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Native species exhibit physiological habituation to invaders: a reason for hope

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Animals cope with environmental perturbations through the stress response, a set of behavioural and physiological responses aimed to maintain and/or return to homeostasis and enhance fitness. Vertebrate neuroendocrine axis activation in response to environmental stressors can result in the secretion of glucocorticoids (GCs), whose acute increases may be adaptive, while chronic elevation may be detrimental. Invasive grey squirrels (*Sciurus carolinensis*) act as a stressor eliciting elevation of GCs in native red squirrels (*Sciurus vulgaris*). Here we used 6-year data of variation in faecal glucocorticoid metabolite (FGM) concentrations following invasion by grey squirrels in three red squirrel populations, to identify if red squirrels showed physiological habituation to this stressor. The decrease in FGMs over time was more pronounced shortly after invasion and at high densities of grey squirrels while it decreased less strongly and was no longer influenced by the invader density as time since invasion elapsed. At the individual level, FGMs also decreased more markedly as each red squirrel experienced prolonged contact with the invader. Our study provides compelling new data suggesting that native species in the wild can habituate to prolonged contact with invasive species, showing that they may avoid the potentially harmful effects of chronic elevations in GCs.

1. Introduction

Free-living animals cope with (un)predictable challenges, such as threatening environmental perturbations, through the stress response, a complex of behavioural and physiological adaptive processes aimed to maintain and/or return to homeostasis and enhance fitness [1–3]. One mechanism that facilitates coping with environmental perturbations is activation of the hypothalamic–pituitary–adrenal (HPA) axis, which mediates the neuroendocrine stress response in vertebrates [1,4]. The HPA axis secretes glucocorticoids (GC) which, at low concentrations, regulate metabolic demand and energy acquisition (baseline) [5–7]. However, at sustained relatively high GC concentrations (stress-induced) [4,5,7], the stress response may become energetically costly [8], and can cause inhibition of growth and/or reproduction [9–11], elevation of blood glucose levels [12] and suppression of the immune system [13,14]. Although in wild animals the proximate costs of chronic elevations in GCs are not very well documented, studies in wild primates show that individuals with increased lifetime exposure to GCs have shorter lifespans [15].

These prolonged elevations in GCs and/or frequent exposure to stressors, termed ‘chronic stress’, often produce a GC response whose intensity attenuates over time, and in some cases, an observed decrease in hormonal response intensity has been attributed to physiological habituation [16]. Longitudinal studies, which monitor individuals over their lifespan and across multiple generations, are the most common approach to study long-term variation in GC concentrations [17,18], as well as how free-living animals adapt their hormonal response to changing environmental conditions (i.e. endocrine plasticity) [5,19,20]. Dynamic environments have been documented to elicit an elevation of GCs following changes in food abundance, habitat quality, intraspecific and/or interspecific competition and predation [21–23], and physiological, behavioural and morphological phenotypic coping mechanisms have been observed to occur in wildlife allowing individuals to optimize their fitness [13,24,25]. However, less is known about physiological responses to human-induced rapid environmental changes (HIREC), such as habitat and climate change, exotic species, pollution and human harvesting (discussed in [26] and references therein).

There has been substantial work on how wild animals adjust their behaviour to HIREC [27,28], but do wild animals also adjust their physiological stress response to cope with HIREC? A growing number of studies have explored the diverse effects of human-induced environmental changes on GCs in amphibians, reptiles, birds and mammals [29–35]. However, these studies almost always focus on how average GCs are affected by anthropogenic disturbances at one point in time, and only few studies have explored the role of invasive species in eliciting a stress response in native species [36–41]. No study has investigated whether native species can exhibit physiological habituation to any stress imposed by the presence of the invasive species. Addressing whether animals habituate physiologically, as they do behaviourally, is important given that physiologically habituation might be paramount to enhance coexistence of the native with the invasive species.

Here we used the well-known model system of native-invasive species competition between the Eurasian red squirrel (*Sciurus vulgaris*) and introduced eastern grey squirrel (*Sciurus carolinensis*). The latter is an invasive alien species (IAS) introduced in Europe from North America [42,43], whose rapid spread and detrimental effects on the native red squirrel has been documented in many studies [44–46]. We previously showed that faecal GC metabolite concentrations (FGMs), a non-invasive measure of circulating GCs over a specific period of time [47–50], were higher in areas where the native red squirrels coexist with the invasive grey squirrel than in areas without the latter. FGMs in red squirrels increased after colonization by the invasive species and experimentally reducing the abundance of the invasive grey squirrel resulted in a significant decrease of FGMs in the native red squirrels [41]. This study conclusively demonstrated that the invasive grey squirrel acts as a stressor on native red squirrels. Here, we extend this previous study to six-year period by quantifying FGMs in three populations of Eurasian red squirrels occurring in areas colonized by the invasive grey squirrel. We specifically tested the hypothesis that native red squirrels exhibit physiological habituation to prolonged exposure to invasive grey squirrels, as has been documented in captive animals [16]. We made the following two predictions that would support this hypothesis.

(1) In a given population of red squirrels that was colonized by grey squirrels, a reduction in red squirrels’ FGMs after a given period of co-occurrence with the invasive species would suggest that red squirrels habituated to the stressor. We further predicted that this reduction in FGMs would depend upon grey squirrel density (following [41]). To test this, we used a population-level measure for the presence of the invasive species acting as a stressor: the number of days since the invasion of a given study site (red squirrel population) by the grey squirrels. Hence, this time variable, hereinafter called ‘days since invasion’, is related to the day each red squirrel faecal sample was collected, therefore identical for all samples (FGM measures) of a given study site on a given date, regardless of how long an individual red squirrel was already present in the population. If habituation to the stressor (invasive grey squirrel) occurs, we expected that FGMs will increase during and shortly after colonization by the grey squirrel, but will gradually decrease with the number of days since invasion. If grey squirrel density significantly influences FGMs, we expected a more pronounced reduction in FGMs of red squirrels at higher than at lower densities of the invasive species, in particular in the early phase of reduction.

(2) Next we tested whether the reduction of FGMs in relation to the persistence of the stressor (invasive grey squirrel) is dependent on how long each individual red squirrel has been in contact with grey squirrels (individual-level experience with the invasive species), and if distinct individual red squirrels respond differently to this stressor (i.e. is there within-individual variation in FGMs plasticity) [5,19]. Hence, we ‘weighed’ the time of co-occurrence for each individual red squirrel, based on when it first interacted with grey squirrels, which was derived from the individual’s first day of capture in the study site. We used the term ‘grey squirrel experience’ for this number of days of contact for each individual red squirrel with the invasive species (stressor). We expected that FGMs in red squirrels will decrease as the individual experience with the stressor increases and that this relationship will differ with grey squirrel density since red squirrels’ FGMs vary in relation to invasive species density [41]. Moreover, we also tested if this relationship would differ for individual red squirrels, following a reaction norm approach.

2. Material and methods

(a) Study sites, trapping and handling squirrels

We trapped Eurasian red squirrels in three study sites in northern Italy (Castelbarco, 61 ha, 45°35′N, 9°31′E; Vanzago, 76 ha, 45°31′N, 8°58′E; Passatempo, 18 ha, 45°00′N, 7°78′E), from December 2013 to October 2019. Two out of three sites were colonized by the invasive species during the study (Castelbarco and Vanzago), while in Passatempo the invasive species was already present. Grey squirrels trapped during the study were euthanized by CO_2 inhalation, following the EC and AVMA guidelines [51–53]. Capture-mark-recapture (CMR) sessions of red squirrels were carried out one to three times per year (except in 2013 for Vanzago, but see electronic supplementary material, figure S1 for details). We used single capture live traps (model 202, Tomahawk Live Trap Co., Hazelhurst, WI, USA), with a fine mesh added underneath to limit contamination between urine and faeces, placed on the ground or at breast height against tree trunks. The number of traps varied for each study site (Castelbarco: mean = 34; range = 18–41 traps; Vanzago: mean = 27, range = 16–30 traps; Passatempo: mean = 21; range = 18–23 traps). Traps were pre-baited with hazelnuts three to four times over a 30 day period, then baited and set for three to 5 days. Traps were activated and checked three times per day to reduce the time squirrels were confined in a trap and to minimize time since defecation (max 3 h). Each captured red squirrel, at first capture, was individually marked using a numbered metal ear-tag (Monel 1005 1L1, National Band & Tag Co. Newport, KY, USA), weighed to the nearest 5 g using a spring-balance (Pesola AG, Baar, Switzerland) and the length of the right hind foot (without nail, 0.5 mm) was measured to the nearest 0.5 mm with a thin ruler [54]. Hind foot length is a reliable proxy for squirrel body size [55]. Sex and reproductive condition were determined from external genitalia (as detailed in [48]). After each capture, the trap, mesh and the ground under the trap were cleaned to remove possible remains of faecal material.

(b) Extraction and quantification of FGMs

Over the 6 years, we analysed a total of 302 faecal samples of 129 different squirrels (75 males and 54 females; 73 individuals had at least two FGM measurements), captured in Castelbarco ($n = 133$; ID = 66), Vanzago ($n = 108$; ID = 36) and Passatempo ($n = 61$; ID = 27). In the present study, we assayed 114 samples, while the other 188 samples were assayed in previous studies conducted in the same study sites, analysed using the same methodology and in the same laboratory (identical experimental design; see [41,48]). Methods of extraction of FGMs and enzyme immunoassay (EIA) validation for Eurasian red squirrels can be found in detail elsewhere [41,48]. Briefly, fresh faecal samples (less than 3 h) of trapped squirrels were collected from underneath the traps, stored dry at −20°C and classified as being taken in the morning (10.00–13.00 h) or in the afternoon (15.00–18.00 h) to account for potential variation in FGMs over the 24-h cycle [47,56,57]. We used a 5α-pregnane-3β, 11β, 21-triol-20-one EIA to measure FGM concentrations (ng g^{-1} dry faeces; [58]). This EIA detects GC metabolites with a 5α–3β, 11β-diol structure (for cross-reactivity see [58]). Assay validation in this species showed that faecal samples collected from traps represent an integrated measure of glucocorticoids, with peak concentrations occurring 24 to 36 h after initial captivity [48]. Hence, we sampled only squirrels that had not been trapped or handled within 72 h prior to capture to exclude effects of capture stress on FGM concentrations. Samples were analysed in duplicate. Intra-assay CVs were 8.4% ± 7.0% (mean ± s.d.). Pools of red squirrel faeces extracts were used as intra-assay controls at dilutions of 1 : 200 (approx. 30% binding) and 1 : 1600 (approx. 60% binding). Average inter-assay coefficients of variation (CVs) were 14.6% and 14.9%, respectively, for pools diluted 1 : 200 and 1 : 1600. Average number of samples per individual (±SE) was 2.25 ± 0.09 (range: 1 to 9). Overall, mean FGM (± SE) was 51 185 ± 3182 ng g^{-1} dry weight of faeces, ranging from 1119 to 290 278 ng g^{-1} dry weight.

(c) Estimating squirrel densities, days since invasion and grey squirrel experience

The two squirrel's species were monitored with different techniques: CMR for red squirrels; capture and removal for greys. Since we needed a population density estimate that was comparable for both species, we used the catch per unit effort (CPUE), calculated in each study site and trapping session, as our abundance index. CPUE was calculated by dividing the number of individual squirrels captured in a given trapping session by the effort, in terms of number of available traps*number of capture days. It was then scaled to a density index (electronic supplementary material, figure S1) by dividing CPUE by the size (ha) of the study site and multiplying this number by 1000:

$$\text{Density index} = \frac{\text{no. ind. captured}}{\text{no. traps} * \text{no. capture days}} \times \frac{1}{\text{size}} \times 1000.$$

Hence, if squirrel density is high, more animals will be captured for a given capture effort than at low densities, when, with a similar effort, fewer squirrels will be trapped. The red squirrel density index varied from 0.61 to 9.92 (mean ± SE = 2.91 ± 0.14), that of grey squirrels ranged from 0 to 9.88 (mean ± SE = 1.98 ± 0.14). Both indices fluctuated strongly depending on study site and grey squirrel control activities (electronic supplementary material, figure S1).

In order to test our first prediction, we calculated a population-level measure based on the number of days since invasion by the first grey squirrels in a given study site (days since invasion: mean ± SE = 989 ± 43; range −89 to 2969 days). Days since invasion can be either a positive value (sample taken after invasion by grey squirrels) or a negative one (sample collected before the invasion by grey squirrels). The approximate date of invasion was estimated for each study site based on the date of the first capture of a grey squirrel.

Next, we calculated an individual-level measure for all red squirrels, based on the number of days of experience with grey squirrels present in a given study site (grey squirrel experience: mean ± SE = 321 ± 21; range 0 to 1875 days). This variable: (a) has a value of zero for red squirrels faecal samples collected when the invasive species was not yet present in a given study site; (b) has a value of 1 when a faecal sample was collected at the first capture of an individual red squirrel in a given study site where the invasive species was already present; and (c) has a number x in the case when a faecal sample was collected x days after an individual red squirrel's first capture in a given study site where the grey squirrel was already present. Hence, the grey squirrel experience measure weighed differently for each individual red squirrel based on its persistence in a given population. We should note that in the majority of cases with a value of grey squirrel experience equal to 1, this will not be exactly the first day of a red squirrel appearance in a given population, since an animal might be already present for a few days, weeks or even months, depending on the frequency of capture sessions and the propensity of a naive red squirrel to get caught. However, we are confident that this potential bias does not influence our predictions since: (1) the predicted reduction in FGMs is likely to be a relatively slow process (FGMs decreased after approx. 6 to 10 weeks in a grey squirrel removal experiment [41]); and (2) it is the relative magnitude of the grey squirrel experience measure that is of importance in affecting the trend in variation in FGMs.

(d) Statistical analyses

The difference between the two approaches described here below was that to test the first prediction we used days since invasion as the explanatory variable describing the time of co-occurrence with grey squirrels, while to test the second prediction we used grey squirrel experience as explanatory variable, describing the individual experience with the invasive species. For the first prediction, we performed a linear mixed-effects model (LMM) with FGMs (transformed using the natural logarithm, ln of ng g^{-1} dry faeces) as the dependent variable and squirrel identity (ID) as random intercept term to account for repeated measures of FGMs for a given individual. Sex, reproductive condition nested in sex (males: abdominal or scrotal testes; females: non-breeding, post-oestrus and pregnant, lactating; details in [41]), season (spring–summer, autumn or winter), daytime (sample collected in the morning or in the afternoon) and study site were added as fixed effects to account for potential changes in FGM concentrations [41,48,49]. Body condition at each capture (the score of the second component from a PCA of body mass and foot length [50]); as well as population density indices for both red and grey squirrels, measured for each study site and trapping session, and days since invasion were included as continuous explanatory variables. Furthermore, since relationships of days since invasion with FGMs could differ among study sites and with fluctuations in grey squirrel densities, we also included first-order interactions of study site with days since invasion, as well as the interactions of days since invasion with grey squirrel density (see electronic supplementary material, table S1).

To consider possible nonlinear (curvilinear) effects of days since invasion on FGMs, its second-order orthogonal polynomial effect was included in the model. We then used the log-likelihood ratio test (LRT) to compare the fit of the full model with the polynomial term with the fit of the same full model without the polynomial term. The same procedure was used for the reduced model, selected by a stepwise backward elimination of non-significant ($p > 0.10$) parameters [56,59]. The models with the second-order orthogonal polynomial effect had the best fit (full model: LRT = 57.7, d.f. = 4, $p < 0.001$; reduced model: LRT = 29.8, d.f. = 2, $p < 0.001$), thus we only present this reduced model in the results.

For the second prediction and following the reaction norm approach described by Guindre-Parker *et al*. [19], we first tested if individual variation in FGMs was better explained using random regression models [60,61] and then we performed model selection on the best supported model. Four linear mixed models were fitted by maximum likelihood and the best supported model was identified using Akaike's information criterion. Top model was determined using *bbmle* function to calculate model AICc scores and model weights [62]. This approach was applied at a full model with identical fixed effects structure than that used to test the first prediction, except for days since invasion, which here was replaced by grey squirrel experience as continuous explanatory variable. Also in this case, the model with second-order polynomial effect had the best fit (full model: LRT = 38.7, d.f. = 4, $p < 0.001$). Hence, we compared the following models: Model 1, the null model including only the fixed effects of the full model with grey squirrel experience; Model 2, built on Model 1 adding a random intercept for each individual; Model 3, with the same structure as Model 2 but including also a random slope for grey squirrel experience, included only as linear predictor; Model 4, similar to Model 3, but allowing correlation between random intercept and slope. Models 3 and 4 tested the individual variation in FGMs plasticity depending on grey squirrel experience, with the addition that Model 4 also tested whether and individual's endocrine phenotype affected the likelihood they exhibited weaker or stronger plasticity [19] (see electronic supplementary material, table S2). Non-significant parameters ($p > 0.10$) of the best-supported model with grey squirrel experience were then eliminated (best-supported full model with grey squirrel experience reported in electronic supplementary material, table S3). The selected reduced model with second-order polynomial effect had the best fit (reduced model: LRT = 7.99, d.f. = 1, $p = 0.005$) and thus we only present this in the results.

All continuous explanatory variables, except body condition (already an index with mean = 0), were standardized ([x − mean]/s.d.) prior to analysis to reduce multicollinearity in the presence of a polynomial term [56,63]. Residuals were visually inspected to verify the assumptions of normality and homoscedasticity [63]. Where necessary, significance of pairwise comparisons was assessed using estimated marginal means (R package *emmeans*, v. 1.7.2, [64]). All the statistical analyses were performed in R [65] using the function *poly* for orthogonal polynomial effects, *lme4* and *lmerTest* packages [66,67].

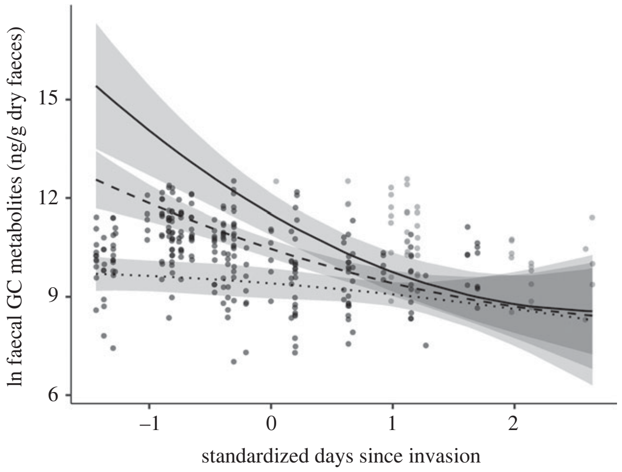

Figure 1. Relationship between FGMs and days since invasion in red squirrels at different levels of grey squirrel density. The line represents the predicted relationship (calculated from the selected model excluding the interaction of sex with reproductive conditions) at low (dotted line, value equal to −1), medium (dashed line, value equal to 0) and high (solid line, value equal to 1) grey squirrel density. Shaded areas represent the 95% CIs, symbols show observed values (full circles).

3. Results

(a) Variation in FGMs with days since invasion

The final model showed that native red squirrels exhibited physiological habituation as the number of days since invasion by grey squirrels increased (figure 1). Specifically, FGMs in native red squirrels were initially high soon after invasion by grey squirrels and then declined over time (figure 1). However, the magnitude of this physiological habituation depended upon the density of invasive grey squirrels, as indicated by the significant interaction between polynomial days since invasion and grey squirrel density index (table 1). Specifically, the reduction of FGMs in red squirrels with increasing days since invasion was more pronounced at higher grey squirrel densities with respect to medium and/or lower grey squirrel density, but with further increase in days since invasion, the curves converged, meaning the relationship between FGMs and days since invasion no longer depended on grey squirrel densities (figure 1). FGMs were higher in native red squirrels with lower body condition (table 1) and lactating females had higher FGMs than non-breeding ones (estimate ± SE: 0.70 ± 0.20, d.f. = 282, $t = 3.49$, $p = 0.006$; all other comparisons $p > 0.05$). FGMs varied among the three study sites: FGMs were significantly higher in Passatempo than in Castelbarco (estimate ± SE: 1.02 ± 0.40, d.f. = 288, $t = 2.53$, $p = 0.03$) and Vanzago (estimate ± SE: 1.25 ± 0.41, df = 279, $t = 3.08$, $p = 0.007$). There was no effect of season, daytime or red squirrel density index on FGMs in native red squirrels (electronic supplementary material, table S1). Interaction of study site with polynomial days since invasion was not significant (electronic supplementary material, table S1) and removed during model selection.

(b) Variation in FGMs with grey squirrel experience

We identified a best-supported model (Model 2; electronic supplementary material, table S2) with a weight of 71%, which included only the random intercept. The other models had a ΔAIC greater than 2 indicating that they were not

Table 1. Selected minimal model with days since invasion explaining the observed variation in ln FGM in red squirrels from three study sites. All the continuous variables were standardized prior to analysis (except body condition, see 'Statistical analyses' section for details). Squirrel ID included as a random intercept to account for repeated measures.

explanatory variables	parameter estimate (± SE)	analysis of variance	p
sex*	0.04 ± 0.24	$F_{1,\ 261} = 0.03$	0.86
reproductive cond. in sex		$F_{3,\ 284} = 5.74$	<0.001
body condition	−0.24 ± 0.07	$F_{1,\ 287} = 9.96$	0.002
days since invasion	−19.75 ± 2.25		
days since invasion^2	2.73 ± 2.40	$F_{2,\ 283} = 40.57^{‡}$	<0.001
study site Vanzago[a]	−0.24 ± 0.15		
study site Passatempo[a]	1.02 ± 0.40	$F_{2,\ 134} = 5.24$	0.007
grey sq. density	1.30 ± 0.25	$F_{1,\ 278} = 27.83$	<0.001
grey sq. density * days since invasion	−13.99 ± 3.16		
grey sq. density * days since invasion^2	4.13 ± 1.17	$F_{2,\ 279} = 10.46^{‡}$	<0.001

*Sex, female held as reference level.

[a] Study site, Castelbarco held as reference level; ‡combined test for the quadratic polynomial function of standardized days since invasion.

Figure 2. Relationship between FGMs and grey squirrel experience in red squirrels ($n = 302$; ID = 129). The bold face line represents the predicted relationship (calculated from the selected model excluding the interaction of sex with reproductive conditions). Shaded areas represent the 95% CIs, symbols show observed values (full circles).

Table 2. Selected minimal model with grey squirrel experience explaining the observed variation in ln FGM in red squirrels from three study sites. All the continuous variables were standardized prior to analysis (except body condition, see 'Statistical analyses' section for details). Squirrel ID included as a random intercept to account for repeated measures (among-individual variance ± s.d. = 0.37 ± 0.61; residual variance ± s.d. = 0.87 ± 0.93).

explanatory variables	parameter estimate (± SE)	analysis of variance	p
sex*	−0.15 ± 0.30	$F_{1,\ 272} = 0.25$	0.62
reproductive cond. in sex		$F_{3,\ 272} = 4.60$	0.004
body condition	−0.18 ± 0.09	$F_{1,\ 291} = 4.49$	0.04
grey sq. experience	−4.48 ± 1.24		
grey sq. experience^2	−0.49 ± 1.14	$F_{2,\ 282} = 6.57^{‡}$	0.002
season spr-sum[a]	−0.58 ± 0.25		
season win[a]	−0.51 ± 0.25	$F_{2,\ 247} = 2.73$	0.07
grey sq. density	0.19 ± 0.07	$F_{1,\ 191} = 6.38$	0.01

*Sex, female held as reference level.

[a] Season, autumn held as reference level; ‡combined test for the quadratic polynomial function of standardized grey squirrel experience.

supported by our data (electronic supplementary material, table S2). Thus, we did not find support for within-individual variation in FGMs plasticity with grey squirrel experience, or for a correlation between random intercepts and slopes (electronic supplementary material, table S2).

The selected model showed that native red squirrels exhibited physiological habituation as the number of days of individual experience with grey squirrels increased (figure 2), as indicated by the significant polynomial effect of grey squirrel experience (table 2). Specifically, FGMs in native red squirrels with low experience in contact with invasive grey squirrels were initially high and there was no or very little decrease. As the individual red squirrel experience with the invasive species increased, FGMs declined more markedly over time (figures 2 and 3).

FGMs were higher in native red squirrels with lower body conditions and positively related with the grey squirrel density index (table 2). Lactating females had higher FGMs than non-breeding ones (estimate ± SE: 0.83 ± 0.23, d.f. = 274, $t = 3.63$, $p = 0.003$; all other comparisons $p > 0.05$). and FGMs were higher in autumn than in spring-summer (estimate ± SE: 0.58 ± 0.25, d.f. = 255, $t = 2.34$, $p = 0.02$) and in winter (estimate ± SE: 0.51 ± 0.25, d.f. = 251, $t = 2.05$, $p = 0.04$). There was no effect of study site, red squirrel density index, or daytime on variation in FGMs (electronic supplementary material, table S3). Interactions of study site with polynomial grey squirrel experience and of grey squirrel density index with polynomial grey squirrel experience were not significant (electronic supplementary material, table S3) and removed during model selection.

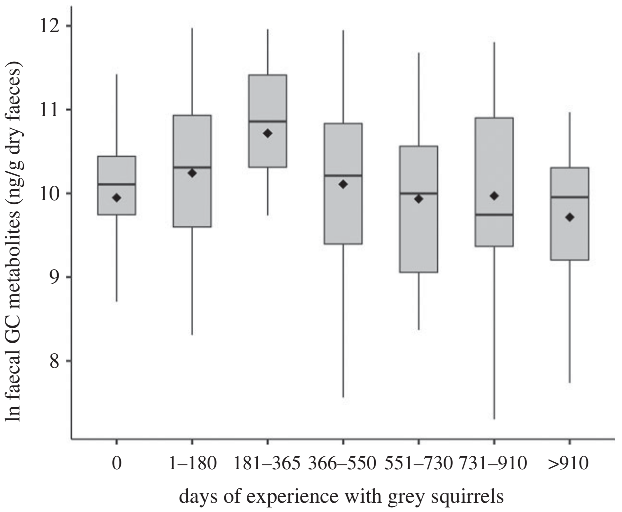

Figure 3. Concentrations of FGMs at increasing number of days of grey squirrel experience, data grouped by six-months periods. Before arrival of grey squirrels (grey squirrel experience = 0). Boxplots show median (solid horizontal line), mean (black diamonds) and 1st (25%) and 3rd (75%) quartiles.

4. Discussion

Using data collected from Eurasian red squirrels over a six-year period, we found strong support for our hypothesis that native red squirrels exhibit physiological habituation to a novel stressor (invasive grey squirrels). At the population level, FGMs increased following early phases of invasion by grey squirrels and then gradually decreased over time, supporting our first prediction. Also, as predicted, the decrease in FGMs in red squirrels was stronger shortly after invasion and more pronounced at high densities of grey squirrels, while it decreased less strongly and was no longer influenced by grey squirrel density as more days since invasion elapsed. In the second model, where time of co-occurrence with the invasive species was determined for each individual separately, FGMs decreased more markedly as the individual red squirrels experienced prolonged contact with invasive grey squirrels, while with little experience with the invasive grey squirrel there was no or very little reduction in FGMs.

When grey squirrels colonized areas occupied by native red squirrels, the latter mounted a physiological stress response as indicated by a marked increase in FGMs (see also [41]). After a period of high levels of FGMs in the early phase of co-occurrence, red squirrels gradually reduced adrenocortical activity and FGMs showed a nonlinear decrease with days since grey squirrel invasion. The slope of this decrease depended on local grey squirrel densities: in the early phase of the decrease FGMs were higher and decreased steeper at high than at low grey squirrel densities, but when days since invasion further increased FGMs tended to have lower values that were independent of the grey squirrel density index. Conversely, there was no effect of native red squirrel density on FGMs, as also seen in a contemporary study on red squirrels in areas without the IAS [50]. By contrast, a relationship between GCs and density has been observed in female North American red squirrels [19,68], where a perceived stressor (increased population density) elicited higher FGMs in females. These studies suggest that density of competitors, either of the same or a different species, can act as environmental stressor in arboreal squirrels. Hence, here we show that the density of an invasive species is perceived as a stressor, but that with increasing time its effect no longer influences the FGMs suggesting hormonal habituation occurs as days since invasion increases (e.g. habituation [16]).

This is confirmed (prediction 2) where FGMs decreased at increasing individual experience of grey squirrels, a measure of co-occurrence with the IAS tuned on each individual red squirrel. Our model comparisons suggested that the model with only random intercept best fitted our data. However, care must be taken with the interpretation of Models 3 and 4 (random slope and correlation between intercept and slope) because our within-individual sample size of FGMs were small (as discussed by [69]). In any case, we showed that the individual stress response attenuates over time as the perceived stressor persists, suggesting that red squirrels habituate to the IAS, more or less one year after first contact with grey squirrels (figure 3). Cyr & Romero [16] proposed a set of criteria and tests that should be considered to disentangle if habituation arises in wild animals. Despite the difficulties in conducting such tests in field studies, we are confident that considering the following aspects we are showing habituation in our system. (1) FGMs increased with elevated density of the invasive species, independently from grey squirrel experience, which suggests that the HPA axis of red squirrels was still capable of mounting a stress response. (2) FGMs decreased as red squirrel body condition increased, suggesting that at least animals in good condition are not having health consequences, even when chronic exposure to stressor is expected to determine health costs (e.g. [8,9,11]). Moreover, (3) FGMs in red squirrels in study sites colonized recently by the invasive grey squirrel are on average three times higher than in populations where only red squirrels occur [41], and here we showed that at increasing grey squirrel experience, FGMs tend to return at similar values than those observed in areas without the stressor [50]. (4) We observed FGMs variation on a long time scale (up to 5 years), so for the majority of an adult squirrel's lifespan (1–6 years [70]), and our model showed that animals continued to undergo changes in the FGMs that varied seasonally (both sexes) and with breeding condition (in females). Hence, despite the impossibility to conduct ACTH injection due to logistical constraints, we are confident that these results suggest habituation and not physiological desensitization or exhaustion [16].

Although several studies have shown that the presence of an invasive species can result in an increase in GCs [37–41], as far as we know, no studies in the field have explored variation in GCs in a native species over a longer time scale and in relationship to the duration of experience with an IAS. Whether habituation of the endocrine stress response in a native species will promote coexistence with the invasive species depends on the relative impact of physiological stress with respect to other mechanisms of interspecific competition (i.e. food resource competition, niche overlap). Such interplay between different processes is inevitably linked to the species and competition mechanisms that are involved in the study system. Here, we observed a decrease in FGMs as red squirrels' body condition increases, suggesting a positive outcome of the hormonal habituation, which ultimately might enhance their fitness. Indeed, having lower FGMs over long time periods could possibly reduce the risk associated with prolonged chronic stress; such as metabolic disorders and related decrease in body condition [8], impaired immune system efficiency [13,14,56], or reduced reproductive output [40]. Moreover, in many mammals that are income breeders, such as Eurasian red squirrels, a good body condition (high body mass) is positively related to lifespan and/or reproductive success [54,71–73]. Hence, although hormonal habituation appears an adaptive physiological response because it reduces the impact of invasive grey squirrels on native red squirrels, other processes of competition are ongoing, such as behavioural adaptations [49,74], food resource competition [43,46] and parasite-mediated competition [44,45,75,76], which ultimately drive the local populations of the native species to extinction [43]. In conclusion, the observed hormonal habituation might limit the deleterious impact of invasive alien species on native species (on a longer time scale), but the final outcome of competition still depends on the relative importance of all competition mechanisms involved and their ultimate effect on the persistence of populations of the native species.

Ethics. Trapping, marking and handling of red squirrels were carried out in accordance with the Guidelines for the treatment of animals in behavioural research and teaching [77]. Removal of grey squirrels was mandatory following European Regulation 1143/2014. Approval and legal requirements according to the Italian Wildlife Protection and Hunting Law L.N. 157 from 1992 and authorizations N. 180–14 616 of 06/07/2011 (2011–2013) and N. 294–34 626 of 12/09/2014 (2014–2016) from the Provincia di Torino and N. 62–3025 (2017–2019) from the Città Metropolitana di Torino, and Decreto N. 11 190 (29/11/2013) and decrees N. 9523 of 15/10/2014 and N. 198 (13/01/2017) from Direzione Generale Agricoltura, Regione Lombardia.

Data accessibility. The data are provided in electronic supplementary material [78].

Authors' contributions. F.S.: conceptualization, data curation, formal analysis, investigation, methodology, writing—original draft, writing—review and editing; L.A.W.: conceptualization, investigation, methodology, project administration, writing—review and editing; B.D.: methodology, resources, supervision, validation, writing—review and editing; R.P.: resources, supervision, validation, writing—review and editing; C.T.: investigation, methodology; D.P.: software, supervision, writing—review and editing; A.M.: project administration, supervision, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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