



Research Article

Physiological stress and spatio-temporal fluctuations of food abundance and population density in Eurasian red squirrels

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Abstract

In continuously changing environments, variation of different ecological factors could affect the functioning of the hypothalamic-pituitary-adrenal (HPA) axis in wild mammals, increasing the secretion of glucocorticoids (GCs). In different animal species, GC concentrations are often used as a measure of the physiological stress response to environmental pressures, such as fluctuations in food abundance, population density, intra- and interspecific competition, and predation risk. However, previous studies reported contrasting results or did not find clear associations between physiological stress and environmental variables. Here, we used concentrations of faecal glucocorticoid metabolites (FGMs) as an integrated measure of physiological stress in wild Eurasian red squirrels (*Sciurus vulgaris*) from three study areas in the Italian Alps, to investigate whether variations in conifer-seed crop size and/or population density affected HPA axis activity. Squirrel density was estimated in each trapping session using the minimum number of animals alive, and annual counts of fresh cones from different conifer species were used to estimate annual food abundance (MJ/ha). We expected higher FGMs in response to increasing population density and/or decreasing food abundance, since these two variables could act as environmental stressors. Our results showed a lack of association between population density and FGMs and a significant effect of food abundance on FGMs. When conifer seed-crops were poor to moderate, FGMs increased with food abundance, while in the range of high seed-crops, FGMs remained first constant and then slightly decreased with a further increase in seed abundance. We also found differences in FGMs among seasons, as previously observed in this species. Our study adds further evidence that physiological stress can be influenced in different ways by environmental pressures and that long-term studies using individually marked animals are needed to disentangle the potential adaptive outcome of the physiological stress response in pulsed resource systems.

Introduction

Free-living vertebrates cope with different environmental pressures through a suite of physiological and behavioural responses mediated in part by the action of the hypothalamic-pituitary-adrenal (HPA) axis (Sapolsky et al., 2000; Romero, 2004). Ultimately, the activation of HPA axis involves the release of glucocorticoids (GCs), whose short-term elevation (acute stress) can help the individual to overcome dangerous situations (Wingfield et al., 1998; Sapolsky et al., 2000; Romero, 2004). Conversely, a chronic increase in GCs concentrations may alter behaviour and energy balance, inhibit growth and reproduction (Cabezas et al., 2007; Sheriff et al., 2009), elevate blood glucose levels (Caso et al., 2008), compromise immune system and suppress the inflammatory response (Romero, 2004; St. Juliana et al., 2014). However, long-term elevated GCs are not always deleterious, but instead can produce adaptive responses to ecological pressures (Denver, 1997; Boonstra, 2013; Dantzer et al., 2020).

In different animal species, measurement of GCs has been frequently used to assess the physiological response to stressful stimuli (Sher-

iff et al., 2011; Shutt et al., 2014; Casas et al., 2016; Fauteux et al., 2017; Sánchez-González et al., 2018) caused by environmental pressures, that can be related to changes in food abundance, habitat quality, intraspecific and/or interspecific competition, predation, or the arrival of invasive species (Herring et al., 2011; Clinchy et al., 2013; Creel et al., 2013; Santicchia et al., 2018). Also, social status of an individual can influence GC levels and several studies on vertebrate species explored the relationship between GCs and intraspecific interactions (Creel et al., 2013). For instance, population density of meadow voles (*Microtus pennsylvanicus*) was positively correlated with free corticosterone levels (Boonstra and Boag, 1992), and in free-living deer mice (*Peromyscus maniculatus*) in North-Central Idaho, estimated population size was positively associated with faecal glucocorticoid metabolite (FGM) levels (Harper and Austad, 2004). In territorial North American red squirrel (*Tamiasciurus hudsonicus*), experimental elevation of actual and perceived population density increased the physiological stress response of adult females (Dantzer et al., 2013), though there is considerable individual variation in the GC response to squirrel density (Guindre-Parker et al., 2019).

Moreover, Dantzer et al. (2016a) suggested that the mechanisms which induce a heightened HPA axis activity at high population density

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may be more complex, and future long-term studies following marked individuals over their lifetime should take into consideration potential confounding effects, such as antagonistic interactions with conspecifics, decreases in food availability or changes in predator pressure. For instance, Chapman et al. (2015) studying Tana River red colobus (*Procolobus rufomitratus*), showed that the increase of faecal glucocorticoid metabolites (FGMs) caused by a poor diet was enhanced in degraded forest. Also, a study on koalas (*Phascolarctos cinereus*) in southwestern Queensland showed that higher faecal cortisol metabolite concentrations was linked to lower rainfall levels typical for low-quality habitats (Davies et al., 2013). Hence, temporal fluctuations in population density, food abundance and spatial differences in habitat quality can, potentially, affect variation in physiological stress measured through GCs concentration, but it might be difficult to decouple the effects of food and density without experimental manipulations (e.g. Dantzer et al., 2020).

In a review of research on studies of GCs in wild primates, Beehner and Bergman (2017) argue the need for comparative studies that measure fluctuating environmental variables, the related changes in GC secretion, and the potential fitness consequences of the variation in GCs to increase our understanding of the adaptive nature of the stress response. Despite the high demand of studies that investigate the adaptive nature of the physiological stress response in wild mammals (e.g. Dantzer et al., 2020), the first appropriate step is to explore factors that cause GCs variation in a specific environment (e.g. pulsed-resource systems; Boutin et al., 2006; Wauters et al., 2008). Therefore, as part of a long-term study on producer-consumer dynamics, we monitored FGMs in three populations of Eurasian red squirrels in subalpine conifer forests over a six-years period. These habitats are pulsed-resource systems, and space use and density of red squirrels vary with fluctuating tree-seed production (Boutin et al., 2006; Wauters et al., 2008, 2021; Mazzamuto et al., 2020). Spatio-temporal dynamics of cone crops and squirrels can show extreme (up to > 10-fold) fluctuations, making this an interesting system to investigate if and how animals change GC secretion in response to fluctuating density and/or food supply. The Eurasian red squirrel is a solitary species, with males having larger home ranges than females. The core-area of a male's home range overlaps with that of other males and with female core-areas, but adult females have food-based home ranges and defend their core-areas against other females (Wauters and Dhondt, 1992; Romeo et al., 2010; Di Pierro et al., 2011). Hence, space use and social organization are different from the territorial system of *Tamiasciurus hudsonicus* where both females and males defend exclusive territories year-round (Dantzer et al., 2020). In subalpine forests, red squirrels tend to have a single litter per year, with parturition between March and July (Wauters et al., 2008), and reproductive success is strongly affected by the female's body condition and by food availability (Wauters and Dhondt, 1995; Wauters et al., 2007; Rodrigues et al., 2010). Red squirrel numbers fluctuate seasonally and between years, with a positive correlation between food abundance and density (Wauters et al., 2008). Most animals disperse as juveniles and subadults during the autumn dispersal peak (from 4 to 10 months old, Wauters et al., 2010).

Several studies on tree squirrels have used faecal glucocorticoid metabolites (FGMs; Sheriff et al., 2011; Dantzer et al., 2010), a combination of both baseline and stress-induced GC levels, as a non-invasive integrated measure of short-term physiological stress (*Sciurus vulgaris*: Dantzer et al., 2016b; Santicchia et al., 2018, 2022; *Sciurus carolinensis*: Romeo et al., 2020; Santicchia et al., 2020; *Tamiasciurus hudsonicus*: Dantzer et al., 2010, 2013). We collected fresh faecal samples from marked individuals to explore whether variation in FGM concentrations was affected by population density of red squirrels (squirrels/ha) and/or by fluctuations in the size of the conifer seed-crop (MJ/ha). In pulsed-resource systems, both squirrel density and tree-seed production vary markedly among years (Boutin et al., 2006; Wauters et al., 2008) and therefore high conspecific densities and/or low food abundance could act as environmental stressors (see also Dantzer et al., 2013; Raulo and Dantzer, 2018). Hence, we predicted that FGMs will increase with density and/or decrease when food abundance is higher.

Also, possible combinations of high density with low food abundance (food by density interaction) should result in high FGMs.

Materials and methods

Study sites

We trapped red squirrels in three study sites in the Italian Alps (Bormio, Cancano, Valfurva) located in Lombardy, Northern Italy. These study sites are characterized by subalpine mixed conifer forests that differ in the dominant tree species. Bormio (46°27' N, 10°30' E, elevation from 1950 m to 2130 m a.s.l.; size 92 ha) is dominated by Arolla pine, *Pinus cembra* (73.2%), with larch, *Larix decidua* (17.8%) and scarce Norway spruce, *Picea abies* (8.0%) and dead trees (1.0%) (Wauters et al., 2007; Zong et al., 2014; Santicchia et al., 2021). Cancano (46°33' N, 10°15' E, elevation from 1940 m to 1970 m a.s.l.) extends over 54 ha in a high-elevation valley and it is part of a monospecific forest of dwarf mountain pine, *Pinus mugo*, whose seeds are the only major food resource for red squirrels (Rodrigues et al., 2010; Romeo et al., 2010). Valfurva (46°27' N, 10°31' E, elevation from 1650 m to 1870 m a.s.l.) is located on an east-north-east slope in Valfurva valley and covers 78 ha (Zong et al., 2014). It is characterized by Norway spruce (88.9%) with a lower presence of Arolla pine (6.2%), dead trees (3.0%) and larch (1.9%) (Wauters et al., 2007; Santicchia et al., 2021).

Trapping and handling squirrels

Capture-mark-recapture (CMR) sessions were carried out each year in May-June (spring-summer) and in September-October (autumn), from 2014 to 2019 (details in Tab. 1). We used single live traps (Tomahawk trap model 202, Tomahawk Live Trap Co., Tomahawk, Wisconsin, U.S.A.) placed with baling wire on tree trunks at breast height or on the ground (Mazzamuto et al., 2016). Traps were more or less homogeneously distributed over the study site, with distances of 75–150 m between them and average trap density ranging from 0.22 to 0.33 traps/ha¹ (details in Tab. 1).

Pre-baiting started 4 weeks before each trapping session: hazelnuts were placed inside the blocked traps and each trap was pre-baited about once a week. Trapping sessions lasted 4 to 5 days and traps were checked two to three times per day to reduce the time a squirrel was confined in the trap (Romeo et al., 2021). A trapped squirrel was flushed from the trap in a zipper-tube handling bag. At first capture, each squirrel was individually marked with a numbered metal ear tag (Monel 1005 1L1 National Band and Tag Co, Newport, Kentucky, USA). Sex and reproductive condition were determined based on external genitalia; males were classified as reproductively active (scrotal testes) or non-active (abdominal testes); females were classified as non-breeding (anoestrus), pregnant (post-oestrus), or lactating (enlarged nipples) (Wauters and Lens, 1995; Wauters et al., 2007). At each capture, an animal was weighed to the nearest 5 g with a spring balance (Pesola AG, Baar, Switzerland), and the length of the right hind foot (nails excluded) was measured (0.5 mm precision) with a thin wing ruler (Wauters et al., 2007; Mazzamuto et al., 2017a; Romeo et al., 2021). Hind foot length is strongly correlated with total head and body length and thus is considered a reliable proxy for body size (Wauters and Dhondt, 1989).

Red squirrel density, cone counts and food abundance

As in previous studies on tree squirrels (e.g. Kenward et al., 1998; Boutin et al., 2006; Wauters et al., 2008, 2021; Mazzamuto et al., 2017b), we estimated population density, in each study site and trapping session, using the minimum number of animals known to be alive (MNA) from capture-mark-recapture data divided by study site size (ha). Each year, we estimated food abundance counting cones in the canopy of marked sample trees in 20 by 20 m vegetation sampling plots (one plot at each trap site, n=20 plots in every study site; see Salmaso et al., 2009; Romeo et al., 2010 for details). Using the average number of seeds per cone for each conifer species, combined with the species-specific energy content per unit of seed dry mass (Salmaso et al., 2009; Romeo et al., 2010), we transformed the annual cone counts in an av-

erage seed production per ha (in MJ/ha) in each study site and for each cone-crop (year). Analyses of the among plot variation in seed production showed that the number of plots was sufficient to capture forest heterogeneity in food abundance (Wauters et al., 2008; Salmaso et al., 2009). As reported by Wauters et al. (2008), squirrels start feeding on immature green cones in summer (July-August) and continue to consume conifer seeds throughout autumn-winter and until cones open in April-May of the next spring. Thus, cone counts were carried out early August, and the cone-crop of year *t* was considered available for squirrels captured between August year *t* and July year *t* + 1. Subsequently, we defined food abundance in spring-summer of year *t* as the seed-crop size estimated in year *t* – 1, while the seed-crop size estimate of year *t* was our measure of food abundance in autumn of year *t*.

Extraction and quantification of FGMs

Fresh faecal samples (<3 h) of trapped squirrels were collected from underneath the trap using forceps and placed into 1.5 ml vials, that were stored dry at –20 °C within 3–4 hours after collection (Dantzer et al., 2010). To account for potential daily fluctuations in FGMs, each faecal sample was classified as being taken in the morning (10.00–13.00 h) or in the afternoon (15.00–18.00 h; Palme, 2019). To measure FGM concentrations (ng/g dry faeces), we used a 5 α -pregnane-3 β , 11 β , 21-triol-20-one enzyme immunoassay (EIA) which detects GC metabolites with a 5 α -3 β ,11 β -diol structure (for cross-reactivity see Touma et al., 2003). A previous study validated this EIA for the study species and showed that a stressor is reflected in FGM levels between 24 to 36 hours after its application (Dantzer et al., 2016b). Therefore, to avoid effects of capture stress on FGM concentrations, we collected faeces only from squirrels that had not been trapped or handled within 72 hours prior to capture (Dantzer et al., 2016b). Methods of extraction of FGMs and enzyme immunoassay validation for Eurasian red squirrels can be found in detail elsewhere (Dantzer et al., 2016b; Santicchia et al., 2018). We analyzed 159 samples in duplicate. Intra-assay CVs were 8.4%±7.0% (mean±SD). Pools of red squirrel faeces extracts were used as intra-assay controls at dilutions of 1:200 (≈30% binding) and 1:1600 (≈60% binding). Average inter-assay coefficients of variation (CVs) were 14.6% and 14.9%, respectively, for pools diluted 1:200 and 1:1600. To further increase our dataset we also included 95 samples assayed in a previous study from the same study sites and analysed using the same methodology and in the same laboratory (identical experimental design; see Tab. 1 in Dantzer et al., 2016b).

Statistical analyses

To test our predictions, we explored the effects of red squirrel population density (ind./ha) and of fluctuations in the size of the conifer seed-crop (MJ/ha) on variation in FGMs. Since a possible trend in FGMs over time could be a confounding effect (Palme, 2019), we added Julian date as a continuous explanatory variable. Based on our earlier studies, we included sex, reproductive condition nested in sex (males: abdominal or scrotal testes; females: non-breeding, post-oestrus and pregnant, lactating; details in Santicchia et al., 2018), season (spring-summer or autumn) and daytime (sample collected in the morning or in the afternoon) as categorical explanatory variables, since, potentially, they can affect FGMs (Dantzer et al., 2016b; Santicchia et al., 2018). Scores from the second component of a principal component analysis (PCA) on squirrel body mass (g) and right hind foot length (mm) (calculated as average between each measure taken during adulthood) were used as a body condition variable (second component loadings: 0.707 body mass — 0.707 foot length). Hence, for squirrels of the same size, heavier ones have a higher score than those that weigh less. Body condition, calculated at each capture, was included in the models as a continuous explanatory variable. We also considered first-order interactions of season with squirrel density and with food abundance, and a squirrel density by food abundance interaction to test for possible interactions between density and food in affecting FGMs. Squirrel identity nested in study site was added as a random effect to account for repeated measures of FGM for a given individual. We used a linear mixed model (LMM) with Gaussian error distribution and FGM (ln transformed using the natural logarithm, ln of ng/g dry faeces) as dependent variable. We started from a saturated model including all the explanatory variables and interactions indicated above, and selected a minimal adequate model by a stepwise backward elimination of non-significant (*p*>0.15) parameters. To consider possible curvilinear effects of food abundance on FGMs, its second-order orthogonal polynomial effect was included in a second model. We then used the log-likelihood ratio test (LRT) to compare the fit of the full model with the polynomial term of food abundance with the fit of the same full model without the polynomial term. The same procedure was used for the final selected minimal model (Lewis et al., 2011; Romeo et al., 2020). In both cases, the model with the second-order orthogonal polynomial effect of food abundance had the best fit (full model: LRT=7.835; df=3; *p*=0.049; selected minimal model: LRT=7.909; df=2; *p*=0.019), thus we only present these models in the results. All continuous explanatory variables, except body condition (already an index with mean=0), were standardized ($\frac{x-\text{mean}}{\text{sd}}$) prior to analysis to reduce multicollinearity in the presence of a polynomial term (Zuur et al., 2010; Romeo et al., 2020).

Table 1 – Trapping session, red squirrel density (individuals ha⁻¹) estimated using MNA (see methods for details) within each trapping session, food abundance expressed in energy-content of conifer seeds per ha (MJ ha⁻¹, mean±SE), in the three study sites per each season. Size of the study site (ha) and trap density (traps ha⁻¹) between brackets. The seed-crop size estimate in year *t* – 1 is the estimate of food abundance in spring-summer of year *t*, while seed-crop size estimate in year *t* is the estimate of food abundance in autumn of year *t*. Sample sizes (n) and number of different animals (ID) between brackets.

Season	Trapping session	Bormio (92.4 ha) (0.22 traps ha ⁻¹) (n=32; ID=26)		Cancano (54 ha) (0.33 traps ha ⁻¹) (n=46; ID=28)		Valfurva (77.8 ha) (0.26 traps ha ⁻¹) (n=176; ID=92)	
		Density	Food abundance	Density	Food abundance	Density	Food abundance
Autumn (n=99; ID=84)	Sept-2014	0.24	2773 ± 322	0.15	637 ± 116	0.59	1669 ± 263
	Sept-2015	0.24	1182 ± 108	0.20	158 ± 32	0.42	13 ± 5
	Sept-2016	0.13	2301 ± 299	0.17	128 ± 13	0.40	2863 ± 328
	Sept-2017	0.13	1605 ± 208	0.19	294 ± 35	0.49	1361 ± 103
	Sept-2018			0.20	243 ± 58	0.30	1559 ± 270
	Sept-2019			0.07	234 ± 32		
Spring-summer (n=155; ID=107)	June-2014					0.44	897 ± 81
	June-2015	0.24	2773 ± 322	0.15	637 ± 116	0.63	1669 ± 263
	June-2016	0.15	1182 ± 108	0.11	158 ± 32	0.37	13 ± 5
	June-2017	0.13	2301 ± 299	0.20	128 ± 13	0.40	2863 ± 328
	June-2018			0.09	294 ± 35	0.37	1361 ± 103
	June-2019					0.32	1559 ± 270

Table 2 – Full model explaining the observed variation in ln FGM in red squirrels from three study sites in the Italian Alps. All the continuous variables were standardized prior to analysis (except body condition, see “Statistical analyses” section for details). Squirrel ID included as a random factor to account for repeated measures (n=254 of 146 ID).

Explanatory variables	Parameter estimate (\pm SE)	Analysis of variance	<i>p</i>
Sex ^a	0.12 \pm 0.26	$F_{1,232}=0.22$	0.64
Body condition	-0.14 \pm 0.10	$F_{1,227}=1.91$	0.17
Julian date	-0.02 \pm 0.06	$F_{1,225}=0.11$	0.74
Squirrel density	-0.15 \pm 0.10	$F_{1,171}=2.22$	0.14
Food	1.63 \pm 1.42		
Food ²	-0.20 \pm 1.58	$F_{2,232}=5.067^d$	0.007
Season ^b	-0.40 \pm 0.15	$F_{1,220}=7.70$	0.006
Daytime ^c	0.06 \pm 0.12	$F_{1,231}=0.24$	0.63
Reprod. cond. in sex		$F_{3,237}=0.51$	0.68
Season \times density	0.07 \pm 0.12	$F_{1,222}=0.35$	0.55
Season \times Food	2.16 \pm 1.83		
Season \times Food ²	-2.76 \pm 1.90	$F_{2,214}=1.75^d$	0.18
Density \times Food	-1.55 \pm 1.47		
Density \times Food ²	-1.06 \pm 1.51	$F_{2,224}=0.58^d$	0.54

^a Sex, female held as reference level;

^b Season, autumn held as reference level;

^c daytime, afternoon held as reference value;

^d combined test for the quadratic polynomial function of standardized food abundance.

Sample sizes reproductive condition: males, 86 non breeding, 82 breeding; females 47 non breeding, 16 pregnant (post-oestrus), 23 lactating.

Unless otherwise specified, all values and parameter estimates are reported as mean \pm SE. All the statistical analyses were performed in R 4.0.5 (R Core Team, 2021) using the function `poly` for orthogonal polynomial effects and `lme4` package (version 1.1.23; Bates et al., 2015).

Results

Squirrel population density differed between the three study sites. Red squirrels occurred at higher densities in Valfurva (0.30–0.63 individuals/ha), than in Cancano (0.07–0.20 individuals/ha) and Bormio (0.13–0.24 individuals/ha; details in Tab. 1). Also, food abundance varied between the study sites, with Valfurva characterized by a larger and more variable production of tree seeds (13–2863 MJ/ha), than Cancano (128–637 MJ/ha), while food abundance was intermediate at Bormio (1182–2773 MJ/ha; details in Tab. 1).

We obtained a total of 254 faecal samples from 146 different red squirrels (93 males and 53 females) captured in the three study sites between 2014 to 2019. Overall, mean FGM (\pm SE) was 20760 \pm 1066 ng/g dry weight of faeces, ranging from 1452 to 108354 ng/g dry weight (Fig. 1). When calculated per study site, the mean varied from 18865 \pm 2008 in Cancano (n=46), over 20234 \pm 1278 in Valfurva (n=176) to 26372 \pm 3617 ng/g dry weight in Bormio (n=32). At a seasonal scale, mean FGM (\pm SE) was 24733 \pm 1864 in autumn (n=99) and 18221 \pm 1240 in spring-summer (n=155).

Regarding full model results, there was no effect of sex, reproductive condition, body condition, daytime or Julian date on variation in FGMs (Tab. 2). Interactions of squirrel density with food abundance or with season, or the direct effect of density were not significant (Tab. 2) and removed during model selection. The minimal adequate model showed that FGMs were lower in spring-summer than in autumn (Tab. 3; Fig. 2). There was a significant polynomial effect of food abundance on FGMs, while the interaction of food abundance with season was not significant (Tab. 3). FGMs increased with increasing food abundance when seed-crop size was poor to moderate, but with further increasing food abundance in periods with high tree-seed production, FGMs remained constant and even slightly decreased (Fig. 3).

Discussion

In this study we measured FGM concentrations to investigate the physiological stress response of free-living Eurasian red squirrels under two different environmental pressures: fluctuations in population

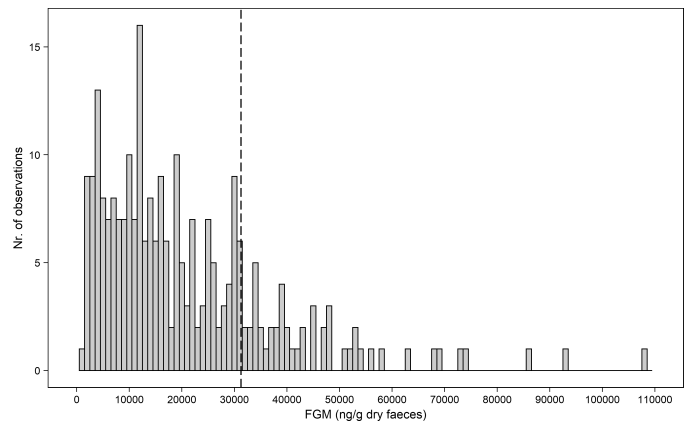


Figure 1 – Frequency distribution of FGM concentrations (ng/g dry faeces) in faecal samples (n=254) from Eurasian red squirrels, *Sciurus vulgaris*. Dashed line indicates the 80th percentile.

density and in food abundance. Contrary to the hypothesis, our results indicated that population density did not affect FGM concentrations, while we found a non-linear positive relationship between food abundance and FGMs that, however, changed in periods of high tree-seed availability.

In the majority of studies reported in a review by Creel et al. (2013), there was a positive correlation between GC levels and population density. Among them, only in two cases FGMs were used as a measure of GCs: in deer mice (*Peromyscus maniculatus*) and in North American red squirrels (*Tamiasciurus hudsonicus*) (Harper and Austad, 2004; Dantzer et al., 2013); while in wild populations of meadow voles (*Microtus pennsylvanicus*), snowshoe hares (*Lepus americanus*), red-backed voles (*Clethrionomys rutilus*), and root voles (*Microtus oeconomus*), physiological stress level was measured using plasma GCs (Boonstra and Boag, 1992; Boonstra et al., 1998; Novikov and Moshkin, 1998; Bian et al., 2011). Plasma GCs obtained from captured or handled animals are usually increased due to acute stress related to the trapping event, whereas FGMs are an integrated measure of circulating GCs over some time prior to trapping and sampling, with the time window being species specific (Dantzer et al., 2016b; Palme, 2019).

Hence, although several studies in literature reported a positive association between GCs and population density, this relationship was not always found. Indeed, as suggested by Boonstra (2013), patterns of variation in physiological stress in wild animals are influenced by the continuous coping with a variety of changing environmental vari-

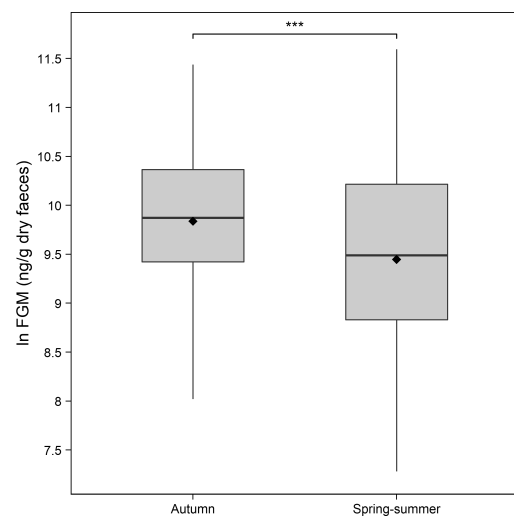


Figure 2 – Comparison of FGM concentrations (ln ng/g dry faeces) in Eurasian red squirrels captured in Autumn (n=99) and Spring-summer (n=55). Boxplots show median (solid horizontal line), mean (black diamond) and 1st (25%) and 3rd (75%) quartiles. ****p*<0.001.

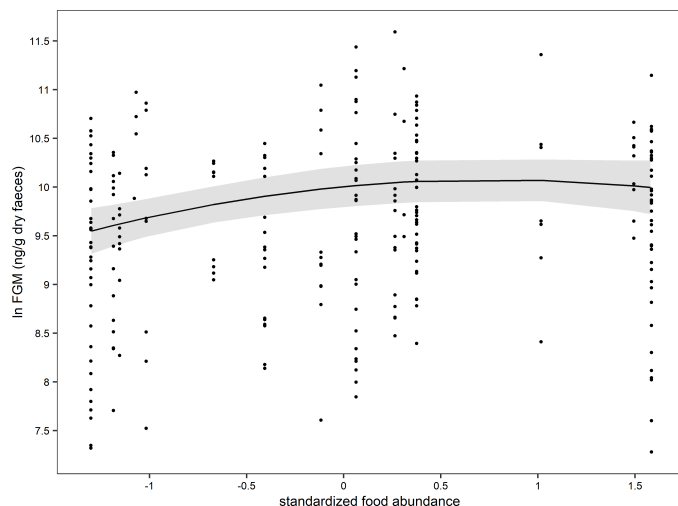


Figure 3 – Relationship between FGM concentrations (ln ng/g dry faeces) and food abundance (MJ, standardized values) in Eurasian red squirrels. The line represents the predicted relationship (calculated from the minimal adequate model excluding the interaction of food abundance with season). Shaded areas represent the 95% confidence intervals, symbols (full circles) show observed values.

ables (e.g. weather, food, competitors, predators), ultimately resulting in adaptive responses. For instance, higher FGMs in wild red colobus (*Procolobus rufomitratus*) groups of larger size may be an adaptive response to cope better with elevated within-group competition for food (Chapman et al., 2007, 2015). Unfortunately, the results of these studies on colobus are questionable, since they used non-validated methods to estimate FGMs. However, it must be considered that population density does not directly affect fluctuations in FGMs, instead the interaction between density and per-capita food availability might influence FGM concentrations. In the present study, we explicitly tested if the food by density interaction affected FGM concentrations, but did not find a significant result. Furthermore, in free-living prairie voles (*Microtus ochrogaster*), FGM levels had the opposite trend than usually predicted, or rather, were higher at lower population densities (Blondel et al., 2016). Indeed, according to Blondel et al. (2016) this tendency of increased GCs at lower densities could be related to high energetic costs of larger home ranges, the effort of searching for potential mates or a general increase in vigilance caused by the higher pro-capita predation risk at lower density. Thus, the predicted relationship between FGMs and population density may be more complex and/or mediated by other factors, or changes in density might influence FGMs only above a lower threshold level of population density.

In this study we did not find a significant relationship between population density and FGM concentrations, in agreement with previous studies on tree squirrels that considered potential effects of squirrel density on FGMs (*Sciurus vulgaris* in Santicchia et al., 2022; *Sciurus carolinensis* in Santicchia et al., 2020). This lack of association may be due to the type of physiological stress measure used in these studies, that could obscure the relationship between density and GCs. In Eurasian red squirrels, FGMs are a measure of GC secretion 24 to 36 hours before defecation (Dantzer et al., 2016b). Hence, the effect of population density may be masked by other stressful events at that time, causing short-term elevations in FGMs that mask the association between density and FGMs. Although FGMs have proven to be a reliable measure and play an important role in mediating the individual endocrine response to environmental pressures (Palme, 2019), measuring only FGMs to characterize physiological stress might be problematic since the stress response is more complex (MacDougall-Shackleton et al., 2019; Gaidica and Dantzer, 2020) and FGMs correspond to circulating GCs over a species-specific period of time before the sample is collected (Sheriff et al., 2011; Palme, 2019).

Another, non-mutually exclusive, reason for the lack of a positive association between GCs and density is that in two of the three study sites, Bormio and Cancano, red squirrels' densities were low and the

Table 3 – Selected minimal model explaining the observed variation in ln FGM in red squirrels from three study sites in the Italian Alps. All the continuous variables were standardized prior to analysis. Squirrel ID included as a random factor to account for repeated measures (n=254 of 146 ID).

Explanatory variables	Parameter estimate (± SE)	Analysis of variance	p
Food	1.07 ± 1.34		
Food ²	-0.41 ± 1.39	F _{2,245} =6.08 ^b	0.003
Season ^a	-0.45 ± 0.11	F _{1,227} =17.37	<0.0001
Season × Food	2.82 ± 1.71		
Season × Food ²	-2.57 ± 1.74	F _{2,223} =2.61 ^b	0.08

^a Season, autumn held as reference level;

^b combined test for the quadratic polynomial function of standardized food abundance.

range of fluctuations during our study period was limited (see Tab. 1). Indeed, densities in Bormio varied from a minimum of 0.13 ind./ha and a maximum of 0.24 ind./ha, and in Cancano among 0.07 and 0.20 ind./ha (Tab. 1). These densities were lower than in Valfurva (max. of 0.63 ind./ha, Tab 1) and than those reported in high-density stable populations in Western Europe (long-term average around 1 squirrel/ha, Wauters et al., 2004). They are also much lower than long-term density variation reported for American red squirrels in the Yukon, Canada (1.32 to 2.61 squirrels/ha; Dantzer et al., 2020). Therefore, the population densities found in this study were likely too low to induce high levels of intraspecific competition for food resources and/or to result in frequent aggressive encounters between conspecifics. Consequently, it was unlikely to detect any significant variation in FGM levels associated with these limited temporal changes in population density. Thus, our results are in contrast with high-density populations of North American red squirrels, in which long-term manipulations of food availability (supplemental feeding) and of social cues of elevated density (frequency of territorial calls) were carried out. The results of these studies showed that both ecological factors can induce similar life-history plasticity, and that changes in the levels of GCs, in response to variation in food and density, is one of the physiological mechanisms behind this plasticity (Dantzer et al., 2020).

We found a significant difference in FGMs among seasons, with lower concentrations in spring-summer than in autumn. This result agreed with a previous study on the same species that found significantly lower FGMs in faeces collected in summer than those collected during winter (Dantzer et al., 2016b). Higher FGMs in autumn and winter were explained as physiological responses to the autumn dispersal peak and to a higher predation risk in winter, due to the increase in foraging on the ground (Dantzer et al., 2016b).

The relationship between seed-crop size and FGMs of red squirrels measured in autumn of the same year or in spring-summer of the following year is a complex one. Similarly, several previous studies found contrasting and/or complex relationships between GCs and measures of food availability. Indeed, some studies in birds reported a negative effect of food abundance on FGM concentrations. For instance, an experimental research on physiological stress response of white ibises (*Eudocimus albus*) to variation in food availability (prey biomass estimated with throw trapping), documented that plasma and FGM levels increased in food-poor periods (Herring et al., 2011). Moreover, also in free living common murres (*Uria aalge*), studied in different food conditions, corticosterone was negatively correlated with food abundance, in this case estimated with the total catch per unit effort, averaged by site and day (Kitaysky et al., 2007).

However, this negative relationship was not a rule for all species. In fact, among mammals, Le Saout et al. (2016) monitored FGMs of Sitka black-tailed deer (*Odocoileus hemionus sitkensis*) over 1 year in three islands of the Haida Gwaii archipelago (Canada). In just one of these islands, where animals were exposed to hunting events, deer took advantage of lower population density and rich understory, while in the other two islands deer were subjected to high population density and a very poor understory, caused by a lack of hunting. Even if authors predicted that FGMs would be higher in island safe from hunting but with

low food abundance, their results showed the opposite trend. The lower FGMs in food-poor islands were explained by the potential low sensitivity of their method to detect small differences in GCs, the limited diversity in environmental conditions between these three islands, or by the hypothesis that lower GC levels may be an adaptation to overcome a situation of poor resource availability. Similarly, this last argument was used by Taillon and Côté (2008) to explain the results of their research on white-tailed deer (*Odocoileus virginianus*). Also, in this species, FGMs were lower under an experimental controlled poor diet, maybe as a physiological adaptation to survive in resource limited periods, for instance during winter.

Our findings suggest yet another pattern with different effect on FGMs based on food abundance level: when seed-crop size was poor to moderate, we found an increase of FGMs with increasing food abundance. This first positive correlation may be due to the fact that, in periods with poor to moderate tree seeds production, an increase of food abundance could induce more concentrated feeding by the red squirrels in the patches with the highest availability of high-energy tree seeds, resulting in higher FGMs. Hence, greater FGMs may be a consequence of the increasing intra- and inter-specific food competition in the best quality patches, and could also be, at least partly, associated with personality-dependent space use (Wauters et al., 2021). Secondly, we found that in high tree-seed production years, a further increase of food abundance resulted in more stable or even slightly decreasing FGMs (see Fig. 3). Probably, this weak negative association between food abundance and FGMs was evident only during high tree-seed production periods, because with these abundant seed-crops, the greater food availability resulted in a reduced competition for food and or space (e.g. Wauters et al., 2005; Romeo et al., 2010; Di Piero et al., 2011).

Hence, environmental pressures can affect the physiological stress response in different directions, based on the type of pressure, season, type of measure of GCs, overall habitat quality and, also, study species. Here, we report that physiological stress levels, measured with FGMs, in Eurasian red squirrels were influenced by variation in food abundance, but not by the range of fluctuations in population density observed in this study. We are aware that our results are not definitive and that future studies should aim to measure fluctuations in FGMs and density more frequently (at shorter time-intervals), and consider the potential effect of spatial variation in local density. In conclusion, our findings enhance the idea that the effects of environmental pressures on physiological status of wild animals are highly heterogeneous and, as recently suggested (Beehner and Bergman, 2017; Dantzer et al., 2020), further investigations using long-term studies on individually marked animals are needed to disentangle the adaptive significance of the stress response in free-living mammals. 🐿️

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