the sample size was smaller than the selection models, and because we predicted that the effects of predator cues on squirrel glucocorticoid responses would not necessarily depend on the number or presence of alternate prey. We additionally controlled for biological factors that may potentially influence FGM production in this population (i.e., age as both a linear and quadratic term, and reproductive status as breeding or not breeding; Dantzer et al., 2010). We also included fixed effects of study grid (to account for any potential differences between the two study grids not captured by our models) and sample collection date (linear and quadratic), as well as a random effect of individual ID. We did not include year as a random effect because it did not improve model fit (i.e., variance = 0, singularity introduced into the model) likely because our fixed factors of temperature, predation risk, squirrel density, and food availability largely captured year-to-year variation in the dataset.

2.4. Ethics statement

This research and associated protocols and procedures were approved by the University of Alberta, Michigan State University, the University of Guelph, and the University of Michigan.

Table 2

Squirrel density, food availability, and predation risk, but not overwinter temperature predict female fitness (annual reproductive success or ARS) and patterns of selection on life history traits. Table shows results from models assessing whether selection on life history traits (parturition timing, litter size, or pup growth rate) shifts depending on multiple aspects of the environment for female North American red squirrels (*Tamiasciurus hudsonicus*). Life history variables were standardized to a mean of zero and expressed in standard deviations within each grid and year. All continuous environmental variables were transformed to z-scores across the global dataset prior to analysis. The dataset contained 1104 litters produced by 623 females across 32 years. Non-significant interactions were removed to construct the final model (see methods for details). The random effect of maternal identity explained 0.00 variance and year explained 0.11 variance. Bold lettering indicates significant relationships ($P < 0.05$).

Independent terms	Dependent: ARS			
	Estimate	SE	Z	P
grid (SU)	-0.01	0.08	-0.1	0.883
maternal age	-0.21	0.17	-1.2	0.229
maternal age ²	0.10	0.17	0.6	0.570
parturition date	-0.17	0.06	-2.9	0.003
litter size	0.11	0.05	2.2	0.031
litter size²	-0.08	0.03	-2.5	0.014
growth rate	0.11	0.04	2.8	0.006
squirrel density	-0.35	0.09	-3.9	<0.001
mast (yes)	0.69	0.19	3.6	<0.001
cone availability	-0.12	0.09	-1.3	0.185
mean overwinter temperature	-0.05	0.08	-0.6	0.549
hare-lynx cycle (peak)	0.01	0.23	0.0	0.979
hare-lynx cycle (1-year post peak)	-0.94	0.27	-3.5	<0.001
hare-lynx cycle (2-years post peak)	0.00	0.23	0.0	0.998
squirrel density * parturition date	-0.21	0.06	-3.5	0.001
squirrel density * litter size	0.02	0.07	0.4	0.727
squirrel density * growth rate	0.04	0.05	0.7	0.515
mast (yes) * parturition date	0.06	0.10	0.7	0.515
mast (yes) * litter size	0.25	0.10	2.6	0.010
mast (yes) * growth rate	0.00	0.10	0.0	0.973
prior year cone availability * parturition date	-0.04	0.05	-0.8	0.411
prior year cone availability * litter size	0.06	0.06	1.0	0.316
prior year cone availability * growth rate	-0.02	0.05	-0.3	0.743
temperature * parturition date	-0.02	0.04	-0.4	0.675
temperature * litter size	-0.06	0.04	-1.3	0.190
temperature * growth rate	0.04	0.04	1.0	0.336
hare-lynx cycle (peak) * parturition date	0.06	0.12	0.5	0.606
hare-lynx cycle (1-year post peak) * parturition date	-0.56	0.20	-2.7	0.007
hare-lynx cycle (2-year post peak) * parturition date	-0.06	0.11	-0.6	0.555
hare-lynx cycle (peak) * litter size	0.14	0.13	1.1	0.283
hare-lynx cycle (1-year post peak) * litter size	-0.08	0.19	-0.4	0.680
hare-lynx cycle (2-year post peak) * litter size	0.17	0.12	1.4	0.160
hare-lynx cycle (peak) * growth rate	0.01	0.13	0.1	0.952
hare-lynx cycle (1-year post peak) * growth rate	0.07	0.20	0.4	0.710
hare-lynx cycle (2-year post peak) * growth rate	0.01	0.13	0.0	0.965

3. Results

3.1. Fluctuations in potential ecological agents of selection

Over the course of our 32-year study period (1989–2019), female red squirrels experienced dramatic fluctuations in overwinter temperature, conspecific density, food availability, and predation risk. Ambient overwinter temperatures averaged $-11.6\text{ }^{\circ}\text{C}$ ($SD = 2.05$) across years (Fig. 2A). The availability of new spruce cones for red squirrels to eat oscillated dramatically from year to year: cone indices (transformed raw cone counts calibrated to the actual number of cones per tree; Boutin et al., 2006; LaMontagne et al., 2005) were as high as 6 in years in which cone production was superabundant (i.e., “mast years”) and as low as

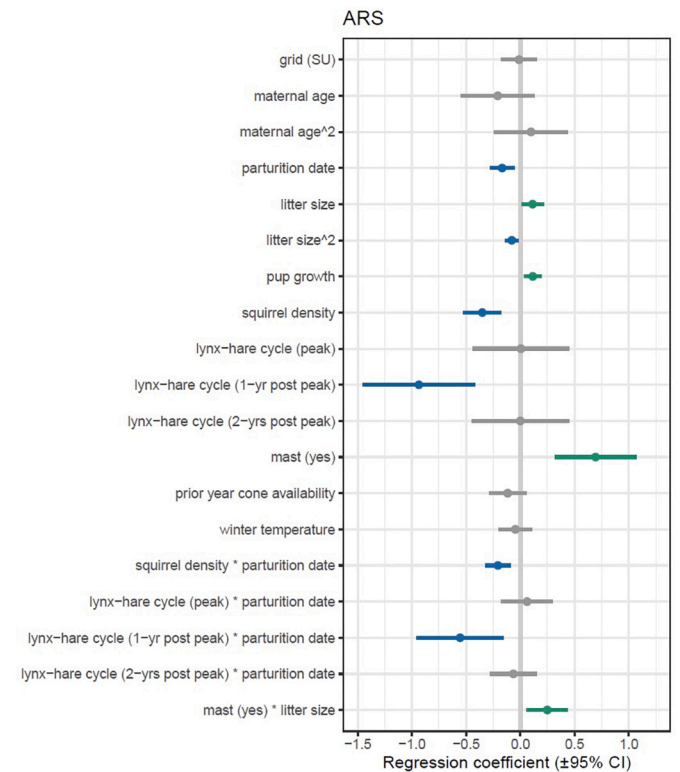


Fig. 3. Shifts in environmental variables predict variation in female reproductive success and patterns of selection on life history traits. Forest plot of model assessing whether selection on life history traits (parturition timing, litter size, or pup growth rate) shift depending on multiple aspects of the environment for female North American red squirrels (*Tamiasciurus hudsonicus*). Life history variables were standardized to a mean of zero and expressed in standard deviations within each grid and year. Continuous environmental variables were transformed to z-scores across the global dataset prior to analysis. The dataset contained 1104 litters produced by 623 females across 32 years. Non-significant interactions were removed to construct the final model (see methods for details). The random effect of maternal identity explained variance = 0.00 and year explained variance = 0.11. Nonsignificant effects are shown in grey; positive effects denoted in green, and negative effects in blue.

zero in some years immediately following the mast year ($SD = 1.65$). Spruce masting cycles resulted in fluctuations in the population densities of red squirrels living on our study grids, and thus in the rate of intra-specific competition (Fig. 2C). Squirrel densities averaged 1.72 squirrels per hectare ($SD = 0.71$) but ranged from 0.53 squirrels/ha in the lowest density years to 4.28 squirrels/ha in the highest density years immediately following mast events (Fig. 2C). Finally, female red squirrels experienced pronounced shifts in predator abundance and predation risk. Estimated abundances of Canada lynx varied from 2.62 to 82.30 (mean snow tracks per 100 km transect, mean = 27.50, $SD = 20.59$, Fig. 2D). Because the population of lynx closely follows the density of their preferred prey, snowshoe hares, prey-switching to red squirrels often occurs years following the peak in hare density (i.e., hare crash, Fig. 2D, Krebs et al., 2001b; O’Donoghue et al., 1998).

3.2. Selection across environmental gradients

3.2.1. Effects of environmental factors on female reproductive success

Female annual reproductive success (ARS) decreased by 0.35 (± 0.09) recruits (i.e., pups surviving their first winter) for every 1 SD (0.8 squirrels/ha) increase in squirrel density ($z = -3.8$, $p < 0.001$, Table 2, Fig. 3). Annual reproductive success nearly tripled from non-mast years (raw mean = 0.53 recruits) compared to mast years (raw mean = 1.57 recruits; model estimate: 0.69 recruits ± 0.19 , $z = 3.6$, $p < 0.001$,

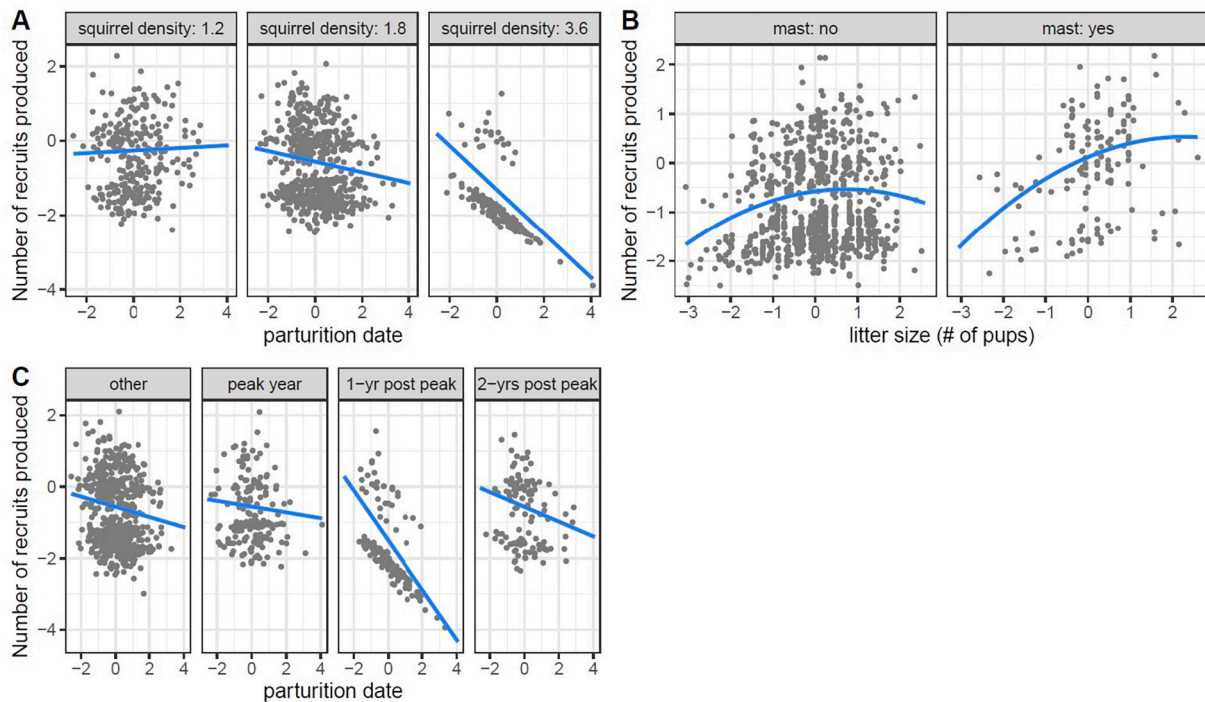


Fig. 4. Intraspecific competition, food availability, and predation risk predict patterns of selection on female red squirrel life history traits. Partial regression plots showing the relationships between life-history traits and the number of recruits produced by female North American red squirrels (*Tamiasciurus hudsonicus*). Lines represent linear regressions extracted from selection analyses testing whether directional selection shifts across environments. For (A), the left panels indicate the regression line at the 10th percentile of squirrel density, the middle panel represents the regression at the 50th percentile, and the right panel indicates the regression at the 90th percentile. Each residual is plotted only once in the panel that indicates the environment of closest proximity.

Table 3

Production of fecal glucocorticoid metabolites is associated with all four environmental fluctuations. Table shows results from linear mixed-effects model testing whether variation in potential ecological agents of selection predict fecal glucocorticoid metabolites (FGMs, ng/g, log-transformed dependent variable). Continuous predictors were standardized to zero mean and unit variance prior to analysis. Model included 1298 FGM measures from 165 squirrels collected across 7 years. Bold lettering indicates significant relationships ($P < 0.05$).

Independent terms	Dependent: Fecal glucocorticoid metabolites (FGMs)			
	Estimate	SE	t	P
maternal age²	-0.14	0.06	-2.16	0.031
maternal age	0.12	0.07	1.72	0.086
reproductive status (non-breeding)	-0.19	0.04	-4.97	<0.001
squirrel density	0.1	0.03	3.46	0.001
mast (yes)	-0.12	0.05	-2.53	0.012
lynx abundance	0.07	0.03	2.59	0.010
mean overwinter temperature	-0.08	0.02	-3.67	<0.001
grid (SU)	0.15	0.05	2.73	0.007
prior year cone availability	0.3	0.03	11.15	<0.001
julian sample date	0.33	0.11	3.1	0.002
julian sample date²	-0.29	0.11	-2.7	0.007

Table 2, Fig. 3). One year after a hare crash, when lynx abundance remained high but hares were low, ARS of female red squirrels decreased by 0.93 (± 0.27) recruits per litter compared to other years in the lynx-hare cycle ($z = -3.5$, $p < 0.001$, Table 2, Fig. 3). There was no difference in female squirrel ARS between peak hare density years (estimate: 0.01 \pm 0.23, $z = 0.0$, $p = 0.964$) or 2-years post peak (estimate: 0.01 \pm 0.24, $z = 0.0$, $p = 0.963$), compared to other years in the cycle (Table 2, Fig. 3). We found no effect of overwinter temperature (estimate: -0.05 ± 0.08 , $z = -0.6$, $p = 0.553$), or cone availability from the preceding autumn (estimate: -0.12 ± 0.09 , $z = -1.3$, $p = 0.186$) on ARS (Table 2, Fig. 3).

3.2.2. Environmental factors influencing patterns of selection on life history traits

Broadly, directional selection generally favored faster pup growth rates ($\beta = 0.11 \pm 0.04$ SE, $z = 2.8$, $p = 0.006$), larger litters ($\beta = 0.11 \pm 0.05$ SE, $z = 2.2$, $p = 0.030$), and earlier parturition dates ($\beta = -0.17 \pm 0.06$ SE, $z = -2.9$, $p = 0.003$) (Table 2). None of the environmental factors tested here affected selection on pup growth rate. Selection on parturition date varied depending on squirrel density and the lynx-hare cycle, and selection on litter size shifted in non-mast to mast years. The strength of selection favoring early parturition dates increased with squirrel density (estimate: -0.21 ± 0.06 , $z = -3.5$, $p < 0.001$, Table 2, Fig. 3), indicating that squirrels who bred earlier in high density years had higher fitness. The strength of selection favoring early parturition dates also increased 1 year after the peak in the hare-lynx cycle (i.e., 1 year after hare and lynx abundances were highest) compared to other years in the lynx-hare cycle (estimate: -0.55 ± 0.20 , $z = -2.7$, $p = 0.007$, Table 2, Fig. 3). The strength of selection favoring larger litters increased in mast years compared to non-mast years (estimate: 0.25 ± 0.10 , $z = 2.6$, $p = 0.010$, Table 2, Fig. 3). Overwinter temperatures were not associated with patterns of selection on any of the three life history traits (Table 2, Fig. 3).

3.3. Endocrine responses to environmental change

We hypothesized that endocrine responses to environmental changes would be specific to ecological agents of selection. To test this hypothesis, we built a linear mixed-effects model to determine the effects of the same environmental variables examined above (food availability, conspecific density, predation risk, temperature) on fecal glucocorticoid metabolites (FGMs). We predicted that cues of an upcoming food pulse, increased squirrel density, and elevated lynx abundance would elicit an endocrine response, but that temperature would have no effect. Instead, we found evidence for a generalized hormonal response to environmental changes regardless of whether they affect selection on life history

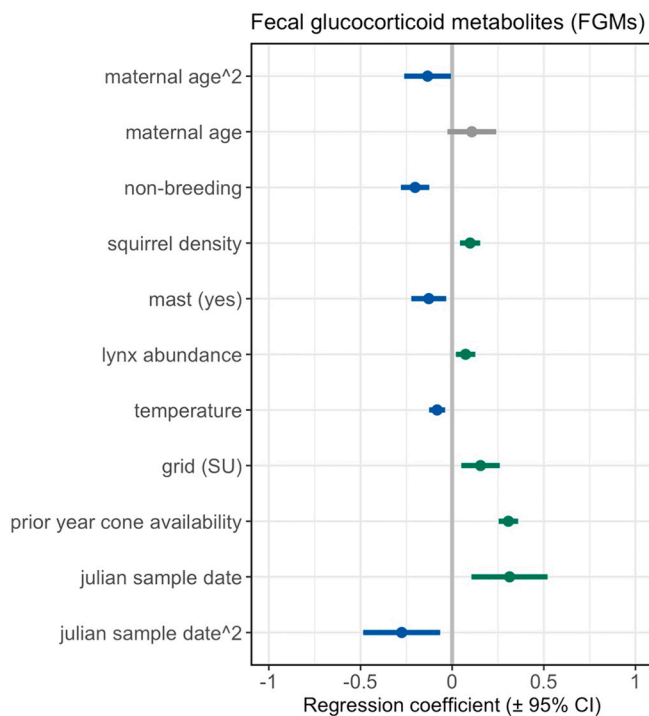


Fig. 5. Female red squirrels exhibit a general, rather than specific, glucocorticoid response to environmental change. Forest plot of model estimates and associated confidence intervals corresponding to linear mixed-effects model testing the effects of the same potential ecological agents of selection from Fig. 4 on concentrations of red squirrel fecal glucocorticoid metabolites (FGMs). The dataset contained 1298 FGM measures from 165 females across 7 years, and the model included maternal ID as a random factor (not shown, explained variance in FGM concentrations 0.03). Continuous fixed variables were standardized to a mean of zero and unit variance, and FGMs were log-transformed to achieve residual normality. We controlled for potential effects of intrinsic factors (i.e., maternal (linear and quadratic) age and reproductive status (breeding or non-breeding) on FGM concentrations. Squirrel density, mast (yes/no), and temperature were assessed the same as in selection models. We included lynx abundance as a continuous variable rather than a categorical variable of lynx-hare cycle as we expected squirrels to exhibit endocrine responses to the presence of lynx regardless of the abundance of hares. Nonsignificant effects are shown in gray; positive effects denoted in green, and negative effects in blue.

traits. As expected, FGMs increased as squirrel densities increased (estimate \pm SE: 0.10 ± 0.03 , $t = 3.46$, $p < 0.001$, Table 3, Fig. 5, Fig. 6), and an upcoming food pulse, or spruce mast, also predicted FGM concentrations such that FGMs were lower in mast years compared to non-mast years (estimate \pm SE: -0.12 ± 0.05 , $t = -2.53$, $p = 0.01$, Table 3, Fig. 5, Fig. 6). FGMs also increased in response to elevated lynx abundance (estimate \pm SE: 0.07 ± 0.03 , $t = 2.59$, $p = 0.010$, Fig. 5, Fig. 6). However, FGMs increased in response to colder overwinter temperatures (estimate \pm SE: -0.08 ± 0.02 , $t = -3.67$, $p \leq 0.001$, Table 3, Fig. 5, Fig. 6), despite temperature exhibiting no effect on patterns of selection, suggesting a generalized, rather than specialized, glucocorticoid response to environmental change.

4. Discussion

For decades, the endocrine system has been viewed as an integrator of both intrinsic physiological shifts and extrinsic environmental and ecological changes. Prior work has largely supported the idea that the endocrine system, and the hypothalamic-pituitary-adrenal (HPA) axis in particular, responds in a generalized way to these changes regardless of their ability to affect patterns of selection on traits associated with fitness. Yet, in highly fluctuating, but predictable, heterogeneous

environments, physiological responses may instead be specific only to those changes that induce adaptive phenotypic shifts. Here, we provide evidence that female red squirrels mount generalized HPA axis responses to environmental stressors regardless of whether or not they affect selection on life history traits. Our findings support the hypothesis that the HPA axis is generalized rather than specialized, but limitations of our study underscore a need for future work on the evolution of the endocrine system in fine-grained, heterogeneous environments.

Confirming earlier work in our study population, we found that selection favored earlier parturition dates when competition was high (e.g., elevated conspecific densities, Dantzer et al., 2013) and that the strength of selection favoring larger litters increased during an upcoming spruce mast (McAdam et al., 2019). However, faster pup growth rates were not favored in high conspecific density environments as prior work has shown (Dantzer et al., 2013; Fisher et al., 2017). Our inability to detect this density-dependent selection on pup growth rates suggests that the strength or form of selection in high conspecific density environments fluctuates temporally and may depend on other aspects of the environment. For example, we may have uncovered this effect if we tested for interactions of conspecific density and other environmental variables on the relationship of pup growth rate and fitness (i.e., 3-way interactions on fitness). While the high dimensionality of these tests would better reflect ecological reality, doing so would push the limits of our data and interpretation and is outside of the scope of the current study.

Beyond the effects of food and density, whether changes in temperature and predators affect selection on female life history traits was previously unknown in our study population. In line with prior work in birds demonstrating effects of increased predation risk on life history traits (i.e., clutch size, Travers et al., 2010), we found that selection on the timing of parturition shifted depending on predation risk. One year following a hare crash, when lynx abundance remained high but hare abundance dropped, the strength of selection against later-born litters increased. Predation pressure from lynx is expected to increase through the season as hare numbers continue to drop and lynx switch to squirrels as an alternative food source (O'Donoghue et al., 1998). Thus, selection appears to favor females that breed earlier in the year, likely because their pups are better able to avoid predation by lynx compared to pups from females that breed later in the year.

We did not find an effect of overwinter temperatures on natural selection on litter size, parturition date, or pup growth rate. We therefore predicted that a generalized endocrine response would be characterized by hormonal responses to the three ecological agents of selection (food availability, squirrel density, and predators) as well as temperature. Alternatively, if the endocrine response is specialized and attuned only to ecological agents of selection, we expected that individuals would respond to ecological agents of selection but not to temperature.

As expected, FGMs increased as squirrel densities, and therefore intraspecific competition, increased (Dantzer et al., 2013; Guindre-Parker et al., 2019). Females also exhibited a change in FGMs depending on spruce masting cycles: FGMs were higher in non-mast years when the production of new cones were low but cache sizes, or stored/hoarded cones, were typically greatest. In mast years when a superabundance of new cones is produced, but food caches are depleted, FGMs were lower. New cones produced in mast years, however, are not available to squirrels until autumn, and fecal samples for analyses were collected before these cones were available to the squirrels, suggesting that the relationship between food availability and FGMs may be related to cache size. Alternatively, we have previously shown that experimentally providing squirrels with additional food increases, rather than decreases, FGMs because of the associated increase in conspecific density (Dantzer et al., 2013) and not because of a change in their diet (Dantzer et al., 2011). Finally, the behavioral and reproductive changes unique to spring and summer of mast years (e.g., increased litter sizes, territorial breakdown; McAdam et al., 2019, Webber et al., submitted) may be accompanied by a potential attenuation of HPA axis function. As the

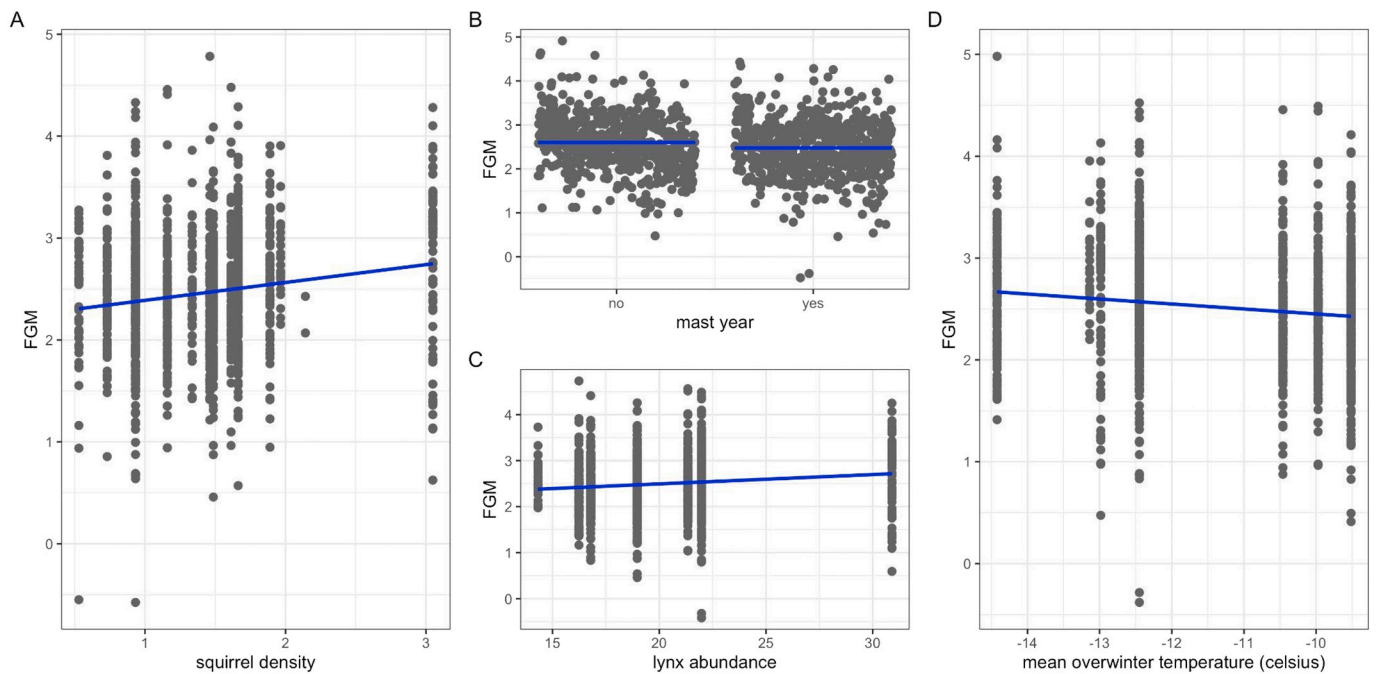


Fig. 6. Higher fecal glucocorticoid metabolite (FGM) concentrations are associated with elevated squirrel densities, non-mast years, increased predators, and colder overwinter temperatures. Partial residual plots demonstrating significant effects of environmental variables on the production of FGMs in female red squirrels. Plots reflect a linear mixed-effects model and each point represents partial residuals for effects of (A) squirrel densities, (B) an upcoming food pulse or mast event, (C) lynx abundance, and (D) mean overwinter temperatures on female FGM production.

energetic demands of increased reproductive output and associated behavioral changes may be expected to increase FGM production, whether lower FGM production in mast years coordinates adaptive shifts in female life history traits remains to be determined in our study population.

We also found that FGMs were elevated when lynx were more abundant regardless of the abundance of their major prey, snowshoe hares. This is aligned with our predictions given the effect of the lynx-hare cycle on selection on the timing of parturition, as well as prior work demonstrating increased glucocorticoids in response to predators and their cues (MacLeod et al., 2018; Sheriff et al., 2009). It also suggests that exposure to predators and their cues can have sustained effects on physiology beyond acute predator attacks (Clinchy et al., 2013). Squirrels are most susceptible to predation by lynx during a hare crash in the year immediately following the peak in hare abundance, but mount a glucocorticoid response to increased lynx abundance regardless. Such a response may reflect an “ecology of fear” among female squirrels that persists despite fluctuations in the likelihood of lynx prey-switching to squirrels (Clinchy et al., 2013).

Finally, cold overwinter temperatures increased FGMs. Despite no evidence of an effect of overwinter temperatures on annual reproductive success or patterns of natural selection in this study, colder winters have been shown to result in higher rates of litter failure (Studd et al., 2015), suggesting that the thermoregulatory and/or food availability costs of low overwinter temperatures may elicit an HPA response that persists into the following year. Moreover, red squirrels exhibit decreased activity at low temperatures (Humphries et al., 2005), and body temperature decrease at low ambient temperatures without effects on heart rate (Menziez et al., 2020). Together, these findings may indicate that cold overwinter temperatures induce broad physiological changes in red squirrels by reducing activity and body temperature while increasing FGM concentrations.

There were a few key limitations of our study. Overwinter temperature was the only environmental variable to not affect patterns of selection on life history traits, thus our interpretation of whether the endocrine response was specialized or generalized hinged on whether or

not female squirrels exhibited a change in FGMs in response to temperature. Moreover, other environmental variables (e.g., precipitation, snow depth, avian predation) that were not quantified in this study may additionally affect both patterns of selection and FGM production. Additionally, environmental covariance, which creates collinearity among environmental variables (Kruuk et al., 2003), can make isolating hormone responses to specific variables complicated (Dantzer et al., 2016). Although the density and detail of our long-term data was a strength of this study, tests of hormonal responses to concurrently shifting variables may be superior when performed in controlled, experimental settings that limit potential environmental covariance. Lastly, the relatively small and shallow FGM sampling in this study precluded our ability to control for the effects of other factors like dietary composition on FGMs, or test directly whether FGM responses themselves coordinate variation in life history traits. For example, do elevated FGMs facilitate earlier breeding in years when predation risk is high? Determining if and how hormone production mediates variation in life history traits in response to environmental shifts is critical to understanding how endocrine responses may drive evolutionary change. We therefore encourage future work to prioritize dense hormone sampling from individuals living in highly heterogeneous environments to permit more direct tests of endocrine mechanisms when possible.

Endocrine responses to environmental change may reflect physiological coping mechanisms that allow organisms to deal with stressors, facilitate adaptive shifts in phenotypic targets of selection, or both. Identifying these effects and their evolution requires an understanding of whether endocrine responses to recurring environmental shifts are generalized, or specialized only to ecological agents of selection. Most commonly, studies assess if individuals exhibit an endocrine response to a single ecological variable at a time, such as exclusively focusing on how predation risk affects hormone production while ignoring that other environmental variables that also may affect hormone production are changing at the same time. Here, we combine detailed data on simultaneous, recurring fluctuations in four environmental factors and provide evidence that the HPA axis may respond in a generalized manner to ecological changes regardless of whether these variables

influence phenotypic targets of selection. Our work joins other research on the role of the endocrine system in broadly integrating information across variable environments (e.g., Potticary and Duckworth, 2020). Our results also further demonstrate the importance of considering how multiple, simultaneously-shifting ecological factors may independently co-opt the HPA axis, potentially to facilitate a return to homeostasis and/or to induce adaptive plasticity in response to environmental change.

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Declaration of competing interest

The authors declare no competing interests.

Data availability

All data and code used for this study is available at the figshare repository: <https://figshare.com/s/0bef9fcf4e963da9712d>.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yhbeh.2022.105262>.

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