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Measurement of fecal glucocorticoid metabolite levels in Eurasian red squirrels (*Sciurus vulgaris*): effects of captivity, sex, reproductive condition, and season

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The causes and consequences of physiological stress in wildlife are of great interest in a wide range of biological disciplines including understanding how environmental changes affect species fate and persistence. In some areas, the Eurasian red squirrel (*Sciurus vulgaris*) faces local extinction because of the invasive Eastern gray squirrel (*Sciurus carolinensis*). Whether or not physiological stress is induced by the presence of invasive species or contributes to local declines in Eurasian red squirrels is unknown. Here, we develop an assay that can be used to quantify physiological stress in fecal samples from Eurasian red squirrels to eventually address these questions. We captured free-living squirrels (6 females, 11 males) and placed them into captivity for 48 h. Fecal glucocorticoid metabolite (FGM) concentrations in female and male squirrels were significantly higher 24 and 32 h after initial captivity with a lag time to peak excretion ranging from 24 to 36 h. We measured FGM concentrations in free-living squirrels (37 females, 45 males) over a 3-year period. Lactating free-living squirrels had higher FGM concentrations compared to nonbreeding or pregnant squirrels but there were no differences in FGM concentrations in males with scrotal and abdominal testes. Free-living squirrels had the highest FGM concentrations in the winter and lowest in the summer (winter > autumn > summer). Squirrels kept in captivity for 4–48 h had significantly higher FGM concentrations than free-living squirrels (111 fecal samples from 82 squirrels). FGM concentrations in captive but not wild squirrels were significantly repeatable. We found no sex differences and no association with body mass in FGM concentrations in captive or free-living squirrels. Our results indicate that this assay can accurately quantify physiological stress in Eurasian red squirrels, which may be useful for future studies to document how the invasive Eastern gray squirrel contributes to local extinction.

Key words: feces, glucocorticoids, repeatability, squirrel, stress

Physiological stress reactions are multifaceted mechanisms exhibited by organisms in response to environmental changes. The causes and consequences of physiological stress reactions are increasingly studied in a wide range of studies in population ecology, behavioral ecology, conservation physiology, and animal welfare (Romero 2004; Palme et al. 2005; Dantzer et al. 2014; Romero and Wingfield 2015). For example, ecological factors such as food availability (Kitaysky et al. 1999), predation risk (Clinchy et al. 2013), or competition (Creel et al. 2013; Dantzer et al. 2013) can induce physiological stress reactions and these changes can affect a range of behavioral (Koolhaas et al. 1999; Mateo 2007; Cockrem 2013) or life history traits (Breuner et al. 2008; Bonier et al. 2009; Crespi et al. 2013). Physiological stress reactions may play a role in determining levels of parasite infection (Chapman et al. 2006; St Juliana et al. 2014), because chronic stress may reduce an

individual's immune response, increasing its susceptibility to infections (Romero 2004). Another field of growing interest is that of conservation physiology, which is an integrative discipline that aims to understand the effects of environmental perturbations and predict the fate (persistence) of wildlife populations or species using physiological measures such as stress hormones (Wikelski and Cooke 2006; Cooke et al. 2013; Dantzer et al. 2014).

Activation of the vertebrate neuroendocrine stress axis initiates a host of behavioral and physiological changes in response to both predictable and unpredictable environmental challenges (Sapolsky et al. 2000; Romero 2004). A major part of the vertebrate neuroendocrine stress response is coordinated by the hypothalamic–pituitary–adrenal (or interrenal) axis and ultimately results in the secretion of glucocorticoid steroid (stress) hormones (cortisol, corticosterone) that can cause a number of pronounced behavioral and physiological responses (Sapolsky et al. 2000; Möstl and Palme 2002; Romero 2004). Although a variety of different techniques exist to assess whether an animal is "stressed" (Sheriff et al. 2011; Dickens and Romero 2013; Romero and Wingfield 2015), measuring the activity of the vertebrate neuroendocrine stress axis usually involves quantifying glucocorticoids. In all species, this can be challenging because the sampling method should not affect or bias the glucocorticoid levels themselves. In all species but especially those of conservation concern, the sampling method should also not in itself negatively influence the individuals sampled. Fortunately, glucocorticoids can also be quantified in biological samples such as feces, urine, or hair which, unlike blood sampling, can be obtained in a relatively noninvasive manner (Sheriff et al. 2011). The measurement of glucocorticoids in fecal samples collected from wild animals has proven to be particularly useful (Sheriff et al. 2011; Romero and Wingfield 2015). Steroid hormones in the blood are metabolized and conjugated by the liver and excreted into the gut via bile where they are additionally metabolized through the gut microbiome before defecation (Taylor 1971; Brownie 1992; Palme et al. 2005). Measures of glucocorticoids in fecal samples therefore represent measures of the metabolites of glucocorticoids but they are closely correlated with circulating glucocorticoid concentrations (Mateo and Cavigelli 2005; Sheriff et al. 2010).

Assays to measure fecal glucocorticoid metabolites (FGM) have now been developed for many vertebrate species over the past 25 years (comprehensive list available from Palme 2016). However, their application must be carefully validated in each species in which they are used, even in closely related species (Touma and Palme 2005; Sheriff et al. 2011; Fanson et al., in press). The benchmark of a biological validation is to show that an experimental increase in circulating glucocorticoid levels is reflected in FGM concentrations (Touma and Palme 2005). Assays to measure FGM are often validated through an application of some type of experimental stressor (e.g., temporary capture—Harper and Austad 2001; Bosson et al. 2013; Hämäläinen et al. 2014) or pharmaceutical challenge that increases adrenal glucocorticoid production (e.g., adrenocorticotropic hormone [ACTH] injection—Dantzer et al. 2010). The latter is sometimes impossible in species of conservation concern because the capture, restraint, and injections may have detrimental effects on free-living study animals. In some species, this is surmountable by conducting pharmaceutical validations (ACTH injections) in captive- or zoo-housed animals of the same species (e.g., Goymann et al. 1999; Dehnhard et al. 2001; Pribbenow et al. 2014). However, the selection and availability of wildlife species in zoos is limited and so biological validations that do not employ ACTH injections such as temporary immobilization or translocation are also commonly used (Touma and Palme 2005; Pribbenow et al. 2014; Munerato et al. 2015). Moreover, ACTH injections require considerably more expertise (injection procedure) and validation procedures to identify the dosage of ACTH that is required. Ideally, a study should conduct both physiological (with ACTH injection) and biological (such as temporary captivity or exposure to predator cues) validations but this is not always possible especially in species of conservation concern (Touma and Palme 2005).

In some parts of Europe, Eurasian red squirrels (*Sciurus vulgaris*) are threatened with local extinction by the introduction and subsequent spread of the Eastern gray squirrel (*Sciurus carolinensis*—Gurnell and Pepper 1993; Martinoli et al. 2010; Bertolino et al. 2014). Consequently, the Eurasian red squirrel has become a species of conservation concern in the British Isles and Italy. The introduction of an invasive species that increases competition for food and other resources may increase physiological stress in the native species. This could directly contribute to declines in reproduction or survival or increase susceptibility of the native species to infection from novel parasites from the invasive species. In Eurasian red squirrels, it is currently not possible to study these questions because of the lack of noninvasive methods to measure stress. The goal of this study was to validate an enzyme immunoassay (EIA) to measure FGM in the Eurasian red squirrel.

We performed a biological validation of this assay where we captured squirrels and temporarily held them in captivity for 48 h. We measured FGM concentrations in samples collected approximately every 4 h over the 48-h period to determine if and when FGM concentrations significantly increased compared to those measured in samples collected at initial capture. We did not perform a physiological validation (ACTH injection) because this species is of conservation concern and therefore ACTH injections were not possible. Biological validations such as temporary captivity that we used here should provide equivalent validation of an assay to measure FGM concentrations. We also measured FGM concentrations in natural populations of Eurasian red squirrels over a 3-year period. We used these samples to explore how sex, reproductive condition, and a measure of body condition (body mass) affected FGM concentrations. These samples were collected throughout the year (summer, autumn, and winter), allowing us to also investigate seasonal patterns of FGM concentrations.

Materials and Methods

Study species.—The Eurasian red squirrel has a social organization that differs between the sexes: adult females tend to

defend exclusive core areas against other females while among neighboring males there exists a dominance hierarchy with older and heavier males occupying the largest home ranges and overlapping with more females than younger males of lower body mass (Wauters and Dhondt 1989, 1992; Di Pierro et al. 2008; Romeo et al. 2010). There is no sexual size dimorphism (Wauters et al. 2007). The mating system is promiscuous, although most females only mate with a dominant male of high body mass (Wauters et al. 1990). Reproduction is seasonal: mating activity peaks in January to February and April to May, with females giving birth to 1–2 litters per year (spring litters born in March to April, summer litters in June to July). These patterns of reproduction are strongly affected by food abundance and, in the mountains, by elevation (Wauters and Lens 1995; Wauters et al. 2008; Rodrigues et al. 2010). At higher elevations, females tend to have a single litter per year, with parturition dates occurring from April to early July based on conifer seed availability (Wauters et al. 2008; Rodrigues et al. 2010). Increased tree-seed availability improves red squirrel body mass, reproductive rate, and survival, resulting in an increase in population growth and density (Wauters et al. 2004; Boutin et al. 2006; Wauters et al. 2008). The species occupies a wide variety of forest types, occurring in both continuous forests and fragmented woodlands. Although red squirrels are well adapted to fragmented habitats and have good dispersal capacities (Wauters et al. 2010), populations inhabiting forest fragments have lower densities and genetic diversity and higher parasite loads than those in continuous forests (Wauters et al. 1994; Santicchia et al. 2015).

Study sites.—We trapped Eurasian red squirrels in 5 study sites in Lombardy, North Italy (Table 1). Two sites (Vanzago, Castelbarco) are lowland mixed deciduous woodlands in the Po plain while the other 3 (Bormio, Cancano, Valfurva) are subalpine conifer forests in the Central Italian Alps (1,620–2,150 m elevation). Detailed data on forest structure and composition of the alpine sites in the Alps are given elsewhere (Wauters et al. 2008; Salmaso et al. 2009; Rodrigues et al. 2010). The lowland sites (160–170 m elevation) are mature mixed deciduous woodlands dominated by oaks (*Quercus robur*, *Q. petraea*, *Q. rubra*) and hornbeam (*Carpinus betulus*), with some birch (*Betula* sp.), bird cherry (*Prunus avium*), elm (*Ulmus minor*), sweet chestnut (*Castanea sativa*), and black locust (*Robinia pseudoacacia*).

Livetrapping, handling, and fecal samples collection from wild squirrels.—We trapped squirrels in 5 sites during trapping sessions that lasted 4–5 days each (sites and trapping dates listed in Table 1). We used Tomahawk traps (model 202, Tomahawk Live Trap Co., Hazelhurst, Wisconsin) with a fine mesh added underneath traps to prevent contamination between urine and feces. We placed traps on the ground or against tree trunks at a height of about 1.50 m and prebaited with hazelnuts 3–4 times over a 1-month period. We checked traps 2–3 times a day. Each trapped squirrel was flushed into a light cotton handling bag with a zipper (Wauters et al. 2007) to minimize stress during handling and individually marked using numbered metal ear-tags (type 1003 S, 10 by 2 mm, National Band and Tag, Newport, Kentucky). We weighed squirrels to the nearest 5 g using a spring-balance (Pesola AG, Baar, Switzerland) and measured the length of the right hind foot (without nail: ± 0.5 mm) with a thin ruler (Wauters et al. 2007). We recorded sex, age, and reproductive condition following Wauters and Dhondt (1995). A female's reproductive status was defined as nonbreeding (anestrous, vulva small, no longitudinal opening, not lactating), postestrous and pregnant (vulva partly or strongly swollen with longitudinal opening, enlarged abdomen during late pregnancy), or lactating (nipples large, milk excretion can be stimulated). We recorded reproductive condition of males (testes size and position) as nonbreeding (testes abdominal or semiscrotal and scrotum small) or breeding (testes scrotal and scrotum large). After capture and handling, fecal samples were collected from underneath the traps using forceps and placed individually into 1.5-ml vials (Dantzer et al. 2010)

Table 1.—Location of study sites in Lombardy, North Italy and periods in which Eurasian red squirrels were captured for collection of fecal samples (sample size: total fecal samples collected and per sex) to measure FGM concentrations. Vanzago is the study site where we trapped squirrels both for the captive experiment (Exp.) and to obtain FGM concentrations of animals trapped and released after handling (Wild). FGM = fecal glucocorticoid metabolite.

Study site	Coordinates	Elevation (m)	Sampling period	Sample size (males, females)
Vanzago (Exp.)	45°31′N, 8°58′E	160–170	2–5 Dec. 2014 19–23 Jan. 2015 17–20 Mar. 2015	17 (11, 6)
Vanzago (Wild)	45°31′N, 8°58′E	160–170	2–5 Dec. 2014 19–23 Jan. 2015 17–20 Mar. 2015	8 (3, 5)
Castelbarco	45°35′N, 9°31′E	155–165	3–6 Dec. 2013	6 (3, 3)
Valfurva	46°27′N, 10°31′E	1,620–1,870	26–29 May 2014 29 Sept. to 2 Oct. 2014 25–28 May 2015 1–4 Oct. 2015	73 (39, 34)
Bormio	46°27′N, 10°30′E	1,950–2,150	9–13 Sept. 2014 30 June to 3 July 2015	10 (7, 3)
Cancano	46°33′N, 10°15′E	1,940–1,970	14–18 Sept. 2014 15–18 June 2015 7–10 Sept. 2015	14 (8, 6)

and the fine mesh and ground under the traps were cleaned to remove any remaining fecal material. We only used fecal samples from squirrels that had not previously been trapped or handled within 72 h prior to capture. We placed fecal samples into an insulated bag with wet ice packs while in the field and then stored them at −20°C within 3–4 h after collection (Dantzer et al. 2010). Trapping and handling squirrels complied with the current laws on animal research in Italy and were carried out under permission of the Region of Lombardy (Decree n. 11190 of 29/11/2013). All of these procedures abided by guidelines of the American Society of Mammalogists (Sikes et al. 2011).

Capture and husbandry of squirrels for laboratory validation experiments.—We trapped 17 red squirrels (6 females, 11 males) between December 2014 and March 2015 at the Vanzago study site that were subsequently held in captivity for 48 h using the structures of the local wild animal rescue center (Centro Recupero Animali Selvatici, WWF Vanzago). Four individuals were used twice (because in the 1st trial they had defecated only a few times) for a total of 21 experiments. Frequent trap checking prevented any squirrel from spending more than 2 h in a trap. Handling (see above) ended by taking the animal completely out of the cotton handling bag and holding it with a glove, checking reproductive condition, and doing a tape-test to look for parasite eggs (see Romeo et al. 2015).

After capture and handling, we released each animal into its own cage, a Tomahawk single door cat/rabbit collapsible trap (66 × 25 × 25 cm, model 205, Tomahawk Live Trap Co.) with a polycarbonate panel covering the entrance to avoid injury. Cages were kept in a warm room (about 10°C), lighted to simulate the natural photoperiod of late winter to early spring. Each cage contained a nest box with bedding material (hay) and was provided with ad libitum food (hazelnuts and fresh apple, the latter providing hydration, for a total of 1,098 kJ for 48 h). Cages were raised from the floor and a mesh underneath each trap prevented feces and urine from contaminating each other. We collected feces from the mesh and only when clearly not mixed with urine also from the polycarbonate board underneath the cage.

We collected fecal samples using forceps approximately every 4 h for a total of 48 h (except from 2200 to 0900 h) and stored in a 1.5-ml vial at −20°C. At the last check of each day (2200 h), we collected all samples and only collected fresh feces the following morning (0900 h) to minimize collection of samples that had been left at room temperature for > 4 h. Because squirrels are diurnal, it is unlikely much defecation happened until the early morning hours. After collection, we cleaned the forceps and any urine and feces underneath the trap with ethanol to avoid contamination. During housing, we did not manipulate squirrels in any fashion to avoid causing any added stress. All female squirrels were reproductively quiescent upon capture, while males had scrotal testes in 9 out of 14 cases. Upon completion of this study (48 h), we weighed all squirrels and returned them to the site of capture.

Extraction of hormone metabolites.—Samples were stored at −20°C until analysis and shipped to the University of Michigan (Ann Arbor, Michigan) on dry ice. Fecal samples were then lyophilized for 14–16 h to remove moisture and then the entire fecal sample was pulverized into a fine powder using a mortar and pestle along with liquid nitrogen. Approximately 0.05 g of dry feces was weighed on an analytical balance (precision to 0.001 g) and placed into a 2-ml microcentrifuge vial. One milliliter of 80% methanol was then added to these samples and they were vortexed on a multitube vortex for 30 min (Touma et al. 2003; Palme 2005; Palme et al. 2013). The samples were then centrifuged at 2,500 × *g* for 15 min. Next, the supernatant was aspirated and stored in individual 1.5-ml tubes at −20°C until analysis.

Enzyme immunoassay.—We used a 5α-pregnane-3β, 11β, 21-triol-20-1 EIA to measure FGM levels (Touma et al. 2003; Dantzer et al. 2010). This EIA detects glucocorticoid metabolites with a 5α-3β, 11β-diol structure (for cross-reactivity, see Touma et al. 2003). The same EIA has been used to measure fecal cortisol metabolite levels in North American red squirrels (*Tamiasciurus hudsonicus*—Dantzer et al. 2010) and has been used successfully to measure FGM in a variety of rodent species (lab mice—Touma et al. 2004; rats—Lepschy et al. 2007, 2010; Columbian ground squirrels *Urocitellus columbianus*—Bosson et al. 2009; Eastern gray squirrels *S. carolinensis*—Bosson et al. 2013; and 3 different chipmunk species—Montiglio et al. 2012, Hammond et al. 2015).

Details of the EIA procedure can be found in Palme and Möstl (1997), Möstl et al. (2005), and Dantzer et al. (2010). Samples were assayed in duplicate, and if the intra-assay coefficient of variation between duplicates was > 15%, these samples were analyzed again. The intra-assay coefficient of variation for all samples was 6.01%. High (~52% binding) and low (68% binding) fecal extract pools were used as quality controls to measure inter-assay precision (Möstl et al. 2005). The inter-assay coefficients of variation for the high and low pooled fecal extracts were 11% and 17%, respectively (n = 9 plates). FGM levels are expressed as ln ng/g dry feces.

Statistical analyses.—We used linear mixed-effects models (LMM) with ln-transformed FGM concentrations to meet assumptions of normality and homeoscedasticity. We first assessed the effects of captivity on the 17 squirrels that were captured and temporarily held in captivity for 48 h. During this time, we collected fecal samples approximately every 4 h except during the night (from 2200 to 0900 h). We included hour of fecal sample collection as a factor and sex in this model to determine if FGM concentrations were higher 4–48 h after capture compared to those in samples collected at initial capture (0 h postcapture).

Second, we used LMM to test if FGM concentrations at the time of capture (0 h after capture) from squirrels differed from those from 1) squirrels that were held in captivity for 4–48 h and 2) wild squirrels that were never held in captivity. In both models, we included a dichotomous (or indicator) predictor variable (captive or wild squirrels) and sex of the squirrel. In these models, there was no a priori reason to expect that males and females would react to the stress of captivity differently and initial analyses indicated no sex-specific interaction between sample type (collected at initial capture, collected 4–48 h into

captivity, or wild caught) and sex. Therefore, we excluded interaction terms between sex and hour in these analyses.

We determined if FGM concentrations measured in wild squirrels differed between females and males, among different reproductive conditions, the different seasons they were collected in, and a measure of body condition (body mass at capture) in 3 separate models. In 1 model for females only, we investigated if FGM concentrations differed among females that were 1) estrous or pregnant; 2) estrous, lactating, or had recently weaned offspring; or 3) those that were anestrous and not lactating (called nonbreeding hereafter). In a 2nd model for males only, we determined if FGM concentrations differed between males with abdominal or scrotal testes. In a model including both females and males, we identified whether FGM concentrations differed between the sexes, varied among 3 distinct seasons (winter [December to March], summer [May to August], or autumn [September to October]) or were associated with body mass at capture (ln transformed). There was no evidence for sex differences or effects of reproductive condition in how season of fecal sample collection or body mass was associated with FGM concentrations so we did not include interaction terms for sex or reproductive condition and season of collection and body mass.

It should be noted that our analyses of season are confounded with differences in habitat as samples collected in the winter months ($n = 14$) were only collected from low-elevation deciduous forests and not high-elevation coniferous forests (Table 1). This is because the high-elevation sites are inaccessible during the winter months and so all of our samples from the autumn and summer months ($n = 97$) come from the high-elevation sites. When we compared our model that contained the 3-level fixed effect for season versus a separate model with the same data that contained habitat type (low or high elevation) instead of season, the models did not differ from each other as assessed with the Akaike information criterion corrected for small sample sizes ($\Delta AIC_c = 0.44$—Burnham and Anderson 2002). Unequal sample sizes between the 2 habitat types (low elevation, $n = 14$; high elevation, $n = 97$) also preclude analyses for habitat type at this point. Our analyses for season should provide an indication of how FGM concentrations vary with season though this confounding factor needs to be mentioned.

FGM concentrations might be affected by factors other than the characteristics of the individual squirrels. We conducted a separate model where we included fecal sample mass as a sole predictor variable to identify if FGM concentrations were affected by mass of the fecal sample we extracted.

All analyses were performed in R version 3.2.1 (R Core Team 2015) using the lme4 package (version 1.1-8—Bates et al. 2015). For each of the models described above, we conducted LMM with FGM concentrations (expressed as ln-transformed ng/g dry feces) as the dependent variable and squirrel identity as a random intercept term to account for repeated samples on the same individuals. We estimated the unadjusted repeatability (R) of FGM concentrations for captive and free-living squirrels using the estimates of within- and among-female variance from our LMMs that did not include any fixed effects (Nakagawa and Schielzeth 2010). We considered these repeatability estimates to be statistically significant if their 95% confidence intervals (*CI*s) did not include 0 (estimated using parametric bootstrapping with 1,000 permutations—Nakagawa and Schielzeth 2010). Finding significant repeatability in this context suggests that some squirrels consistently differ in their FGM concentrations in response to captivity (captive squirrels) or changes in reproductive condition and season (wild squirrels). In the models for the FGM concentrations from free-living squirrels, we also included a random intercept term for year of collection to account for repeated samples in the same years (2013, $n = 6$; 2014, $n = 37$; 2015, $n = 68$). We assessed significance of the pair-wise comparisons either using t-tests (using Satterthwaite approximations to degrees of freedom in R package lmerTest, version 2.0-29) or Tukey's post hoc comparisons test (R package multcomp version 1.4-1—Hothorn et al. 2008).

Results

Effect of temporary captivity on FGM concentrations.—FGM concentrations were influenced by time since initial capture ($F_{8,67} = 2$, $P = 0.06$). Compared to fecal samples collected at initial capture (0 h after capture, $n = 17$ samples, 9.83 ± 0.29 ln ng/g dry feces; Fig. 1), squirrels that were captured and temporarily held in captivity (6 females, 11 males) had FGM concentrations that were 52.8% higher 24 h after initial capture ($n = 16$ samples, 10.52 ± 0.18 ln ng/g dry feces, Tukey honest significant difference [HSD], $P = 0.048$) and 28.7% higher 32 h after initial capture ($n = 10$ samples, 10.45 ± 0.18 ln ng/g dry feces, Tukey HSD, $P = 0.042$). Of the 12 individuals for which we had repeated samples at 0 and 24 h postcapture, 9 had higher FGM concentrations 24 h postcapture (Fig. 2A). All 9 individuals for which we had repeated samples at 0 and 32 h postcapture had higher FGM concentrations 32 h postcapture (Fig. 2B). FGM concentrations were also 34.3% higher 48 h after initial capture ($n = 12$, 10.51 ± 0.14 ln ng/g dry feces), though this was not significant (Tukey HSD, $P = 0.088$). The mean and median lag time from initial capture to peak excretion tended to be from 24 to 36 h (Fig. 1). For those individuals for which we collected a fecal sample at initial capture and thereafter, the time to peak excretion of FGM concentrations was 29.8 ± 11.7 h (mean ± *SD*). There were no significant differences among the other different collection periods (Fig. 1) and there were no sex differences in FGM concentrations ($t_{30} = 1.1$, $P = 0.31$). We obtained an average of 5 estimates of FGM concentrations from each of 17 squirrels over the 48-h period of captivity. FGM concentrations were repeatable within individual squirrels while they were in captivity ($R = 0.52$, 95% $CI = 0.25$–0.69). This can be visualized in Fig. 2 showing that individual squirrels with higher FGM concentrations than other squirrels at initial capture (0 h) also generally had higher FGM concentrations than these same squirrels 24 and 32 h after capture.

Comparing FGM concentrations of captive and wild squirrels.—FGM concentrations from captive squirrels at 0 h after capture ($n = 17$ samples, 9.83 ± 0.29 ln ng/g dry feces) did not differ when compared to FGM concentrations of free-ranging

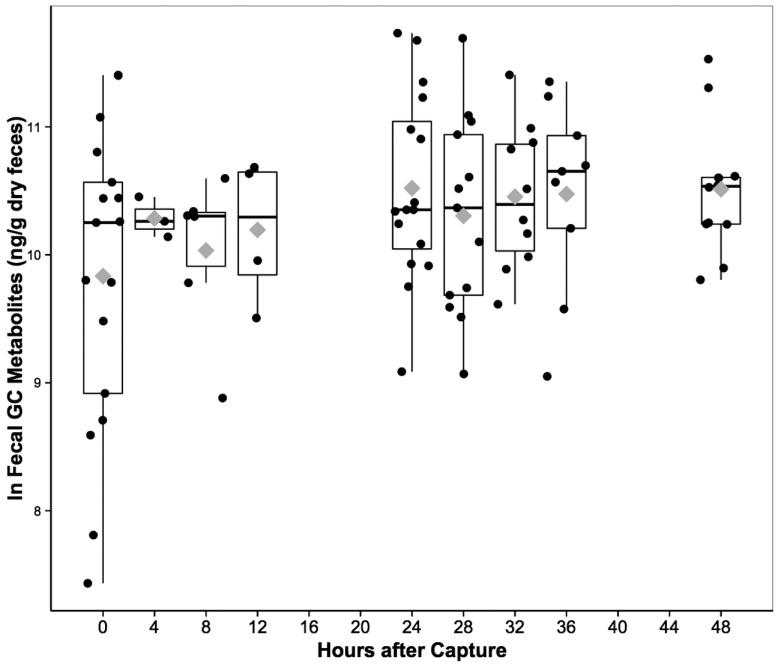

Fig. 1.—Fecal glucocorticoid (GC) metabolite concentrations in Eurasian red squirrels ($n = 11$ males, 6 females) from initial capture (0 h after capture) to 48 h after initial capture. Fecal GC metabolite levels were significantly higher in samples collected 24 h ($P = 0.047$) and 32 h ($P = 0.042$) after initial capture. Boxplots show median (solid horizontal line), mean (gray diamond), and 1st (25%) and 3rd (75%) quartiles. Fecal samples were not collected overnight during captivity (2200–0900 h) so no data are shown for 16, 20, 40, and 44 h after capture.

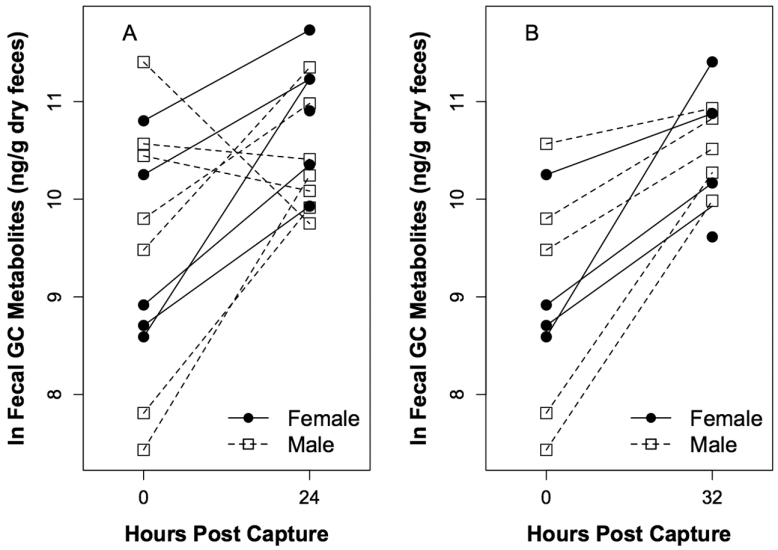

Fig. 2.—Fecal glucocorticoid (GC) metabolite concentrations in Eurasian red squirrels from initial capture (0 h after capture) to A) 24 and B) 32 h after initial capture. Lines connect points of individual squirrels that were sampled at each time period.

squirrels ($n = 111$, 9.81 ± 0.07 ln ng/g dry feces, $t_{126} = 0.01$, $P = 0.92$; Fig. 3) but increased significantly when squirrels remained in confinement for 4–48 h ($n = 73$, 10.4 ± 0.07 ln ng/g dry feces, $t_{86} = 27.2$, $P < 0.0001$; Fig. 3) compared to those from free-ranging squirrels.

Effect of sex, reproductive condition, body mass, and season on FGM concentrations in wild squirrels.—FGM concentrations were influenced by reproductive state in females ($F_{2,45} = 3.4$, $P = 0.044$). Pregnant females ($n = 10$ samples) had significantly lower FGM concentrations than females that were lactating or had recently weaned their offspring ($n = 14$, Tukey HSD, $P = 0.027$; Fig. 4). FGM concentrations in nonbreeding females ($n = 27$) did not differ from those that were pregnant ($n = 10$, Tukey HSD, $P = 0.45$) or lactating/recently weaned (Tukey HSD, $P = 0.22$). Males with abdominal ($n = 33$) or scrotal ($n = 27$) testes had similar FGM concentrations ($t_{57} = -0.96$, $P = 0.34$; Fig. 4).

Overall, FGM concentrations varied significantly among the 3 different seasons ($F_{2,76} = 4.95$, $P = 0.009$; Fig. 5). FGM concentrations in samples collected from squirrels during the summer (May to August) were significantly lower than those collected in the winter (Tukey HSD, $P = 0.015$) and tended

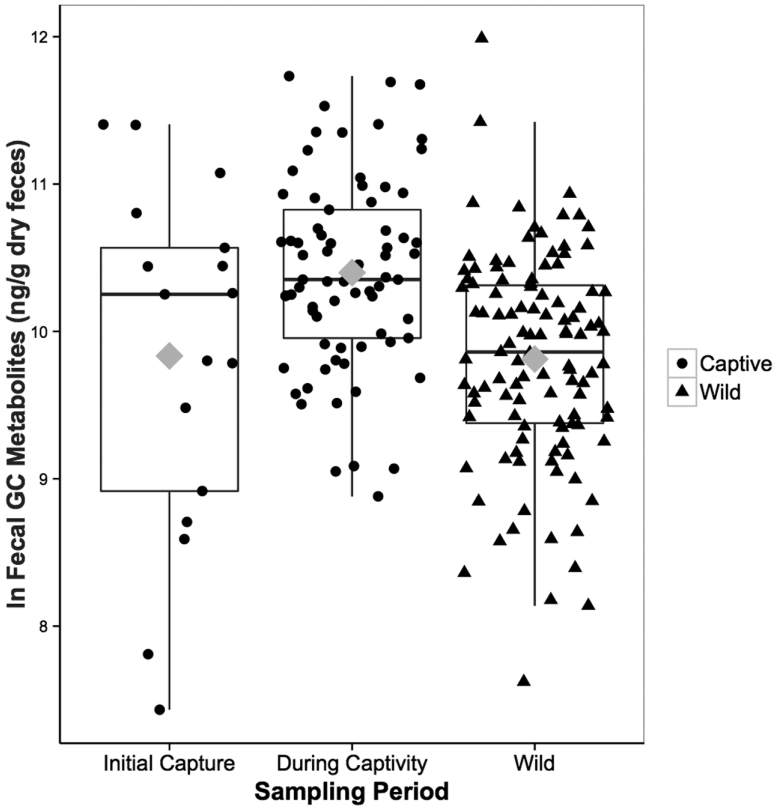

Fig. 3.—Comparison of fecal glucocorticoid (GC) metabolite concentrations in Eurasian red squirrels at initial capture (time 0 of capture, $n = 17$ samples), captive red squirrels collected 4–48 h after initial capture ($n = 73$ samples), and those collected from wild squirrels that were not maintained in captivity after initial capture ($n = 111$). Boxplots show median (solid horizontal line), mean (gray diamond), and 1st (25%) and 3rd (75%) quartiles. Some data points shown represent samples collected from the same individuals.

to be lower than those collected in the autumn (Tukey HSD, $P = 0.062$). FGM concentrations in winter tended to be higher than those in autumn but this was not statistically significant (Tukey HSD, $P = 0.41$; Fig. 5). FGM concentrations did not differ between females ($n = 51$ samples, 9.85 ± 0.08 ln ng/g feces) and males ($n = 60$ samples, 9.78 ± 0.11 ln ng/g feces, $t_{96} = 0.06$, $P = 0.81$) and there was no association between body mass at capture and FGM concentrations ($t_{96} = 0.19$, $P = 0.66$), or interaction between body mass and sex ($t_{96} = 0.06$, $P = 0.81$). FGM concentrations were not repeatable within individual free-living squirrels ($R = 0.12$, 95% $CI = 0–0.45$).

Effect of fecal sample mass on FGM concentrations.—The mass of most samples we extracted was around the target value of 0.05 g ($n = 201$, mean ± SD: 0.047 ± 0.011 g, range = 0.009–0.056 g). Some samples were substantially lighter than our target extraction mass ($n = 34$ samples, 0.009–0.04 g) because that was the maximum amount of sample that we were able to collect. Despite this variation in fecal sample mass, there was no association between mass extracted and FGM concentrations in samples collected from wild and captive squirrels ($t_{199} = -0.8$, $P = 0.42$).

Discussion

We show that livetrapping and temporary capture elevated FGM concentrations in Eurasian red squirrels and that these changes in adrenocortical activity were detected through the use of our EIA using an antibody that detects glucocorticoid metabolites with 5α-3β, 11β-diol structure. FGM concentrations were significantly higher in samples collected 24 and 32 h after initial capture compared with FGM concentrations in samples collected 0 h after initial capture. Squirrels that were in captivity for 4–48 h had significantly higher FGM concentrations than those that were free-ranging and never placed into captivity. Our assay also detected significant repeatable differences in FGM concentrations among captive squirrels. In free-ranging squirrels, pregnant squirrels had lower FGM concentrations than those that were lactating or nonbreeding, whereas there were no differences in FGM concentrations between males with scrotal or abdominal testes. Using 3 years of fecal samples collected during the summer, autumn, and winter months, we show that FGM concentrations are lowest in summer and highest in the winter.

Assays to measure fecal hormone metabolites should be carefully validated (Buchanan and Goldsmith 2004; Touma and Palme 2005; Sheriff et al. 2011; Fanson et al., in press). Two caveats of this study are that we did not use an ACTH challenge and we did not test the sensitivity of our antibody to detect variation in FGM concentrations compared to other available antibodies. An ACTH challenge is often used as a pharmacological validation that a specific EIA to measure FGM concentrations can detect increases in endogenous

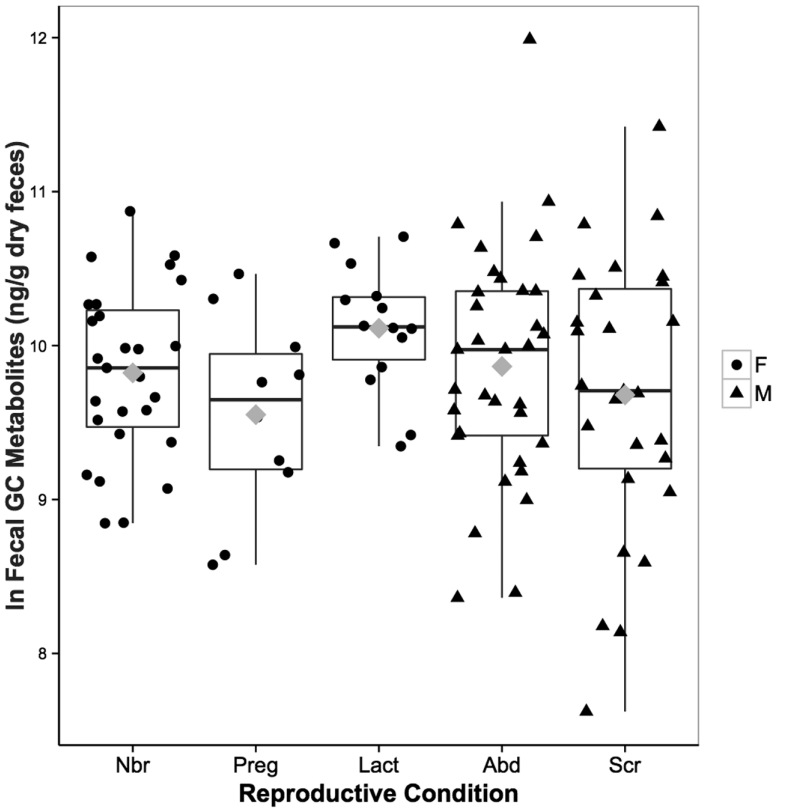

Fig. 4.—Effects of reproductive condition on fecal glucocorticoid (GC) metabolite concentrations in wild-caught Eurasian red squirrels. Fecal GC metabolite concentrations were measured in samples from pregnant females or those in estrous (n = 10, "Preg"), females who were lactating or had recently weaned their offspring (n = 14, "Lact"), nonbreeding females (n = 27, "Nbr"), and males with abdominal (n = 28, "Abd") or scrotal (n = 32, "Scr") testes. Symbols show females ("F") and males ("M"). Boxplots show median (solid horizontal line), mean (gray diamonds), and 1st (25%) and 3rd (75%) quartiles. Some data points shown represent samples collected from the same individuals.

glucocorticoid production from the adrenal glands (Touma and Palme 2005; Sheriff et al. 2011). However, biological validations, such as the use of temporary captivity in our study, are equally suited to validate an assay to measure FGM concentrations because they cause an acute stress response (Touma and Palme 2005). Capture, translocation, and temporary captivity induces profound changes in the hypothalamic–pituitary–adrenal axis (Romero and Wingfield 1999; Dickens et al. 2009, 2010). Within 2–3 min of initial capture, animals mount a physiological stress response where endogenous glucocorticoid production is increased (Kenagy and Place 2000; Romero and Reed 2005; Delehanty and Boonstra 2009) and this increase in plasma glucocorticoid levels can be sustained for the first few hours (Fletcher and Boonstra 2006; Dantzer et al. 2010) or days (Dickens et al. 2009) of captivity (an acute stressor). Previous studies have used a similar biological validation as ours showing that FGM concentrations were elevated after 4 h of captivity (Harper and Austad 2001; Bosson et al. 2013; Hämäläinen et al. 2014) compared to precapture samples and remained elevated for the next 96 h (Hämäläinen et al. 2014). In our case, we show that capture and confinement (an acute stressor) significantly increased FGM concentrations, which indicates that our EIA can detect biologically relevant information about endogenous glucocorticoid production. Ideally both physiological (ACTH) and biological (e.g., temporary captivity) validations should be conducted in the same study but this was not possible here because the species is of conservation concern and not readily available in zoos.

We used a group-specific antibody that detects glucocorticoid metabolites with a 5α-3β, 11β-diol structure. We show that this antibody was effective in detecting significant increases in FGM concentrations in response to temporary capture. Thus, this antibody has now proven to be useful in detecting variation in FGM concentrations in a variety of rodent species (Touma et al. 2003; Lepschy et al. 2007; Bosson et al. 2009; Dantzer et al. 2010; Lepschy et al. 2010; Montiglio et al. 2012; Bosson et al. 2013; Hammond et al. 2015). Furthermore, we found that fecal sample mass was not associated with FGM levels, and that low mass fecal samples (0.009–0.04 g) could be used to assess FGM levels. This is important as some authors report inaccurate results from samples of low mass (< 0.02 g dry weight), and in species where fecal mass may be limited it is important to validate the use of low mass samples (see Millspaugh and Washburn 2004, and references therein).

Recently, some studies have tested the efficacy of multiple antibodies to detect variation in the concentrations of some specific FGM (e.g., Heistermann et al. 2006; Montiglio et al. 2012; Shutt et al. 2012; Hämäläinen et al. 2014; Fanson et al., in press). While valuable, in most cases, these studies conclude that each of the multiple antibodies that they have tested is

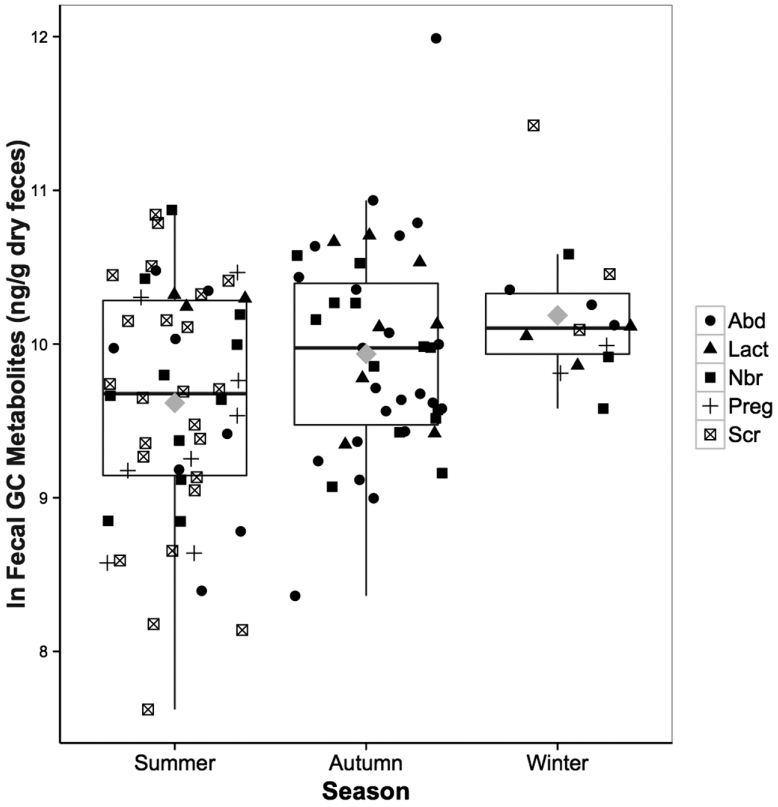

Fig. 5.—Fecal glucocorticoid (GC) metabolite concentrations in female and male Eurasian red squirrels captured in the summer (n = 54, May–August), autumn (n = 43, September–October), and winter (n = 14, December–March). Boxplots show median (solid horizontal line), mean (gray diamonds), and 1st (25%) and 3rd (75%) quartiles. Symbols show reproductive condition for females that were pregnant ("Preg"), lactating ("Lact"), or nonbreeding ("Nbr") and for males that had abdominal ("Abd") or scrotal ("Scr") testes. Some data points shown represent samples collected from the same individuals.

useful in detecting changes in the endogenous glucocorticoid production though some antibodies are more sensitive (show stronger increases in FGM following adrenocortical activation) than others within a species (e.g., Montiglio et al. 2012). Interestingly, studies in diverse taxa suggest that the acute stress response to capture and restraint (Manogue et al. 1975; Narayan et al. 2013; Pakkala et al. 2013) or ACTH injections (Sheriff et al. 2012) may not be as severe as exposure to natural stressor such as a predator (but see Harris et al. 2012). This suggests that the methods that are often used to physiologically (ACTH injection) or biologically (capture, restraint, translocation) validate assays to measure FGM concentrations (Touma and Palme 2005; Sheriff et al. 2011) may produce subtler increases in FGM concentrations than variation in the factors (food and predator abundance, competition, climate, infection status) most workers are interested in. Because our assay is detecting changes in glucocorticoids in response to capture, this suggests that our assay should detect changes in FGM concentrations of Eurasian red squirrels in response to variation in these ecological factors.

We were not able to directly measure gut passage time in this study but we did find that peak FGM concentrations occurred 24–36 h after initial capture. In most individuals, FGM concentrations were significantly higher 24 h after initial capture. This differs from some previous studies in different squirrel species showing that the time until peak excretion of FGM was 8–12 h following an ACTH challenge (Dantzer et al. 2010; Montiglio et al. 2012; Sheriff et al. 2012) or handling stressor (Dantzer et al. 2010). However, several recent studies in other squirrel species also found that FGM concentrations peaked 24 h (Hammond et al. 2015) or 16–40 h (Bosson et al. 2013) after ACTH injection. It is also notable that in our study, FGM concentrations were higher 4, 8, and 12 h after initial capture but these were not statistically significant. This suggests that an acute stress response that elevates endogenous glucocorticoid production in Eurasian red squirrels should be evident in the feces at least 24 h later if not earlier. This also indicates that FGM concentrations measured in fecal samples collected within 4–48 h after capture will be biased by the previous capture and handling event. Because squirrels were continuously held in captivity for 48 h (likely a more chronic form of stress), whereas wild-caught squirrels are only captured and then released, it is likely that FGM concentrations in wild-caught squirrels that are released after capture return to normal more quickly than 48 h after initial capture.

We found no sex differences in FGM concentrations in captive or wild squirrels and no sex difference in the effects of temporary captivity on FGM concentrations. Differences in plasma glucocorticoid levels can be caused by a variety of factors including the effects of gonadal steroid hormones and sex

hormone binding globulins (Handa et al. 1994; Cavigelli et al. 2005; Lepschy et al. 2007; Weiser and Handa 2009). Sex differences in FGM concentrations could be caused by a variety of factors such as sex differences in the metabolism and excretion of hormone metabolites (Touma et al. 2003; Millspaugh and Washburn 2004; Cavigelli et al. 2005; Goymann 2005; Palme et al. 2005; Goymann 2012). While sex differences in FGM concentrations have been shown in some species, they have not been found in others and the results are far from consistent (Touma and Palme 2005; Goymann 2012; Harris et al. 2012). We note that the lack of sex differences in FGM concentrations in our study and in others could likely be due to not having enough samples around the period of time when sex differences in plasma glucocorticoid levels are most obvious such as when females are pregnant or lactating compared to nonbreeding males or females (Kenagy and Place 2000; Boonstra et al. 2001; Reeder et al. 2004; Edwards and Boonstra, 2016). Similarly, the lack of resolution on pregnancy stages in wild animals may obscure any general differences between females and males. For example, measurement of FGM concentrations in females in the early and middle stages of pregnancy may be quite similar to those from nonbreeding males, whereas there may be vast differences between the sexes if the samples were collected from females in the late stages of gestation (Dantzer et al. 2010).

FGM concentrations were repeatable among captive but not free-ranging squirrels. Repeatability in the context of our method of measurement suggests that some squirrels in captivity consistently had higher FGM concentrations across the different sampling periods than others. Wild squirrels did not exhibit significant repeatability of FGM concentrations across changes in reproductive conditions or seasons though the latter may be due to low statistical power (Wolak et al. 2011; but see Bell et al. 2009) given that we obtained < 2 fecal samples for 82 different free-living squirrels (111 total samples). Our results from captive squirrels adds to the growing number of studies showing significant repeatability of either plasma or fecal glucocorticoid levels (Cockrem et al. 2009; Dantzer et al. 2010; Ouyang et al. 2011; Fletcher et al. 2015).

FGM concentrations were highest in females that were lactating or recently finished lactating, whereas they were lowest in pregnant females. Lactation is one of the most energetically demanding activities exhibited by mammals (Naya et al. 2008; Fletcher et al. 2012) and increased production of glucocorticoids may help to mobilize energy stores to facilitate lactation (Kenagy and Place 2000; Romero 2002; Romero and Wingfield 2015). Thus, our results suggest that biologically relevant increases in energetic-expenditure (lactation) can be detected in Eurasian red squirrels by measuring FGM concentrations. Why FGM concentrations were lowest in pregnant females is not clear but other studies in different squirrel species have also found that females have higher FGM concentrations when they are lactating compared to when they are pregnant (Aschauer et al. 2006). This is also possibly related to the low sample sizes (n = 10) we had compared to those from nonbreeding or lactating/postlactating females. We also found no difference in FGM concentrations between males with scrotal or abdominal testes. Although we assume that males with scrotal testes are in breeding condition, it is possible that we would recover some difference in FGM concentrations between breeding and nonbreeding males if we had measured these in samples collected during the peak breeding season (when males were actively mating) and nonbreeding season. Of course, in all these cases, it is important to mention that changes in concentrations of fecal hormone metabolites with reproductive condition could reflect a real biological phenomenon (i.e., matching a change in circulating hormone levels). Alternatively, these could reflect a change in metabolism or gut passage time that occurs with differences in reproductive condition (Goymann 2012).

We observed substantial seasonal changes in FGM concentrations where they increased from the spring/summer (May to August) until the winter months (December to March). Autumn trapping coincided with the autumn dispersal peak of both subadult and adult red squirrels, while during winter months animals were engaged in mating chases and/or, for some females, had started lactating (Wauters and Lens 1995; Wauters et al. 2008, 2010). Thus, winter FGM concentrations may have been elevated because the squirrels were breeding (Romero 2002). However, there is not a general pattern in mammals as glucocorticoid levels are elevated during the breeding season compared to the pre- or postbreeding seasons in some species (Vera et al. 2013; Corlatti et al. 2014; Jachowski et al. 2015) but not in others (Place and Kenagy 2000; Romero et al. 2008; Delehanty and Boonstra 2011; Bauer et al. 2014; Fletcher et al. 2015). Although speculative, increased FGM levels in autumn and winter could thus be related to more frequent intraspecific interactions linked to dispersal, higher predation risk when foraging on the ground and reduced food quality and/or more extreme weather conditions (cold, snow cover) in winter. In autumn and winter, Eurasian red squirrels tend to forage more on the ground to cache tree seeds (September to October—Wauters and Casale 1996) which could conceivably increase their risk of predation. Also, late winter may be a period of food shortage, at least in some years, with squirrels feeding more often on poor-quality (low energy and higher fiber content) resources, such as buds and fungi growing under the bark of dying or injured branches (Lurz et al. 2005). In contrast, during summer, Eurasian red squirrels forage mainly in the tree canopy on flowers and maturing seeds. The seasonal changes we observed in FGM concentrations may also be caused by changes in diet (Dantzer et al. 2011). An alternative explanation is presented by a confounding factor in our study. Specifically, 2 of our sites were in low-elevation deciduous forest, whereas 3 of our sites were in high-elevation coniferous forests (Table 1). Because the high-elevation sites were inaccessible during the winter months, we were unable to collect fecal samples from squirrels in these areas during the winter. As such, the seasonal changes we observed could be due to differences between squirrels inhabiting these different types of habitats, though at present we cannot address this without further study where samples are collected from the 2 different types of habitats during the autumn, winter, and summer months.

In conclusion, we validate an EIA to measure FGM concentrations in Eurasian red squirrels by showing that capture and temporary captivity significantly elevate FGM concentrations and that this assay can detect changes in FGM concentrations in response to changes in reproductive condition or seasonal/habitat factors. As in other species, this assay will be useful to detect how ecological factors induce physiological stress in wildlife and associations between measures of physiological stress and survival and reproduction (Bonier et al. 2009). We expect this assay will prove to be particularly useful in understanding whether the presence of an invasive species (gray squirrels, *S. carolinensis*) are causing stress in Eurasian red squirrels that are contributing to their decline and local extinction throughout parts of Europe (Gurnell and Pepper 1993; Martinoli et al. 2010; Bertolino et al. 2014).

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