



## Physiological stress response to urbanisation differs between native and invasive squirrel species

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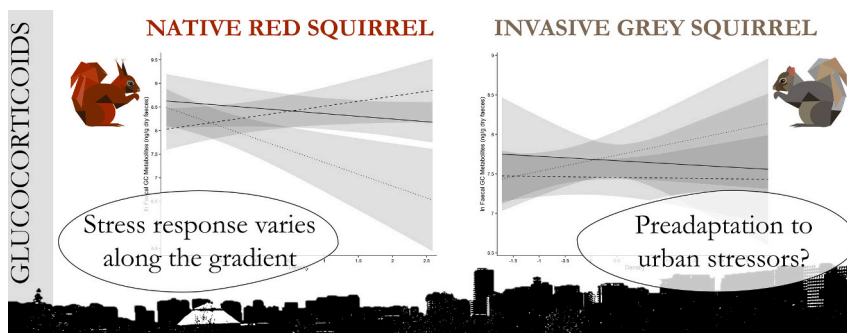
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### HIGHLIGHTS

- Urbanisation can influence physiological stress response of wild animals.
- Glucocorticoid concentrations in native and invasive squirrels
- Synergic effect of conspecific density and urbanisation
- Native red squirrels' stress response varies along rural-urban gradient.
- Invasive grey squirrels might be pre-adapted to urban stressors.

### GRAPHICAL ABSTRACT



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### ABSTRACT

Novel pressures derived from urbanisation can alter native habitats and ultimately impact wildlife. Coping with such human-driven changes might induce shifts in species phenotypic traits, such as physiological responses to anthropogenic stressors. Preadaptation to face those challenges has been suggested to favour settlement and spread of invasive alien species in urbanised areas which, consequently, might respond differently than ecologically similar native species to stressors posed by urbanisation. The activation of the hypothalamic-pituitary-adrenal (HPA) axis and the subsequent release of glucocorticoids (GCs) has been suggested to mediate responses to anthropogenic disturbance in vertebrates. Furthermore, intraspecific competition, in conjunction with stressors related to urbanisation, might affect invasive and native species physiological stress responses differently. Using a parallel pseudo-experimental study system we measured faecal glucocorticoid metabolite (FGM) concentrations of the native Eurasian red squirrel and the invasive alien Eastern grey squirrel along a rural-urban gradient and in relation to conspecific density. The two species responded differently to challenges posed by the synergic effect of urbanisation and intraspecific competition. Association of FGMs and

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conspecific density in native red squirrels varied between rural and suburban sites, potentially depending on differential HPA axis responses. In urban sites, this relationship did not differ significantly from that in rural and suburban ones. Conversely, invasive grey squirrels' FGMs did not vary in relation to conspecific density, nor differed along the rural-urban gradient. Improving knowledge about native and competing invasive species' physiological responses to anthropogenic stressors can support conservation strategies in habitats altered by man. Our findings suggested that the invasive squirrels might be preadapted to cope with these challenges in urbanised areas, potentially increasing their success under the future global change scenario.

## 1. Introduction

Human-induced changes associated with urbanisation can alter native habitats forming novel ecosystems through variation of vegetation characteristics, availability and type of food resources, landscape structure (i.e. built fraction), as well as biotic and abiotic interactions (Alberti, 2015; Alberti et al., 2017; Santangelo et al., 2018). All these human-driven changes ultimately impact wildlife. For instance, light pollution was shown to shape animals' activity patterns and interactions, besides their space use and spatial distribution (Ciach and Fröhlich, 2019; Hoffmann et al., 2019). Noise, generally originated by road traffic, can affect traits such as intraspecific communication and vigilance (Kaiser et al., 2011; Sweet et al., 2022) and the traffic itself may increase the rate of collisions between wildlife and vehicles. Air pollution, measured as heavy metal contamination in great tit feathers, increases long-term stress in individuals inhabiting heavily polluted urbanised environments (Bauerová et al., 2017). Consequently, the continuous and rapid expansion of urbanisation is leading to the degradation and reduction of natural and diverse ecosystems, negatively affecting biodiversity (McKinney, 2006; Li et al., 2021).

To cope with such novel pressures, wild animal species can change some phenotypic traits (i.e. phenotypic plasticity), increasing their probability of surviving in urban environments; that could subsequently entail adaptation if the animals have advantages in some fitness components (McDonnell and Hahs, 2015). For instance, several studies reported variation in phenotypic traits, such as morphology, behaviour and cognition, space use, and physiology, between urban and rural settings (Bonier, 2012; Lowry et al., 2013; McDonnell and Hahs, 2015; Ritzel and Gallo, 2020; Sarkar and Bhadra, 2022). However, responses to urbanisation are species-specific, based on geographical areas and the characteristics (i.e. shape, size, human population density) of the city where the species occurs (i.e. Tranquillo et al., 2023). Also, species preadapted to live in cities thrive in areas where the built-up fraction is high (i.e. pigeons and rats), while other species are unable to adapt to the new environment and are forced to migrate or become locally extinct (McDonnell and Hahs, 2015). Preadaptation has been suggested as one of the multiple causes of successful settlement and subsequent spread of invasive alien species (IAS) in urbanised areas, which are known as one of the major introduction pathways (Cadotte et al., 2017; Wauters and Martinoli, 2018; Borden and Flory, 2021). In particular, IAS seems to be favoured in urban areas since cities are islands of reduced competition and escape from natural enemies (Cadotte et al., 2017), although there is no consensus about the impact of predation in urbanised areas (see Fischer et al., 2012). Also, this should be included in the context of the life-history theory (reported by Santana Marques et al., 2020) where nutritional trade-offs, influenced by food availability, reproduction, predator avoidance and competition, might drive the invasive potential of IAS in urbanised areas (i.e. invasiveness). Moreover, IAS traits variation in urban habitats can enhance their competitive ability an exacerbate impact on native species (Borden and Flory, 2021). Therefore, studying and comparing native and invasive species responses to cope with pressures imposed by urban habitats conditions is mandatory, especially to provide knowledge about potential adaptation to the future global change scenario (Alberti et al., 2017; Santangelo et al., 2018; Borden and Flory, 2021).

As previously described, species responses to urbanisation have been

explored from different perspectives, in particular physiological response mechanisms (reviewed in Bonier, 2012; Iglesias-Carrasco et al., 2020) can be involved in species stress responses to cope with challenges of an urbanised environment (see also Santicchia et al., 2022a and references therein). Specifically, in vertebrate species, the activation of the hypothalamic-pituitary-adrenal (HPA) axis and subsequent release of glucocorticoids (GCs) mediates responses to environmental perturbations (Santicchia et al., 2022a, 2022b; Dantzer, 2023) as further confirmed by a meta-analysis showing a positive link between GC levels and anthropogenic disturbance (Dantzer et al., 2014). For instance, studies on amphibians, reptiles, birds and mammals explored the effects of human-induced stressors on GCs secretion, showing how response to anthropogenic disturbance can be species-specific and context-dependent (Formenti et al., 2018; Narayan, 2019; Carbillet et al., 2020; Iglesias-Carrasco et al., 2020; Injaian et al., 2020; Kaisin et al., 2021; Shidemantle et al., 2022; Robertson et al., 2023). Thus, not only investigating the GC response of native species facing stressors posed by urban habitats can determine conservation strategies (Dantzer et al., 2014), but also considering how invasive species respond to similar stressors might inform managing programs in urban systems (see Gaertner et al., 2017).

To explore physiological stress responses of invasive and native species to the challenges posed by urbanisation, we used a parallel (within species) pseudo-experimental study system with replicated study sites along a rural-urban gradient. We measured faecal glucocorticoid metabolite (FGM) concentrations, an integrated measure of both baseline and stress-induced GC levels, which has been used as a proxy of an animal's HPA axis activity over a specific period of time (Dantzer et al., 2016b; Santicchia et al., 2018, 2022a, 2022b; Palme, 2019). We explored differences in FGM levels among rural, suburban and urban areas, in two squirrel species, since they occur in sites located along the urbanisation gradient and are also common in urban parks: the Eurasian red squirrel (*Sciurus vulgaris*), a native sciurid, and the Eastern grey squirrel (*Sciurus carolinensis*), an invasive alien congener, known to replace the native species in several countries across Europe (Wauters et al., 2023). In each study sites only one squirrel species occurs.

As detailed in previous studies, GCs secretion can be elicited by a variety of environmental challenges, among which intraspecific competition (often measured using conspecific density), influencing an animal's physiological response to external pressures (Dantzer et al., 2013; Cox, 2020; Dantzer and Newman, 2022; Petruccio et al., 2022). As such, Tranquillo et al. (2022) highlighted how FGM levels might be linked to intraspecific interactions, but the mechanism of increased HPA axis activity in relation to conspecific density might be more complex and potentially associated with the synergic effect of food availability (Dantzer et al., 2016a, 2020), which can vary also depending on the supplemental food provisioning known to occur in urban areas (Snell-Rood et al., 2015).

Here we explored whether the native species and its invasive congener showed different patterns in FGM concentrations in response to urbanisation (rural < suburban < urban), and in relation to intraspecific competition (conspecific density). In native red squirrels, we expected a positive relationship between conspecific density and FGM concentrations as urbanisation level increases, since at higher human-disturbance, red squirrels are potentially subject to a synergic effect of urbanisation and intraspecific competition. Conversely, since invasive

species are favoured in urban areas (i.e. less interspecific competition, potential preadaptation to human-induced stressors), we expected that FGM concentrations in invasive grey squirrels will not be associated with variation in conspecific density, and will not be affected by the level of urbanisation.

## 2. Materials and methods

### 2.1. Study sites, trapping and handling squirrels

We trapped red squirrels in two rural (Calusco, Golasecca), two suburban (Pineta, Varesesub) and two urban (Gallarate, Vareseurb) study sites located in Lombardy, Northern Italy (Table A.1), from October 2020 to September 2022. We also trapped grey squirrels in two rural (Commande, Piobesi), two suburban (Adda Nord, RS) and two urban (ILO, TO) study sites located in Lombardy and Piedmont, Northern Italy (Table A.1), from May 2020 to February 2022. As in previous studies, the proportion of built-up fraction in a circular area with radius of 200 m was used to classify the study sites as rural, suburban or urban (Tranquillo et al., 2024). Moreover, we considered urban those sites completely surrounded by an urban matrix, rural sites are separated from cities (> 1 km) and surrounded by agricultural land, while suburban sites are only partly surrounded by an urbanised matrix, thus presenting intermediate characteristics between the other two site types. Built-up fraction was high for urban sites, low for rural, and intermediate for suburban ones (Tranquillo et al., 2024). Some study sites had more than one trapping grid (Table A.1). In each site, we first carried out three to 10 Capture-Mark-Recapture (CMR) sessions (approximately one every two to four months) lasting two to six days. CMR sessions were then followed by a final removal session in all the six study sites where grey squirrels were present. We trapped squirrels using single capture live traps (model 202, Tomahawk Live Trap Co., Hazelhurst, WI, USA), homogeneously distributed in each trapping grid. Traps were placed on the ground or at breast height against tree trunks and a fine mesh was added underneath to limit contamination between urine and faeces. Traps were baited with hazelnuts 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). At first capture, each squirrel was individually marked using a numbered metal ear-tag (Monel 1005 1 L1, National Band & Tag Co. Newport, KY, USA) and weighed to the nearest 5 g using a spring-balance (Pesola AG, Baar, Switzerland). Sex and reproductive condition were determined from external genitalia and nipples inspection (as detailed in Dantzer et al., 2016b; Santicchia et al., 2018). After capture and handling, faecal samples were collected from underneath the traps using forceps and placed individually into 1.5-ml vials (Dantzer et al., 2016b). The trap, mesh and the ground under the trap were cleaned to remove possible remains of faecal material. We only used faecal samples from squirrels that had not previously been trapped or handled within 72 h prior to capture. We placed faecal samples into an insulated bag with wet ice packs while in the field and then stored them at  $-20^{\circ}\text{C}$  within 3–4 h after collection (Dantzer et al., 2016b).

### 2.2. Ethical statement

Trapping, marking and handling of squirrels were carried out in accordance with the Guidelines for the treatment of animals in behavioural research and teaching (Animal Behaviour, 2020, 159, I–XI; <https://doi.org/10.1016/j.anbehav.2019.11.002>). Removal of grey squirrels was mandatory following European Regulation 1143/2014. Grey squirrels were euthanized by  $\text{CO}_2$  inhalation following international animal welfare guidelines (Close et al., 1996, 1997; Leary et al., 2013). Approval and legal requirements according to the Italian Wildlife Protection and Hunting Law L.N. 157 from 1992 and authorizations N. DD 1015 of 20/03/2020 from Città Metropolitana di Torino, decree N. 1938 of 18/02/2020 from Direzione Generale Agricoltura, Alimentazione e Sistemi Verdi, Regione Lombardia, authorizations of Parco

Lombardo della Valle del Ticino, Parco Pineta di Appiano Gentile e Tradate, Parco Adda Nord, Comune di Gallarate and Comune di Varese.

### 2.3. Extraction and quantification of FGMs

We analysed 206 faecal samples of 106 red squirrels (RS; 57 males and 49 females; 51 individuals had at least two FGM measurements) and 226 faecal samples of 156 grey squirrels (GS; 77 males and 79 females; 50 individuals had at least two FGM measurements). Samples were oven dried ( $80^{\circ}\text{C}$ ) overnight, ground up under liquid nitrogen, homogenized and weighed to 0.05 g ( $\pm 0.004$  g), and extracted using 80 % methanol, shaking at 1500 r.p.m. on a multivortex for 30 min, centrifuging at 2500g for 15 min. An aliquot of the supernatant was diluted in assay buffer (1 + 9) and assayed using a  $5\alpha$ -pregnane- $3\beta$ , 11 $\beta$ , 21-triol-20-one enzyme immunoassay (EIA) to measure FGM concentrations (ng g<sup>-1</sup> dry faeces; Touma et al., 2003; Santicchia et al., 2018; Romeo et al., 2020). This in-house EIA detects GC metabolites with a  $5\alpha$ - $3\beta$ , 11 $\beta$ -diol structure (for cross-reactivity see Touma et al., 2003). Methods of EIA validation for Eastern grey squirrels and Eurasian red squirrels can be found in detail elsewhere (Bosson et al., 2013; Dantzer et al., 2016b). Samples were analysed in duplicate. Intra- and inter-assay CVs were 2.2 % and 3.3 %, respectively.

### 2.4. Statistical analyses

To test our predictions, we performed two separate linear mixed-effects models (LMM) with the same model structure, one for each squirrel species, using FGMs (transformed using the natural logarithm,  $\ln$  of ng/g dry faeces) as the dependent variable. To test for the best random effects models structure we first performed a log-likelihood ratio test (LRT) to compare the fit of the following models: Model 1, a null model, including only fixed effects; Model 2, adding squirrel identity (ID) as random intercept term to Model 1, to account for repeated measures of FGMs for a given individual; Model 3, adding ID and study site nested in urban type both as random intercepts to Model 2 (Table A.2). For both species, Model 2 was selected as the best model (Table A.2). LMMs included urban type (rural, suburban, urban), sex, reproductive condition nested in sex (males: abdominal or scrotal testes; females: non-breeding, post-oestrus and pregnant, lactating; Santicchia et al., 2018), season (spring-summer, autumn or winter) and daytime (sample collected in the morning or in the afternoon) as fixed effects (Dantzer et al., 2016b; Santicchia et al., 2018, 2022b). Body mass, measured at each capture, and squirrel density, measured for each trapping session and grid in a given study site (Table A.1), were included as continuous explanatory variables. Red squirrel density was calculated as the ratio of the minimum number known to be alive (MNA) in each trapping session on trapping grid size (ha). Grey squirrel density was calculated as the ratio of the catch per unit effort (CPUE) in each trapping session, as detailed in Santicchia et al. (2022a), on trapping grid size (ha). The different methods to estimate density were applied because for grey squirrels no long-term CMR data were available due to the removal sampling design in the last trapping session. Also, since relationships of squirrel density with FGMs could differ depending on anthropogenic pressures, we included first-order interaction of density with urban type (Table A.3). Model selection was performed through stepwise backward elimination of non-significant ( $p > 0.10$ ) parameters (Table 1; Lewis et al., 2011) and without eliminating those variables specifically related to our predictions. Where necessary, comparisons of factors with more than two levels were carried out changing the reference level in the model. Slope estimates of the interaction between density and urban type were obtained removing the main effect of density from the selected models, for each squirrel species. All continuous explanatory variables were standardized ( $[x - \text{mean}]/\text{SD}$ ) prior to analysis and residuals were visually inspected to verify the assumptions of normality and homoscedasticity (Zuur et al., 2010). All the statistical analyses were performed in R (R Core Team, 2022).

**Table 1**

Selected models on red and grey squirrel data. All the continuous variables were standardized prior to analysis (see “Statistical analyses” section for details). Squirrel ID included as a random intercept to account for repeated measures.

Explanatory variables	Parameter estimate (± SE)	df	t value	p
<b>Red squirrel</b>				
Sex M – Sex F	0.61 ± 0.23	193	2.61	0.01
Sex F: lactating – Sex F: post-oestrus/pregnant	−0.13 ± 0.22	193	−0.57	0.57
Sex F: non-breeding – Sex F: post-oestrus/pregnant	0.16 ± 0.19	193	0.83	0.41
Sex F: non-breeding – Sex F: lactating <sup>a</sup>	−0.29 ± 0.19	193	−1.53	0.13
Sex M: scrotal – Sex M: abdominal	−0.46 ± 0.18	193	−2.47	0.02
Season spring-summer – Season autumn	0.44 ± 0.14	193	3.24	0.001
Season winter – Season autumn	0.50 ± 0.14	193	3.56	<0.001
Season winter – Season spring-summer <sup>a</sup>	0.06 ± 0.13	193	0.46	0.64
Daytime morning – Daytime afternoon	0.57 ± 0.13	193	4.41	<0.001
Density	−0.42 ± 0.16	193	−2.57	0.01
Urban type suburban – Urban type rural	0.37 ± 0.15	193	2.46	0.02
Urban type urban – Urban type rural	0.60 ± 0.20	193	3.05	0.003
Urban type suburban – Urban type urban <sup>a</sup>	−0.23 ± 0.18	193	−1.29	0.20
Density * Urban type suburban – Density * Urban type rural	0.59 ± 0.20	193	2.92	0.004
Density * Urban type urban – Density * Urban type rural	0.31 ± 0.19	193	1.60	0.11
Density * Urban type suburban – Density * Urban type urban <sup>a</sup>	0.29 ± 0.15	193	1.92	0.057
<b>Grey squirrel</b>				
Sex M – Sex F	0.28 ± 0.11	154	2.58	0.01
Density	0.14 ± 0.10	185	1.34	0.18
Urban type suburban – Urban type rural	−0.21 ± 0.15	132	−1.38	0.17
Urban type urban – Urban type rural	0.02 ± 0.16	160	0.10	0.92
Urban type suburban – Urban type urban <sup>a</sup>	−0.23 ± 0.15	163	−1.50	0.14
Density * Urban type suburban – Density * Urban type rural <sup>b</sup>	−0.14 ± 0.13	204	−1.15	0.25
Density * Urban type urban – Density * Urban type rural <sup>b</sup>	−0.17 ± 0.19	206	−0.92	0.36
Density * Urban type suburban – Density * Urban type urban <sup>a,b</sup>	0.03 ± 0.18	215	0.16	0.87

<sup>a</sup> Estimated reorganizing levels of the factor.

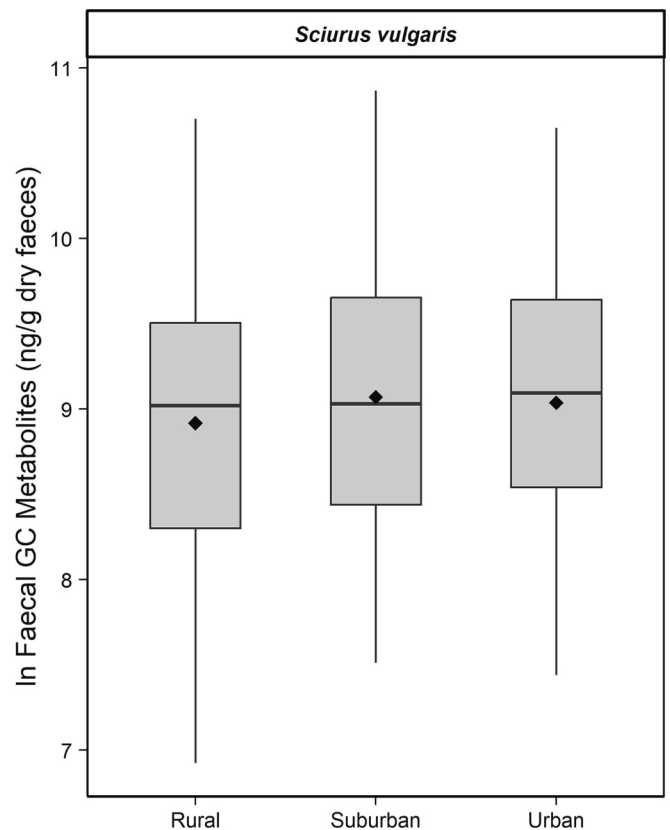
<sup>b</sup> Removing Density \* Urban type interaction does not improve the model fit.

### 3. Results

#### 3.1. Red squirrels

Individual variation in FGMs was high in all sites (Fig. 1). Average number of samples per individual (± SE) was 1.87 ± 0.08 (range: 1 to 6). Comparisons of FGM concentrations of red squirrels among rural, suburban and urban sites are given in Fig. 1.

A significant interaction between urban type and density in the selected model (Table 1) indicated that the relationship between density and FGMs changed according to the type of area (Fig. 2). In rural sites, FGMs decreased with increasing squirrel density (slope estimate ± SE: −0.42 ± 0.16, df = 193, t = −2.57, p = 0.01), while not significant relationships between density and FGMs were observed in urban (slope estimate ± SE: −0.11 ± 0.10, df = 193, t = −1.16, p = 0.25) and suburban sites (slope estimate ± SE: 0.18 ± 0.12, df = 193, t = 1.53, p = 0.13). The association between density and FGMs in suburban sites was significantly different than in rural ones (Table 1) with a trend of higher FGMs at high densities in suburban than in rural sites (Fig. 2). In



**Fig. 1.** FGM concentrations (ln ng/g dry faeces) of Eurasian red squirrels (*S. vulgaris*) in rural (n = 78; ID = 40), suburban (n = 80; ID = 39) and urban sites (n = 48; ID = 27). Boxplots show median (solid horizontal line), mean (black diamond) and 1st (25 %) and 3rd (75 %) quartiles.

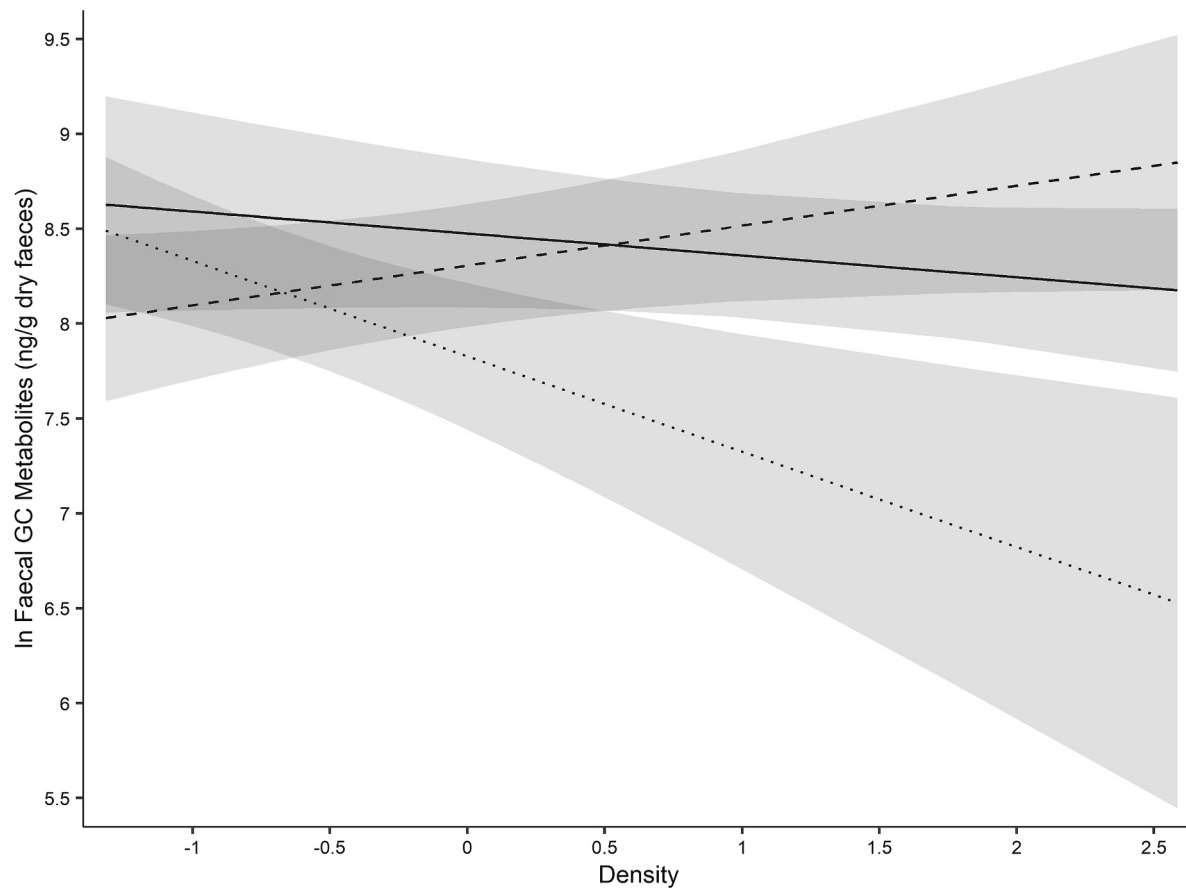
contrast, in urban sites, there was no difference in the association between density and FGMs respect to rural ones (Table 1), while there was a marginally significant difference compared to suburban sites (Table 1). Furthermore, males had on average higher FGMs than females, and FGMs from samples collected in the morning were higher than from those collected in the afternoon (Table 1). Seasonally, FGMs were higher in spring-summer and winter than in autumn (Table 1), while there was no difference between spring-summer and winter seasons (Table 1). Males with scrotal testes had lower FGMs than those with abdominal testes (Table 1), while there was no effect of reproductive condition in females (Table 1). There was no effect of body mass on FGMs in native red squirrels (Table A.3) thus it was removed during model selection.

#### 3.2. Grey squirrels

Also in the invasive species, individual variation in FGMs was high in all sites (Fig. 3). Average number of samples per individual (± SE) was 1.40 ± 0.04 (range: 1 to 4). Comparisons of FGM concentrations of grey squirrels among rural, suburban and urban sites are given in Fig. 3.

The final model showed that grey squirrels' FGMs did not vary in relation to density in urban (slope estimate ± SE: −0.04 ± 0.16, df = 212, t = −0.23, p = 0.82), suburban (slope estimate ± SE: −0.008 ± 0.07, df = 219, t = −0.11, p = 0.91) or rural sites (slope estimate ± SE: 0.14 ± 0.10, df = 185, t = 1.34, p = 0.18). In contrast with the native species, the association between density and FGMs was not different among urban types (Table 1). Males had on average higher FGMs than females (Table 1). However, there was no effect of reproductive condition, body mass, season and daytime (Table A.3), and these variables were removed during model selection.





**Fig. 2.** Relationship between red squirrel density (standardized) and FGMs in different urban types. The lines represent the predicted relationship (calculated from the selected model excluding the interaction of sex with reproductive conditions) in rural (dotted line), suburban (dashed line) and urban (solid line) sites. Shaded areas represent the 95 % CIs.

#### 4. Discussion

Using a parallel replicated study design, we explored physiological stress response of a native species and an invasive congener to similar stressors along a rural-urban gradient. Improving knowledge about native species responses to human-induced stressors can support conservation strategies, while studying invasive species responses to similar stressors can add insights in their invasive potential and inform management programs in face of increasing urbanisation (Dantzer et al., 2014; Alberti et al., 2017; Gaertner et al., 2017). Our results suggest no association between population density and FGMs and no changes in FGMs with urban type in the invasive grey squirrel. In contrast, in native red squirrels we found complex relationships between density and FGMs that furthermore varied between rural, suburban and urban sites. These patterns differed from relationships documented for both species concerning morphological changes (mainly body mass), where red as well as grey squirrels were heavier in the urban than in the rural sites (Tranquillo et al., 2024).

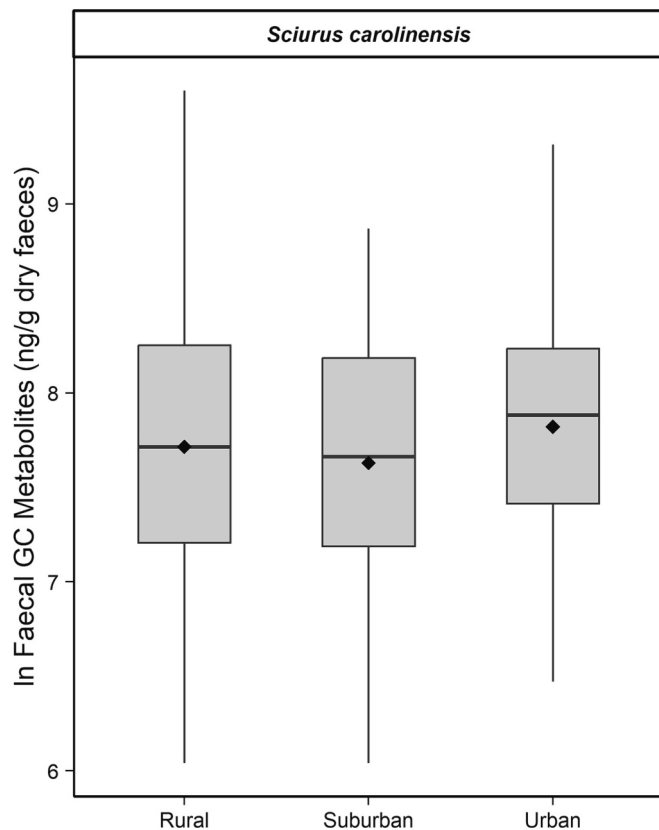
##### 4.1. Native species

Physiological stress along the urbanisation gradient was assessed in some native vertebrates, however, the results are not consistent among species, sexes, locations or even ages (Scheun et al., 2015; Price et al., 2018; Brunton et al., 2020). For instance, Shimamoto et al. (2020) showed that FCM levels were not significantly different between urban and rural red squirrel populations, while Stothart et al. (2019) reported higher FGMs in urban than in rural areas for grey squirrels in their native range. Moreover, two studies on chipmunks (*Tamias striatus*) found

contrasting patterns: Lyons et al. (2017) reported lower FCMs in urban compared to rural areas, while Ouellette et al. (2023) showed that chipmunks in more urban habitats exhibit higher levels of FGMs than conspecifics in less urban habitats. Another study, on eastern grey kangaroos (*Macropus giganteus*), found differences in stress levels between urban and rural individuals, however, the direction depended on the region where the study was carried out (Brunton et al., 2020). While, a study by Creel et al. (2013) showed that FGM concentrations were lower for African lions (*Panthera leo*) in a conservation area, and increased in zones with higher anthropogenic activity. Overall, studies in literature reported inconsistent findings, but a recent meta-analysis suggests, although conclusions are premature, that urban areas are no more stressful for wildlife than rural or non-urban areas (Iglesias-Carrasco et al., 2020).

Moreover, intraspecific competition has been suggested to covary with anthropogenic stressors, eliciting an increase in circulating GCs. However, previous studies reported contrasting results, with positive associations between GCs and population density in some rodents (root vole *Microtus oeconomus*: Bian et al., 2011; North American red squirrel *Tamiasciurus hudsonicus*: Dantzer et al., 2013; Eurasian red squirrel *Sciurus vulgaris*: Haigh et al., 2017; Algerian mice *Mus spretus*: Navarro-Castilla et al., 2017), while others reported a negative relationship (Prairie voles *Microtus ochrogaster*: Blondel et al., 2016).

Considering our predictions, where we expected that red squirrel FGM concentrations at high conspecific density will increase along rural-urban gradient, this was only confirmed in suburban sites where at high densities FGMs were higher than in rural ones. Conversely, the density – FGM relationship in urban sites was not significantly different from either rural or suburban sites. A possible explanation for the lack of



**Fig. 3.** FGM concentrations (In ng/g dry faeces) of Eastern grey squirrels (*S. carolinensis*) in rural ( $n = 66$ ; ID = 40), suburban ( $n = 72$ ; ID = 44) and urban sites ( $n = 88$ ; ID = 72). Boxplots show median (solid horizontal line), mean (black diamond) and 1st (25 %) and 3rd (75 %) quartiles.

a strong, positive effect of density on FGMs in urban sites could be that red squirrels subject to a continuous exposure of stressors produced in more urbanised sites, might show physiological habituation, desensitisation or exhaustion (Cyr and Romero, 2009; Santicchia et al., 2022a). Thus, these animals might not perceive increased competitor densities as a stressor and might not show an increase in FGMs. Nevertheless, to be demonstrated, these processes related to decreased intensity of the physiological stress response, require long-term monitoring of red squirrels in urban areas to investigate a possible hormonal response reduction and disentangle the proximate causes. Alternatively, red squirrels occurring in urban study sites might not perceive increased conspecific density as a stressor, when a constant availability of palatable high-energy seeds (tree species with different phenology; Tranquillo et al., 2024) is likely to reduce the pressure of intraspecific competition for food resources.

Red squirrels inhabiting suburban sites, which had intermediate levels of urbanisation, showed a density - FGM association significantly different than those in rural sites, with higher FGMs at high squirrel densities. However, the density - FGM relationship was not different from that in urban sites. A tentative explanation could be that these individuals in suburban sites faced a higher amount (i.e. type, intensity, frequency) of stressors than those occurring in rural sites, but they are less used to them respect to red squirrels in urban sites (see above). Consequently, they might be more sensitive to the synergic effect of anthropogenic stressors and intraspecific competition (see Tranquillo et al., 2024). Such complex associations between GCs and population density according to the degree of urbanisation are further supported by several studies which highlighted how HPA axis reactivity can be influenced by early-life exposure to GCs or stressful experiences, which might promote (or not) a downregulation of the HPA axis through

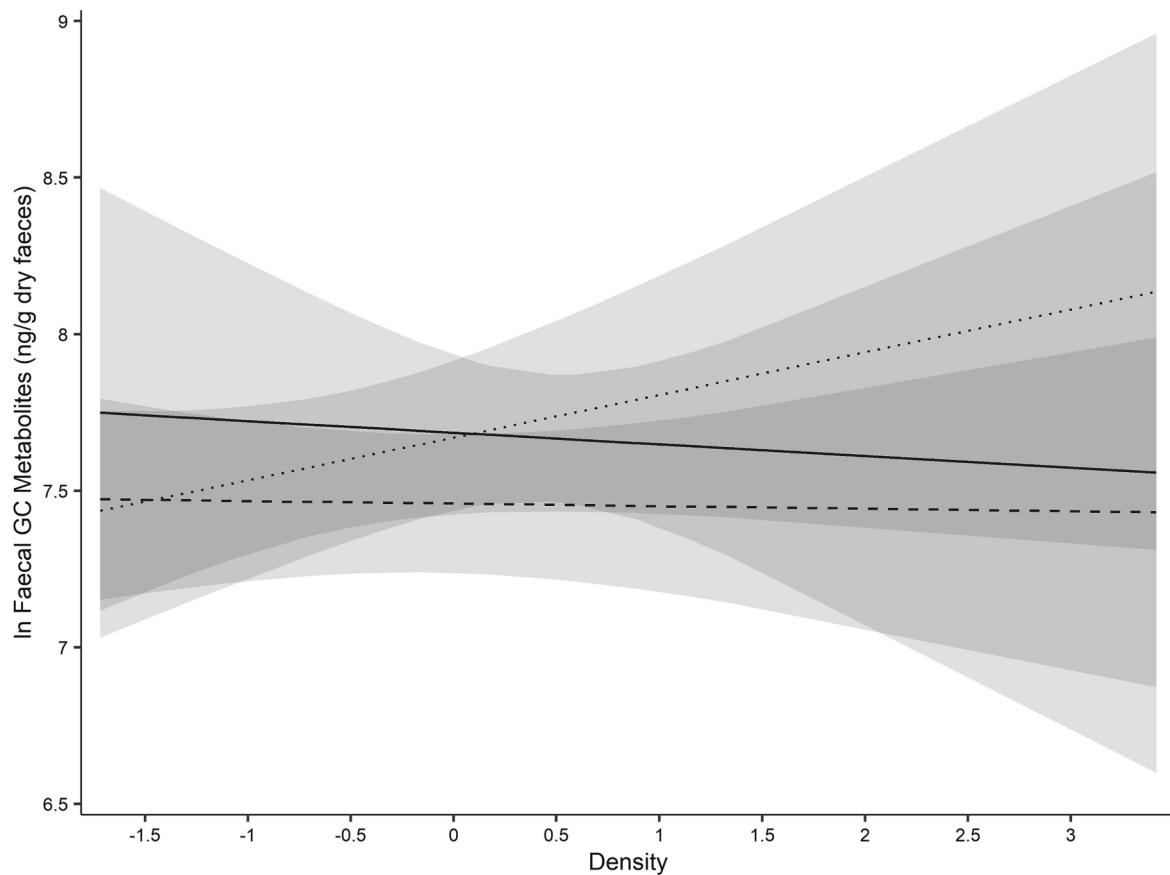
adulthood. This process can further vary among species and sexes, (Marasco et al., 2012; Grace and Anderson, 2018; Dantzer et al., 2019; Grace et al., 2020) and, to some extent, might differ in relation to constraints posed by increasing levels of urbanisation. However, two studies on aquatic vertebrates reported contrasting results: two species of fish coped with urban habitats through negative feedback on the hypothalamic-pituitary-inter-renal (HPI) axis which suppressed their GC stress response (Kolonin et al., 2022); while in common toad tadpoles (*Bufo bufo*), the negative feedback produced a higher stress response in populations living in anthropogenic habitats (Bókony et al., 2021). Nevertheless, demonstrating if regulation of the HPA axis and consequent lower or higher stress response occur in red squirrels inhabiting urban or suburban sites, also in relation to the potential additive stressor of intraspecific competition, would require a long-term study on the effects related to GCs secretion across squirrels' life-time, coupled with experimental manipulation of the HPA axis responsiveness (Westrick et al., 2021).

Considering red squirrels living in rural areas, with no or little anthropogenic disturbance, we found that FGMs decreased at higher densities. Lower stress at high densities in the rural sites might be affected by a positive relationship between density and food availability, hence, FGMs could decrease when high energy food resources are easily acquired (Tranquillo et al., 2022). Finally, as in some previous studies (Dantzer et al., 2016b), several other variables affected variation in FGMs, the squirrel's sex, reproductive condition, and temporal factors (e.g. time of day of faeces collection, season), which could partly mask the urbanisation effects.

#### 4.2. Invasive species

Invasive alien species, which seems to be advantaged in urbanised areas (Cadotte et al., 2017), are the perfect candidates to thrive and spread in such novel ecosystems (Alberti et al., 2017; Santangelo et al., 2018). To the best of our knowledge, no studies explored the physiological stress response, measured with GC concentrations, of invasive species along the urbanisation gradient, which was unexpected, considering the detrimental role of these IAS in the invaded ecosystems. As for the native species (see above), we assessed the FGM concentrations in face of similar stressors such as urbanisation and conspecific density, which might influence their invasive potential (sensu Santana Marques et al., 2020). As far as we know, only one study was conducted on physiological stress response of an invasive rodent species in its range of introduction which highlighted that there was no covariation among FGM concentrations and conspecific density (*Sciurus carolinensis*: Santicchia et al., 2020).

Our prediction that grey squirrel FGM concentrations did not vary in relation to conspecific density, nor that they differed among more or less urbanised sites (rural-urban gradient), was confirmed (see Table 1 and Fig. 4). This trend differs from observations in their native range, where grey squirrels living in areas with relatively little vegetation and more human-made materials and structures had lower hair cortisol concentration (HCC) compared to those living in less urbanised areas such as urban parks and forest sites (Rimbach et al., 2022). Moreover, grey squirrels in the native range are present in natural forests as well as in urban parks or cities (Benson, 2013; Shuttleworth et al., 2016), often in co-occurrence with other sciurid species with which they share part of their ecological niche (Don, 1983; Riege, 1991; Koprowski, 1994; Wauters et al., 2023). Thus, in the non-native ranges where they have been introduced, grey squirrels might not perceive urbanisation as an adverse ecological factor eliciting an increase in GCs, indicating a sort of preadaptation to this type of stressor (McDonnell and Hahs, 2015; Borden and Flory, 2021). This supports the proposed framework of "Anthropogenically induced adaptation to invade" (Hufbauer et al., 2012), where the authors suggested that species exposed to human-altered habitats and anthropogenic stressors in the native range become adapted to cope with them and this confers advantages in



**Fig. 4.** Not significant relationship between grey squirrel density (standardized) and FGMs in different urban types. The lines represent the predicted relationship in rural (dotted line), suburban (dashed line) and urban (solid line) sites. Shaded areas represent the 95 % CIs.

similarly-altered habitats in the novel range.

## 5. Conclusions

Our parallel (within-species) study highlighted that native red squirrels and invasive grey squirrels responded differently to similar challenges posed by the synergic effect of urbanisation and intraspecific competition. Despite the limitations of the study, such as the need of a more precise quantification of sources of human-induced stressors, we showed that red squirrels' physiological stress response varied in relation to levels of intraspecific competition potentially depending on differential responsiveness of the HPA axis in face of stressors in suburban sites respect to rural ones. Conversely, grey squirrels' GC response was not affected by population density or urban type. This suggests, that the invasive grey squirrels might be preadapted to cope with these challenges (sensu Hufbauer et al., 2012) and thrive in urbanised areas, ultimately resulting in higher competitive ability and enhanced invasive potential, all facets which can exacerbate their success, especially under the future global change scenario.

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## CRediT authorship contribution statement

**Francesca Santicchia:** Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Claudia Tranquillo:** Conceptualization, Data curation,

Methodology, Writing – original draft, Writing – review & editing. **Lucas A. Wauters:** Conceptualization, Data curation, Methodology, Supervision, Writing – review & editing. **Rupert Palme:** Conceptualization, Formal analysis, Methodology, Writing – review & editing. **Mattia Panzeri:** Data curation, Writing – review & editing. **Damiano Pretoni:** Formal analysis, Supervision, Writing – review & editing. **Francesco Bisi:** Writing – review & editing, Formal analysis. **Adriano Martinoli:** Conceptualization, Methodology, Supervision, Writing – review & editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Dataset is available at <https://doi.org/10.5281/zenodo.10715546>.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2024.171336>.

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