

1 **Stress in biological invasions: introduced invasive grey squirrels increase physiological stress in native**  
2 **Eurasian red squirrels**

3

4 **Running headline:** Invasive species increases stress in red squirrels

5

6 Santicchia Francesca<sup>a\*</sup>, Dantzer Ben<sup>b, c</sup>, van Kesteren Freya<sup>b</sup>, Palme Rupert<sup>d</sup>, Martinoli Adriano<sup>a</sup>, Ferrari  
7 Nicola<sup>e</sup>, Wauters Lucas Armand<sup>a, f</sup>

8 a Environment Analysis and Management Unit - Guido Tosi Research Group - Department of Theoretical and Applied Sciences,  
9 Università degli Studi dell'Insubria, Via J. H. Dunant 3, 21100 Varese, Italy

10 b Department of Psychology, University of Michigan, 530 Church Street, Ann Arbor, MI 48109, USA

11 c Department of Ecology and Evolutionary Biology, University of Michigan, 830 North University, Ann Arbor, MI 48109, USA

12 d Unit of Physiology, Pathophysiology and Experimental Endocrinology, Department of Biomedical Sciences, University of Veterinary  
13 Medicine Vienna, Veterinärplatz 1, 1210 Vienna, Austria

14 e Department of Veterinary Sciences and Public Health, Università degli Studi di Milano, Via Celoria 10, 20133 Milan, Italy

15 f Department of Biology, Evolutionary Ecology Group, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium

16

17 \*Corresponding author (Santicchia F.): Department of Theoretical and Applied Sciences, Università degli  
18 Studi dell'Insubria, via J.H. Dunant, 3 - 21100 - Varese. Italy. Telephone: (+39) 0332 421538. email:

19 [f.santicchia@uninsubria.it](mailto:f.santicchia@uninsubria.it)

20

21

22

23

24 **Abstract**

- 25       **1.** Invasive alien species can cause extinction of native species through processes including predation,  
26 interspecific competition for resources, or disease-mediated competition. Increases in stress  
27 hormones in vertebrates may be associated with these processes and contribute to the decline in  
28 survival or reproduction of the native species.
- 29       **2.** Eurasian red squirrels (*Sciurus vulgaris*) have gone extinct across much of the British Isles and parts  
30 of Northern Italy following the introduction of North American invasive grey squirrels (*Sciurus*  
31 *carolinensis*). We extracted glucocorticoid metabolites from faecal samples to measure whether the  
32 presence of the invasive species causes an increase in physiological stress in individuals of the  
33 native species.
- 34       **3.** We show that native red squirrels in seven sites where they co-occurred with invasive grey squirrels  
35 had glucocorticoid concentrations that were three times higher than those in five sites without the  
36 invasive species. Moreover, in a longitudinal study, stress hormones in native red squirrels increased  
37 after colonisation by grey squirrels. When we experimentally reduced the abundance of the invasive  
38 grey squirrels, the concentration of faecal glucocorticoid metabolites in co-occurring red squirrels  
39 decreased significantly between pre- and post-removal periods.
- 40       **4.** Hence, we found that the invasive species acts as a stressor which significantly increases the  
41 concentrations of glucocorticoids in the native species.
- 42       **5.** Given that sustained elevations in glucocorticoids could reduce body growth and reproductive rate,  
43 our results are consistent with previous studies where the co-occurrence of the invasive grey  
44 squirrel was associated with smaller size and lower reproductive output in red squirrels.

45  
46 **Keywords:** biological invasions impact, glucocorticoids, interspecific competition, invasive alien species,  
47 physiological stress, removal experiment, *Sciurus carolinensis*, *Sciurus vulgaris*

48

49

50

## 51 **1 Introduction**

52           The introduction of non-native species that subsequently become invasive (invasive alien species)  
53 can cause large economic losses and affect human activities and health (Simberloff et al., 2013). Moreover,  
54 invasive species represent a threat to native species worldwide through different ecological processes  
55 (Keller, Geist, Jeschke, & Kühn 2011), such as interspecific competition (e.g. Cadi & Joly, 2003; Gurnell,  
56 Wauters, Lurz, & Tosi 2004), predation (Berger, Wikelski, Romero, Kalko, & Rödl 2007; Banks & Dickman,  
57 2007), transmission of infectious diseases (Daszak, Cunningham, & Hyatt 2000), and even changes in  
58 ecosystem functioning (Strayer, 2012). Such detrimental effects can lead to extinction of native species and  
59 consequent loss of biodiversity (Keller et al., 2011). For example, the introduction of alien species in a new  
60 environment can produce high levels of niche overlap with ecologically similar native species, and  
61 competition for key resources (Glen & Dickman, 2008; Mazzamuto, Bisi, Wauters, Preatoni, & Martinoli  
62 2017), that can ultimately result in exclusion competition and extinction of the native species (Mooney &  
63 Cleland, 2001).

64           In vertebrates, interspecific interactions may also impact populations through sublethal individual-  
65 level effects (Boronow & Langkilde, 2010; Anson, Dickman, Boonstra, & Jessop 2013), that cause an increase  
66 in physiological stress (acute or baseline stress). In fact, vertebrates have a suite of behavioural, hormonal  
67 and physiological mechanisms to cope with harmful environmental stimuli (stressors, Romero, 2004). The  
68 two most important physiological responses are the stimulation of the sympathetic nervous system  
69 (resulting in the release of catecholamines) and the activation of the hypothalamic-pituitary-adrenal (HPA)  
70 axis, resulting in the secretion of glucocorticoids (GCs), lasting several minutes to hours, which helps to  
71 restore homeostasis (Sapolsky, Romero, & Munck 2000). However, while the effects of short-term elevated  
72 GC concentrations (acute stress) can help an individual to escape from life-threatening situations (Wingfield  
73 et al. 1998), activation of the HPA axis and elevated GC concentrations over a longer period of time (several  
74 weeks-months) may have negative effects on body condition, survival or reproductive output (Blas,  
75 Bortolotti, Tella, Baos, & Marchant 2007; Sheriff, Krebs, & Boonstra 2009; Narayan, Jessop, & Hero 2015;

76 Jessop, Anson, Narayan, & Lockwood 2015). If the stressor persists and GCs remain elevated, alterations of  
77 behaviour and/or energy balance, inhibition of growth and/or reproduction (Cabezas, Blas, Marchant, &  
78 Moreno 2007; Sheriff et al., 2009), increase in blood glucose levels, suppression of digestion (Caso, Leza, &  
79 Menchen 2008) and suppression of immunity and the inflammatory response (Romero, 2004; St. Juliana,  
80 Khokhlova, Wielebnowski, Kotler, & Krasnov 2014) can be observed. Competitive food resource exploitation  
81 (Chase, Flynn, & Todgham 2016), parasite-mediated competition (St. Juliana et al., 2014), introduction of  
82 (alien) predators (Berger et al., 2007; Anson et al., 2013), and/or invasive competitors (Boronow &  
83 Langkilde, 2010; Narayan et al., 2015) are all documented cases of direct and/or indirect interspecific  
84 interactions, that show, through different mechanisms, how negative stimuli lead to increased physiological  
85 stress in at least one of the species involved.

86 In wild mammals, baseline GC concentrations can be estimated using faecal glucocorticoid  
87 metabolites, which represent an integrated measure of plasma GCs (FGM concentrations; e.g. Millspaugh &  
88 Washburn, 2004; Sheriff, Dantzer, Delehanty, Palme, & Boonstra 2011; Dickens & Romero, 2013). Hence,  
89 measuring these hormones (stress response) in individuals of a native species in situations with and without  
90 the invasive species (the environmental stressor) can help us understand if and how the stressor affects  
91 animals in native species' populations (Dantzer, Fletcher, Boonstra, & Sheriff 2014).

92 Although there are many studies on the impact of invasive species on native species, only a few  
93 have used glucocorticoid metabolites (before and after the stressor) to investigate the degree of  
94 physiological impact of invasive predators on native species (frogs, *Platymantis vitiana*, Narayan, Cockrem,  
95 & Hero 2013; Narayan et al., 2015; iguanas or lizards, *Amblyrhynchus cristatus*, Berger et al., 2007;  
96 *Urosaurus ornatus*, Graham, Freidenfelds, McCormick, & Langkilde 2012; *Varanus varius*, Anson et al., 2013;  
97 Jessop et al., 2015).

98 Here, we tested the hypothesis that the presence of an invasive species causes an increase in  
99 physiological stress in individuals of a native species. We use the well-known case of competitive  
100 replacement of the Eurasian red squirrel (*Sciurus vulgaris*) by the introduced invasive North American grey  
101 squirrel (*Sciurus carolinensis*) in Europe to explore effects of stress at the individual level using faecal

102 glucocorticoid metabolites (FGM; Sheriff et al., 2011; Dantzer et al., 2014; Dantzer et al., 2016; Haigh,  
103 Butler, O’Riordan, & Palme 2017). Tree squirrels are often successful invaders (Bertolino, 2009; Di Febbraro,  
104 Martinoli, Russo, Preatoni, & Bertolino 2016), and the rapid spread of the invasive grey squirrel and  
105 subsequent decline and widespread extinction of the native red squirrel on the British Isles and in parts of  
106 Italy has been documented in many studies. Both species can produce one to two litters per year between  
107 February and June-July (weaned offspring from April to September) with grey squirrels starting earlier than  
108 red squirrels in most years (Gurnell et al., 2004). Dispersal of juveniles or subadults occurs mainly in autumn  
109 (September-November). The two species have similar space use and activity patterns, and compete for food  
110 resources, resulting in smaller body size, and reduced female reproduction and juvenile recruitment in red  
111 squirrels (Wauters, Tosi & Gurnell 2002a; Wauters, Gurnell, Martinoli, & Tosi 2002b; Gurnell et al., 2004),  
112 ultimately causing a decrease in population size and (local) extinction (Bertolino, Montezemolo, Preatoni,  
113 Wauters, & Martinoli 2014). Food competition is most intense between December and April (Wauters,  
114 Gurnell, Martinoli, & Tosi 2001; Wauters et al., 2002a). On the British Isles, a squirrel pox virus results in  
115 disease-mediated competition, with grey squirrels acting as a reservoir and transmitting the virus to red  
116 squirrels, for which, in most cases, the infection is lethal (Mc Innes et al., 2013; White et al., 2016). In Italy  
117 the virus is not present (Romeo et al., 2018).

118         We first compared FGM concentrations in individual red squirrels co-occurring for more than two  
119 years with grey squirrels (red-grey sites), with FGM concentrations of squirrels in sites not colonised by the  
120 invasive species (red-only sites), and predicted that FGM concentrations in red squirrels will be higher in  
121 red-grey than in red-only sites. However, since the correlative nature of this approach makes it difficult to  
122 isolate the invader as the causal factor driving observed differences (Graham, Freidenfelds, Thawley,  
123 Robbins, & Langkilde 2017), we also tested the direct impact of interactions between invasive and native  
124 species. We did this in two ways: (1) monitoring changes in FGM concentrations of red squirrels in two  
125 study sites that were colonised by the invader during this study (measuring FGM concentrations before and  
126 after colonisation) and comparing them with variation in FGM that occurred over the same time-period in  
127 non-colonised red squirrel populations; and (2) removing grey squirrels in red-grey sites over a period of six

128 months, and concomitantly monitoring changes in FGM concentrations in native red squirrels. If the invader  
129 causes an increase in FGM concentrations in the native species (i.e. the presence of grey squirrels is the  
130 driving factor), we predicted that: FGM concentrations will be higher in red squirrels after the two study  
131 sites were colonised than before the colonisation by the invasive species (experiment 1); and FGM  
132 concentrations in syntopic red squirrels (red-grey sites) will decrease after the removal of the grey squirrels  
133 (experiment 2).

134

## 135 **2 Materials and methods**

136         2.1 *Study sites.* — We trapped Eurasian red squirrels in five study sites without grey squirrels  
137 (historically red-only sites) in Lombardy, North Italy (Supporting Information, Table S1). Two of these sites  
138 (Vanzago, Castelbarco) were colonised by the invasive species during the study; hence they were used as  
139 red-only sites before colonisation (respectively before March 2015 and November 2014), and as red-grey  
140 sites afterwards. We trapped both squirrel species (red-grey sites) in seven study sites in Lombardy and  
141 Piedmont, Northern Italy (Supporting Information, Table S1). All red-grey sites and the two red-only sites  
142 mentioned above (Vanzago, Castelbarco) are lowland mixed deciduous woodlands in the Po plain, while the  
143 other three red-only sites (Bormio, Cancano, Valfurva) are subalpine conifer forests in the Central Italian  
144 Alps (1620-2150 m elevation).

145         2.2 *Live-trapping, handling and faecal sample collection.* — We trapped squirrels in all sites during  
146 trapping sessions that lasted four or five days each (sites and trapping dates listed in Supporting  
147 Information, Table S1). We used Tomahawk traps (model 202, Tomahawk Live Trap, WI, USA) with a fine  
148 mesh added underneath traps to prevent contamination between urine and faeces. We checked traps two  
149 to three times a day. Each trapped squirrel was individually marked using numbered metal ear-tags (type  
150 1003 S, National Band and Tag, Newport, KY, USA). We weighed squirrels to the nearest 5 g using a spring-  
151 balance (Pesola AG, Baar, Switzerland) and measured the length of the right hind foot (without nail, 0.5  
152 mm) with a ruler (Wauters et al., 2007). A female's reproductive status was defined as non-breeding

153 (anoestrous, vulva small, no longitudinal opening, not lactating), post-oestrous and pregnant (vulva partly  
154 or strongly swollen with longitudinal opening, enlarged belly during late pregnancy), or lactating (nipples  
155 large, milk excretion can be stimulated). We recorded reproductive condition of males (testes size and  
156 position) as non-breeding (testes abdominal or semi-scrotal and scrotum small) or breeding (testes scrotal  
157 and scrotum large). We used the minimum number of animals known to be alive from trapping and  
158 observations (MNA, see also Wauters et al., 2008) during each trapping session as an estimate of population  
159 size and squirrel density. Previous studies showed that MNA estimates were strongly correlated with  
160 estimates from capture-mark-recapture models (e.g. MARK, Wauters et al., 2008).

161 To test effects of removal of the invasive species on FGM concentrations in co-occurring native red  
162 squirrels we analysed samples from four study sites (Vanzago, Lambro, Passatempo, Castelbarco; see Table  
163 1) where all grey squirrels that were trapped over three subsequent periods (one period every six to ten  
164 weeks between November 2015 and May 2016) were removed. If the number of grey squirrels removed in  
165 period  $i$  is  $m_i$ , then  $m_1 + m_2 + m_3 = m_{\text{tot}}$  the total number of grey squirrels removed over the entire  
166 experiment. Red squirrel faecal samples collected in period 1 corresponded with high grey squirrel densities  
167 ( $m_{\text{tot}}$ ), those collected in period 2 with intermediate densities of grey squirrels ( $m_2 + m_3$ ), and those collected  
168 in period 3 with the lowest grey squirrel densities ( $m_3$ ) (see Table 1). We used  $m_{\text{tot}}$  to estimate the minimum  
169 number of animals known to be alive (MNA) of the invasive species, considering that no immigration or  
170 recruitment of juveniles occurred during the removal period. Trappability of grey squirrels in these relatively  
171 small woodlands was high (our unpublished data) and visual observations of individuals of this diurnal and  
172 conspicuous species indicated that few animals remained at the end of the removal experiment. Removal of  
173 grey squirrels was part of the LIFE09 NAT/IT/000095 EC-SQUARE project: animals were euthanized by CO<sub>2</sub>  
174 inhalation, following the EC and AVMA guidelines (Close et al., 1996, 1997; Leary et al., 2013).

175 After capture and handling, faecal samples were collected from underneath the traps using forceps,  
176 and placed individually into 1.5 mL vials (Dantzer et al., 2010) and the fine mesh and ground under the traps  
177 were cleaned to remove any remaining faecal material. We only used faecal samples from red squirrels that  
178 had not previously been trapped or handled within 72 h prior to capture to minimize effects of capture

179 stress on FGM concentrations (Dantzer et al., 2016). Faecal samples of grey squirrels were also collected  
180 (data analysed elsewhere). We obtained multiple samples in different seasons/periods from most squirrels,  
181 but not all captured individuals produced faeces within a given trapping session. Each faecal sample was  
182 classified as being taken in the morning (10.00 – 13.00h) or in the afternoon (15.00 – 18.00h) to account for  
183 potential variation in FGM concentration over the 24 h cycle (Millspaugh & Washburn, 2004). We placed  
184 faecal samples into an insulated bag with ice packs while in the field, and samples were stored at -20 °C  
185 within 3-4 h after collection, which should not cause temperature-induced changes in faecal hormone  
186 metabolite concentrations (Dantzer et al., 2010). Trapping and handling of squirrels complied with the  
187 current laws on animal research in Italy, and was carried out with permission of the Region of Lombardy  
188 (Decree n. 11190 of 29/11/2013). All of these procedures abided by ASM guidelines (Sikes & Gannon,  
189 2011).

190         *2.3 Extraction of hormone metabolites and enzyme immunoassay.* —Methods of extraction of FGM  
191 and enzyme immunoassay validation for red squirrels and grey squirrels are described in detail elsewhere  
192 (Bosson, Palme, & Boonstra 2013; Dantzer et al., 2016). Briefly, samples were lyophilized overnight, ground  
193 up under liquid nitrogen and weighed to 0.05 g ( $\pm$  0.004 g), and extracted using 80% methanol, shaking at  
194 1500 r.p.m on a multivortex for 30 min, centrifuging at 2500 g for 15 min, and aspirating the supernatant.  
195 Supernatants were diluted in assay buffer and assayed using a 5 $\alpha$ -pregnane-3 $\beta$ , 11 $\beta$ , 21-triol-20-one  
196 enzyme-immunoassay (EIA) which detects glucocorticoid metabolites with a 5 $\alpha$ -3 $\beta$ , 11 $\beta$ -diol structure (for  
197 cross-reactivity see Touma, Sachser, Möstl, & Palme 2003) to measure FGM concentrations (ng/g dry faeces,  
198 Dantzer et al., 2010, 2016). Samples were analysed in duplicate. We assayed 193 faecal samples on a total  
199 of 22 EIA plates. Pools of grey squirrel faecal extracts were used as intra-assay controls at dilutions of 1:50  
200 (~30% binding) and 1:400 (~70% binding). Average intra-assay coefficients of variation (CVs) were 9.5% and  
201 9.4% respectively for pools diluted 1:50 and 1:400. Inter-assay CVs were estimated from standards of known  
202 concentration with a high (n = 22 plates, 12.3% binding) and low (n = 22 plates, 81.6% binding)  
203 concentration that had inter-assay CVs of 16.1% and 9.3%, respectively. To further increase our dataset, we



204 also included 125 samples assayed in a previous study from the same red-only study sites and analysed  
205 using the same methodology and in the same laboratory (see Table 1 in Dantzer et al., 2016).

206         2.4 *Statistical analyses.* —All analyses were performed in R version 3.3.3 (R Development Core  
207 Team, 2017) using the lme4 package (version 1.1-12, Bates, Mächler, Bolker, & Walker 2015). For each of the  
208 models described, we conducted linear mixed-effects models with FGM concentrations (transformed using  
209 the natural logarithm, ln of ng/g dry faeces) as the dependent variable and squirrel identity (ID) nested in  
210 study site as a random intercept term to account for repeated samples on the same individuals. Sex and  
211 reproductive condition nested in sex were added as fixed effects to account for potential changes in FGM  
212 concentrations with reproductive activity in males and females (Goymann, 2012; Dantzer et al., 2016).  
213 Residuals were visually inspected to verify the assumptions of normality and homoscedasticity (Zuur, Ieno,  
214 & Elphick 2010). Where necessary, we assessed significance of pair-wise comparisons using differences of  
215 least square means (DLSM) with Satterthwaite approximations to degrees of freedom in R package lmerTest  
216 (version 2.0-33, Kuznetsova, Brockhoff, & Christensen 2016). All full models (see below) contained  
217 interactions. When interactions were not significant and including them did not improve model fit ( $\Delta$ BIC  
218 between model with and without interactions  $> 2.0$ ; Schwarz & Gideon, 1978), they were removed from the  
219 models to obtain reliable parameter estimates for the fixed effects.

220         2.4.1 *Comparisons between red-only vs. red-grey sites.* —We first assessed the direct effects of  
221 invasion status (sites with only red squirrels vs. sites with both red and grey squirrels) including also season  
222 (winter [December to March], spring-summer [April to August], or autumn [September to November]) and  
223 daytime (animal sampled in morning or afternoon) as factors, and body mass as a continuous variable. We  
224 also tested the interaction between sex and invasion status to explore whether the effect of the presence of  
225 grey squirrels on FGM concentrations in red squirrels differed between the sexes. Study site nested in  
226 invasion status was added as random intercept to account for potential differences in FGM concentrations  
227 between sites. Since sites with only red squirrels occurred in coniferous as well as in deciduous forests, we  
228 also explored the effect of habitat type on FGM concentrations (models and results in Supporting  
229 Information).

230           2.4.2 *Effect of colonisation by grey squirrels.* —We had two study sites (Vanzago and Castelbarco, 45  
231 km apart) that were colonised by the invasive species during the study, and we explored whether FGM  
232 concentrations in red squirrels changed after the colonisation (samples collected between 6-10 months  
233 after the pre-grey sample collection, see also Table S1). We also used data from three non-colonised study  
234 sites (Valfurva, Bormio and Cancano, see Table S1) that were monitored over the same time-period as  
235 control data to test if differences in FGM were simply time-related. We investigated the effects of  
236 colonisation (yes or no), time-period (before vs. after, the dates of colonisation), and their interaction, and  
237 included sex, reproductive condition nested in sex, daytime, and body mass in the full model. Study site  
238 nested in colonisation status was added as a random intercept to account for potential differences in FGM  
239 concentrations between sites.

240           2.4.3 *Removal experiment.* —We assessed the effects of grey squirrel removal considering  
241 treatment period (initial sampling when no grey squirrels had been removed yet = period 1; intermediate  
242 sampling, with a low proportion of grey squirrels removed = period 2; final sampling with a higher  
243 proportion of grey squirrels removed = period 3; see methods and Table 1), study site, sex, reproductive  
244 condition nested in sex and daytime as factors, and body mass as an explanatory variable, and two factor  
245 interactions between the period by study site, and period by sex, to explore whether the removal of grey  
246 squirrels would result in a significant decrease in FGM concentrations in sympatric red squirrels. In order to  
247 account for the fact that the observed differences in FGM concentrations between removal periods were  
248 influenced by seasonal variation, we also explored a model in which we used season as a fixed effect instead  
249 of removal period (both factors could not be fitted in a single model because of problems with collinearity,  
250 Zuur et al., 2010). We used the difference in BIC value to test which model best fitted the data (Schwarz &  
251 Gideon, 1978).

252

### 253 **3 Results**

254           3.1 FGM concentrations of red squirrels in red-only vs. red-grey sites. — FGM concentrations in  
255 native red squirrels (260 samples from 166 different animals) were three times higher in sites that  
256 contained invasive grey squirrels (n samples = 135, mean  $\pm$  SD = 78133  $\pm$  61074 ng/g dry faeces) than in  
257 sites that only contained native red squirrels (n = 125, 24890  $\pm$  20566 ng/g dry faeces; estimate of the  
258 invasion status effect on ln FGM: 0.97  $\pm$  0.20;  $t_8 = 4.85$ ;  $p = 0.0012$ ). There was no evidence that the  
259 presence of invasive grey squirrels impacted the FGM concentrations of the two sexes differently (sex by  
260 invasion status interaction:  $t_{165} = 0.98$ ;  $p = 0.33$ ; Fig. 1). FGM concentrations did not differ between the sexes  
261 (estimate males against females on ln FGM -0.04  $\pm$  0.25;  $t_{210} = 0.18$ ;  $p = 0.86$ ), and were not affected by  
262 reproductive condition (males, breeding compared to non-breeding: 0.06  $\pm$  0.16;  $t_{247} = 0.40$ ;  $p = 0.69$ ;  
263 females, pregnant compared to lactating: -0.12  $\pm$  0.23;  $t_{249} = 0.52$ ;  $p = 0.60$ ; pregnant compared to non-  
264 breeding: -0.34  $\pm$  0.23;  $t_{226} = 1.50$ ;  $p = 0.14$ ). Native red squirrels had lower FGM concentrations in spring-  
265 summer than in autumn (spring-summer against autumn estimate: -0.33  $\pm$  0.14;  $t_{220} = 2.39$ ;  $p = 0.018$ ; all  
266 other comparisons  $p > 0.05$ ). FGM concentrations did not vary significantly with daytime (hour of faecal  
267 sample collection:  $t_{223} = 0.76$ ;  $p = 0.45$ ) or squirrel body mass ( $t_{226} = 0.30$ ;  $p = 0.76$ ).

268           3.2 Effect of colonisation by grey squirrels. — FGM concentrations in red squirrels increased with  
269 time-period (before versus after the dates of colonisation) and with colonisation status (colonised versus  
270 not colonised), and there was a significant time by colonisation status interaction (Supporting Information,  
271 Table S2). Red squirrels from the three sites without colonisation did not show a significant increase in FGM  
272 concentrations between time-periods (difference in ln FGM estimate 0.21  $\pm$  0.15;  $t_{152} = 1.27$ ;  $p = 0.21$ ; Fig.  
273 2). In contrast, red squirrels in the two sites colonised by the invasive species during our study had a  
274 significant increase in FGM from pre- to post-colonisation time-period (difference in ln FGM estimate 0.74  $\pm$   
275 0.18;  $t_{173} = 3.93$ ;  $p = 0.0004$ ; Fig. 2).

276           In this dataset there was no statistically significant effect of sex, reproductive condition nested in  
277 sex, daytime, or body mass on FGM concentrations (Supporting Information, Table S2).

278           3.3 Removal experiment. — After the removal of invasive squirrels, FGM concentrations in co-  
279 occurring red squirrels decreased significantly in periods 2 and 3 compared to FGM concentrations at the

280 start of the experiment (period 1, Fig. 3 and Table 1). Interactions between period and study site and  
281 between period and sex were not significant, and were removed from the model (Supporting Information,  
282 Table S3). Daytime and body mass also did not influence FGM concentrations and were removed during  
283 stepwise model selection (Supporting Information, Table S3). FGM concentrations in red squirrels decreased  
284 significantly after the first removal session, but there was no further, significant, decrease after the second  
285 removal session (period 2-period 1 estimate  $-0.58 \pm 0.23$ ,  $t_{121} = 2.53$ ;  $p = 0.012$ ; period 3 - period 1 estimate  
286  $-0.50 \pm 0.23$ ,  $t_{121} = 2.18$ ;  $p = 0.031$ ; period 2 - period 3 estimate  $0.08 \pm 0.21$ ;  $t_{121} = 0.35$ ;  $p = 0.73$ ).

287 There was no difference between FGM concentrations of males and females, but in this dataset  
288 breeding males had higher FGM concentrations than non-breeding males (Supporting Information, Table  
289 S3). Variation between study sites was nearly significant only for two sites, with overall lower FGM  
290 concentrations in Lambro than in Passatempo (Table 1; difference in ln FGM estimate  $-0.63 \pm 0.32$ ;  $t_{121} =$   
291  $1.89$ ;  $p = 0.06$ ).

292 The selected model which included 'period' had a lower BIC value than the model which included  
293 'season' ( $\Delta\text{BIC} = 4.53$ ), and differences in FGM concentrations between seasons were not statistically  
294 significant (all  $p > 0.10$ ).

295

## 296 **4 Discussion**

297 We assessed the impact of an invasive alien competitor on faecal glucocorticoid metabolite  
298 concentrations in individuals of a native species. We predicted that the threat posed by invasive grey  
299 squirrels would increase FGM concentrations in co-occurring native red squirrels. FGM concentrations were  
300 significantly higher in sites invaded by grey squirrels than in red-only sites, and in the two areas colonised by  
301 grey squirrels during our study, FGM concentrations in the native red squirrels increased with respect to  
302 pre-colonisation concentrations. Both results support our hypothesis and suggest that the invader is the  
303 causal factor driving observed differences in FGM concentrations (see also Graham et al., 2017). This was  
304 further supported by our grey squirrel removal experiment. Removal of grey squirrels resulted in a  
305 significant decrease in glucocorticoids in co-occurring red squirrels over a two month interval, taking into

306 account seasonal variation in glucocorticoid concentrations and changes in reproductive condition in both  
307 males and females (see also Boonstra et al., 2001; Dantzer et al., 2010). The reduction in glucocorticoids  
308 was more pronounced in two sites: these were the two smallest woodlands that, at the start of the  
309 experiment, had the highest grey squirrel densities (Passatempo and Lambro, Table 1) and where red  
310 squirrel home ranges were more strongly overlapped by individuals of the invasive species (Wauters L.A.  
311 unpublished data 2016).

312         4.1 Comparisons between red-only vs. red-grey sites. — Native red squirrels had higher FGM  
313 concentrations in areas where they co-occurred with the invasive grey squirrels than did those in areas  
314 without the introduced competitor. This pattern is supported by previous studies. Stress levels of native  
315 lizards (*Sceloporus undulatus*) were higher in sites with a long history of co-occurrence with invasive fire  
316 ants (*Solenopsis invicta*) than in sites without the invader (Graham et al., 2012). Similar results were found  
317 in a system of a native lizard (the lace monitor, *Varanus varius*) and an introduced alien predator (red fox,  
318 *Vulpes vulpes*) in Australia. In habitats with high fox densities, lizards produced a significantly greater basal  
319 and capture stress-induced corticosterone response compared to individuals in low-fox density habitat,  
320 suggesting competition with red foxes, perhaps via nutritional stress and increased hypersensitivity of the  
321 adrenocortical axis in lizards (Jessop et al., 2015). So far, it is not clear whether such responses mediate  
322 lizard fitness. Another study examined whether introduced foxes caused elevated glucocorticoid (GC)  
323 hormone concentrations (predator stress hypothesis) on a native predator (*Varanus varius*) and a prey  
324 species (ringtail possum, *Pseudocheirus peregrinus*). No differences were found in glucocorticoid  
325 concentrations or in haemoparasite loads between areas with and without fox control in either of the two  
326 native species (Anson et al., 2013). These types of observational studies reveal the need for experimental  
327 manipulations to investigate the potential causality of the relationship between the presence of invasive  
328 species and increased stress in native species.

329         4.2 Pre - post colonisation. —The colonisation by grey squirrels in two of our study sites can be  
330 considered as a natural experiment of introducing an invasive competitor. The strong increase in FGM  
331 concentrations we detected following colonisation of grey squirrels suggests that grey squirrels are causing

332 the higher levels of physiological stress detected in field samples from the native species. Extra support to  
333 this interpretation is given by the fact that no such increase occurred in red squirrels monitored over the  
334 same time-period in sites that were not colonised. A similar pattern was documented in the only study we  
335 found which used experimental approaches to investigate changes in stress levels in the native and  
336 endangered Fijian ground frog (*Platymantis vitiana*) caused by the presence of introduced cane toads  
337 (*Rhinella marina*) that can prey on the native frogs (Narayan et al., 2013, 2015). Artificial exposure or  
338 introduction of the alien predator resulted in an increase in urinary corticosterone metabolites (Narayan et  
339 al., 2013), and subsequently reduced reproductive success (fewer eggs laid in enclosures which also  
340 contained alien predators than in no-predator enclosures) in the endemic species (Narayan et al., 2015).

341         4.3 *The removal experiment.* —A next step is manipulation of the presence/density of the invasive  
342 species that acts as stressor. Previous studies used an approach where they compared areas without  
343 predator manipulation to areas with predator manipulation (e.g. Anson et al., 2013; Jessop et al., 2015). We  
344 used a new approach: removal over time comparing the observed changes in faecal glucocorticoid  
345 metabolites within populations using sites as independent replicates. In other words the FGM  
346 concentrations in individual red squirrels within a given population/study site are measured under high  
347 pressure of grey squirrels (no removal) and compared with lower pressure of the invasive species (after  
348 removal). This experiment may be confounded by temporal effects on glucocorticoids, such as the season  
349 effect found in the data comparing red-only with red-grey sites, and the time-span from the beginning of  
350 the removal and the measurements. However, our models showed that seasonal variation in FGM measures  
351 was not statistically significant in this dataset and that models with removal period better fitted the data  
352 than models including a season effect. Moreover, the strongest decrease in FGM concentrations of red  
353 squirrels after removal of grey squirrels occurred between period 1 and period 2. FGM data for period 1  
354 were gathered in autumn and winter, and all data for period 2 were taken in winter, and in the large dataset  
355 comparing red-only with red-grey sites there was no significant difference between winter and autumn  
356 levels of glucocorticoids. In a dataset of red squirrels in areas without the invasive species, glucocorticoids  
357 were even higher in winter than in autumn (Dantzer et al., 2016). Hence, these data support our conclusion

358 that the observed decrease in faecal glucocorticoid metabolites in red squirrels was indeed caused by a  
359 reduction of the density of invasive grey squirrels.

360 We found that FGM concentrations in native red squirrels decreased significantly (between the first  
361 and second period) in relation to invasive species removal. FGM levels also remained lower in the third  
362 period (see Fig. 3), but a further decrease with respect to period 2 was observed only in the two smallest  
363 study sites, where, as mentioned above, grey squirrels occurred at the highest densities when the  
364 experiment started (see Table 1). This suggests that the impact of the invasive competitor on individual red  
365 squirrels' FGM concentrations is probably related to the degree of home range (or core-area) overlap, and  
366 the resulting differences in food competition and pilfering of red squirrel caches by overlapping greys  
367 (Wauters & Gurnell, 1999; Wauters, Tosi, & Gurnell 2002a). In fact, in areas of co-occurrence there is no  
368 niche partitioning between the two species (Wauters et al., 2002b), in marked contrast with the habitat  
369 specialization and niche differentiation observed between co-evolved North American red squirrels and  
370 grey squirrels in mixed forests in their natural range (Riege, 1991). Although shifts in diet may be a possible  
371 driver of changes in FGM concentration (Dantzer, McAdam, Palme, Boutin, & Boonstra 2011; Goymann  
372 2012), we believe it is unlikely that this is the main driver in our study for several reasons. First, we  
373 controlled for habitat effects and still found higher FGM concentrations in red squirrels in deciduous woods  
374 with grey squirrels than in similar habitats without the invasive species. Second, the experimental  
375 approaches (colonization and removal) were within-site comparisons. Third, we believe it is unlikely that  
376 within a study area, FGM increases in red squirrels were driven by red squirrels foraging on poorer quality  
377 food items due to food competition with grey squirrels because grey squirrel densities were relatively low in  
378 our study sites. Furthermore, previous studies showed high foraging niche overlap between the two species  
379 in red-grey sites which, however, did not result in diet shifts and/or lower daily energy-intake in red squirrels  
380 during most of the year (see Wauters et al. 2001, 2002b). Finally, it is unlikely that increased hormonal  
381 stress in native red squirrels is caused by direct (aggressive) interactions with grey squirrels, since  
382 interspecific interactions are rare and much less common than intraspecific aggression (Wauters & Gurnell,  
383 1999).

384           4.4 *Physiological relevance and fitness consequences.* —Here, we documented a 3-fold increase in  
385 FGMs in red squirrels in red-grey sites compared to red-only sites, a 2-fold increase in sites that were  
386 colonised by the invasive species and a 1.5-fold decrease when grey squirrels were removed. Although it  
387 may be difficult to assess the direct physiological relevance of increased FGM (Dickens & Romero, 2013),  
388 and comparisons between studies are complicated due to the use of different experimental protocols (e.g.  
389 Fanson et al., 2017) and other variables that may affect FGM concentrations (Dantzer et al., 2011;  
390 Goymann, 2012), we believe that the changes in FGMs that we observed are comparable to chronic stress  
391 paradigms found in other studies, and that they may have other effects on life history traits. For example, in  
392 a communally breeding rodent (*Ctenomys sociabilis*) the difference in baseline glucocorticoid levels  
393 between animals kept in less or more stressful situations was about 1.5 to 2-fold (Woodruff, Lacey, Bentley,  
394 & Kriegsfeld 2013). A previous study in wild North American red squirrels (*Tamiasciurus hudsonicus*) showed  
395 that pregnant females have 30% higher FGMs when high population density conditions were simulated, and  
396 that this was associated with a change in offspring postnatal growth rates (Dantzer et al., 2013). Laboratory  
397 studies have found similar effects, and, in laboratory rats, exposure to a variable chronic stress paradigm  
398 caused a ~1.5 fold increase in plasma corticosterone levels (Herman, Adams & Prewitt 1995).

399           To date, few studies have been able to demonstrate the consequences of prolonged elevations of  
400 glucocorticoids caused by invasive species on native species' fitness components (Narayan et al., 2015). Our  
401 study was too short to reveal fitness consequences of the increase in glucocorticoids levels in red squirrels  
402 in areas invaded by the congener. However, our previous studies clearly demonstrated negative effects of  
403 grey squirrels, exacerbated at high densities of the invasive species, on red squirrel body size and  
404 reproductive rate (Wauters, Gurnell, Preatoni, & Tosi 2001; Gurnell et al., 2004). Both these effects are in  
405 agreement with alterations of the hormonal functions of glucocorticoids in regulating of body growth and  
406 reproduction (Cabezas et al., 2007; Sheriff et al., 2009). Therefore, we conclude that invasive grey squirrels  
407 increase FGM concentrations in native red squirrels and this increase in glucocorticoids is likely to have  
408 physiological impacts on red squirrels, such as affecting growth and reproduction (Wauters et al., 2001;  
409 Dantzer et al., 2013; Gurnell, Lurz & Wauters 2015). Moreover, studies on animal models indicate that  
410 chronic stress can reduce immune-efficiency (Sapolsky et al., 2000), and further research will explore



411 whether this mechanism is involved in the probability that naïve red squirrels acquire an alien parasite,  
412 *Strongyloides robustus*, typical of grey squirrels (Romeo et al., 2015).

413 The interaction between invasive and native species is a phenomenon that occurs through many  
414 different mechanisms (e.g. direct competition, parasite-mediated competition, disease transmission,  
415 predation, habitat destruction or alteration) in a wide variety of ecosystems. This study shows that direct  
416 and/or indirect effects of increases in physiological stress in native species caused by the presence of an  
417 invasive species should be considered as one of these mechanisms. Our FGM data suggest that red squirrels  
418 are chronically stressed by invasive grey squirrels. Although there are wild species in which chronic stress  
419 may be an evolutionary response to stressors such as predation pressure, and is not necessarily deleterious,  
420 (see Boonstra, 2013), we believe this is not the case in our study system. Our results indicate that red  
421 squirrels have a physiological stress response to a threat posed by an invasive competitor to which they  
422 show no evolutionary adaptation. Therefore, the observed increase in glucocorticoid concentrations is likely  
423 to have detrimental consequences for red squirrels (Wauters et al., 2001; Gurnell et al., 2004, 2015; Romeo  
424 et al., 2015). We suggest that future studies should assess if and how changes in stress levels are involved in  
425 species interactions and invasion processes and to what extent it has direct or indirect (e.g. by increasing  
426 susceptibility to parasite infections) fitness consequences for the native species.

#### 427 **Acknowledgements**

428 Thanks to Zainab Almusawi for helping in laboratory analysis, Mattia Panzeri and Greta Colombo for  
429 assistance with the fieldwork. We thank Stelvio National Park to allow research in three study areas and to  
430 natural reserve – sic/zps “Bosco WWF di Vanzago” for access to the woodlands and housing facilities. We  
431 are also grateful to the private land owners for access to their estates. Constructive comments by two  
432 anonymous reviewers and by the associate editor greatly helped us to improve the manuscript. This is  
433 paper no. 29 of the ASPER (Alpine Squirrel Population Ecology Research) project. ESI (European Squirrel  
434 Initiative) funded laboratory analyses.

#### 435 **Authors' contributions**

436 This study is part of FS PhD project supervised by AM. FS, LAW and NF designed the study and analyses.  
437 Fieldwork and data collection were done by FS and LAW. FS and FVK carried out laboratory analyses and BD  
438 supplied laboratory space, equipment, and coordinated laboratory analyses. RP produced and supplied  
439 reagents for lab analyses. FS carried out statistical analyses with the contribution of BD and NF. The  
440 manuscript was written by FS and LAW with improvement and editorial input from all other authors. All  
441 authors gave approval for publication.

442

#### 443 **Data accessibility**

444 Data available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.bp8jf84>

#### 445 **References**

- 446 Anson, J. R., Dickman, C. R., Boonstra, R. & Jessop, T. S. (2013). Stress triangle: Do introduced predators  
447 exert indirect costs on native predators and prey? PLoS ONE, 8, e60916. doi:10.1371/journal.pone.0060916
- 448 Banks, P. B. & Dickman, C. R. (2007). Alien predation and the effects of multiple levels of prey naiveté.  
449 Trends in Ecology & Evolution, 22, 229–230. doi:10.1016/j.tree.2007.02.006
- 450 Bates, D., Mächler, M., Bolker, B. & Walker, S. (2015). Fitting linear mixed-effects models using lme4. Journal  
451 of Statistical Software, 67(1). doi:10.18637/jss.v067.i01
- 452 Berger, S., Wikelski, M., Romero, L. M., Kalko, E. K. V. & Rödl, T. (2007). Behavioral and physiological  
453 adjustments to new predators in an endemic island species, the Galápagos marine iguana. Hormones and  
454 Behavior, 52, 653–663. doi:10.1016/j.yhbeh.2007.08.004
- 455 Bertolino, S. (2009). Animal trade and non-indigenous species introduction: the world-wide spread of  
456 squirrels. Diversity and Distributions, 15, 701–708. doi:10.1111/j.1472-4642.2009.00574.x

457 Bertolino, S., Montezemolo, N. C., Preatoni, D. G., Wauters, L. A. & Martinoli, A. (2014). A grey future for  
458 Europe: *Sciurus carolinensis* is replacing native red squirrels in Italy. *Biological Invasions*, 16, 53–62.  
459 doi:10.1007/s10530-013-0502-3

460 Blas, J., Bortolotti, G. R., Tella, J. L., Baos, R. & Marchant, T. A. (2007). Stress response during development  
461 predicts fitness in a wild, long lived vertebrate. *Proceedings of the National Academy of Sciences USA*, 104,  
462 8880–8884. doi:10.1073/pnas.0700232104

463 Boonstra, R. (2013). Reality as the leading cause of stress: rethinking the impact of chronic stress in nature.  
464 *Functional Ecology*, 27, 11–23. doi: 10.1111/1365-2435.12008

465 Boonstra, R., Hik, D., Singleton, G. R., Tinnikov, A. (1998). The impact of predator-induced stress on the  
466 snowshoe hare cycle. *Ecological Monographs*, 68, 371–394. doi:10.1890/0012-  
467 9615(1998)068[0371:TIOPI]2.0.CO;2

468 Boonstra, R., Hubbs, A. H., Lacey, E. A., McColl, C. J. (2001). Seasonal changes in glucocorticoid and  
469 testosterone concentrations in free-living arctic ground squirrels from the boreal forest of the Yukon.  
470 *Canadian Journal of Zoology*, 79, 49-58. doi:10.1139/z00-175

471 Boronow, K. E. & Langkilde, T. (2010). Sublethal effects of invasive fire ant venom on a native lizard. *Journal*  
472 *of Experimental Zoology Part A: Ecological and Integrative Physiology*, 313A, 17–23. doi:10.1002/jez.570

473 Bosson, C. O., Palme, R. & Boonstra, R. (2013). Assessing the impact of live-capture, confinement, and  
474 translocation on stress and fate in eastern gray squirrels. *Journal of Mammalogy*, 94, 1401–1411.  
475 doi:10.1644/13-MAMM-A-046.1

476 Cabezas, S., Blas, J., Marchant, T. A. & Moreno, S. (2007). Physiological stress levels predict survival  
477 probabilities in wild rabbits. *Hormones and Behavior*, 51, 313–320. doi:10.1016/j.yhbeh.2006.11.004

478 Cadi, A. & Joly, P. (2003). Competition for basking places between the endangered European pond turtle  
479 (*Emys orbicularis galloitalica*) and the introduced red-eared slider (*Trachemys scripta elegans*). *Canadian*  
480 *Journal of Zoology*, 81, 1392–1398. doi:10.1139/z03-108

481 Caso, J. R., Leza, J. C. & Menchen, L. (2008). The effects of physical and psychological stress on the  
482 gastrointestinal tract: lessons from animal models. *Current Molecular Medicine*, 8, 299–312.  
483 doi:10.2174/156652408784533751

484 Chase, D. A., Flynn, E. E. & Todgham, A. E. (2016). Survival, growth and stress response of juvenile tidewater  
485 goby, *Eucyclogobius newberryi*, to interspecific competition for food. *Conservation Physiology*, 4, cow013.  
486 doi:10.1093/conphys/cow013

487 Close, B., Banister, K., Baumans, V., Bernoth, E-M., Bromage, N., Bunyan, J., ... Warwick, C. (1996).  
488 Recommendations for euthanasia of experimental animals: Part 1. *Laboratory Animals*. 30, 293–316.  
489 doi:10.1258/002367796780739871

490 Close, B., Banister, K., Baumans, V., Bernoth, E-M., Bromage, N., Bunyan, J., ... Warwick, C. (1997).  
491 Recommendations for euthanasia of experimental animals: Part 2. *Laboratory Animals*. 31, 1–32.  
492 doi:10.1258/002367797780600297

493 Dantzer, B., Fletcher, Q. E., Boonstra, R. & Sheriff, M. J. (2014). Measures of physiological stress: a  
494 transparent or opaque window into the status, management and conservation of species? *Conservation*  
495 *Physiology*, 2, cou023-cou023. doi:10.1093/conphys/cou023

496 Dantzer, B., McAdam, A. G., Palme, R., Boutin, S. & Boonstra, R. (2011). How does diet affect fecal steroid  
497 hormone metabolite concentrations? An experimental examination in red squirrels. *General and*  
498 *Comparative Endocrinology*, 174, 124–131. doi:10.1016/j.ygcen.2011.08.010

499 Dantzer, B., McAdam, A. G., Palme, R., Fletcher, Q. E., Boutin, S., Humphries, M. M. & Boonstra, R. (2010).  
500 Fecal cortisol metabolite levels in free-ranging North American red squirrels: Assay validation and the  
501 effects of reproductive condition. *General and Comparative Endocrinology*, 167, 279–286.  
502 doi:10.1016/j.ygcen.2010.03.024

503 Dantzer, B., Newman, A. E. M., Boonstra, R., Palme, R., Boutin, S., Humphries, M. M. & McAdam, A. G.  
504 (2013). Density triggers maternal hormones that increase adaptive offspring growth in a wild mammal.  
505 *Science*, 340, 1215–1217. doi:10.1126/science.1235765

506 Dantzer, B., Santicchia, F., van Kesteren, F., Palme, R., Martinoli, A. & Wauters, L. A. (2016). Measurement  
507 of fecal glucocorticoid metabolite levels in Eurasian red squirrels (*Sciurus vulgaris*): effects of captivity, sex,  
508 reproductive condition, and season. *Journal of Mammalogy*, 97, 1385–1398. doi:10.1093/jmammal/gyw095

509 Daszak, P., Cunningham, A. A. & Hyatt, A. D. (2000). Emerging infectious diseases of wildlife—threats to  
510 biodiversity and human health. *Science*, 287, 443–449. doi:10.1126/science.287.5452.443

511 Di Febbraro, M., Martinoli, A., Russo, D., Preatoni, D., Bertolino, S. (2016). Modelling the effects of climate  
512 change on the risk of invasion by alien squirrels. *Hystrix, the Italian Journal of Mammalogy*, 27, 22–29.  
513 doi:10.4404/hystrix-27.1-11776

514 Dickens, M. J. & Romero M. L. (2013). A consensus endocrine profile for chronically stressed wild animals  
515 does not exist. *General and Comparative Endocrinology*, 191, 177–189. doi: 10.1016/j.ygcen.2013.06.014

516 Fanson, K. V., Best, E. C., Bunce, A., Fanson, B. G., Hogan, L. A., Keeley, T., ... Bashaw, M. (2017). One size  
517 does not fit all: Monitoring faecal glucocorticoid metabolites in marsupials. *General and Comparative*  
518 *Endocrinology*, 244, 146–156. doi:10.1016/j.ygcen.2015.10.011

519 Glen, A. S. & Dickman, C. R. (2008). Niche overlap between marsupial and eutherian carnivores: does  
520 competition threaten the endangered spotted-tailed quoll? *Journal of Applied Ecology*, 45, 700–707.  
521 doi:10.1111/j.1365-2664.2007.01449.x

522 Goymann, W. (2012). On the use of non-invasive hormone research in uncontrolled, natural environments:  
523 the problem with sex, diet, metabolic rate and the individual. *Methods in Ecology and Evolution*, 3, 757-  
524 765. doi:10.1111/j.2041-210X.2012.00203.x

525 Graham, S. P., Freidenfelds, N. A., McCormick, G. L. & Langkilde, T. (2012). The impacts of invaders: Basal  
526 and acute stress glucocorticoid profiles and immune function in native lizards threatened by invasive ants.  
527 *General and Comparative Endocrinology*, 176, 400–408. doi:10.1016/j.ygcen.2011.12.027

528 Graham, S. P., Freidenfelds, N. A., Thawley, C. J., Robbins, T. R. & Langkilde, T. (2017). Are invasive species  
529 stressful? The glucocorticoid profile of native lizards exposed to invasive fire ants depends on the context.  
530 *Physiological and Biochemical Zoology*, 90, 328–337. doi:10.1086/689983

531 Gurnell, J., Wauters, L. A., Lurz, P. W. & Tosi, G. (2004). Alien species and interspecific competition: effects  
532 of introduced eastern grey squirrels on red squirrel population dynamics. *Journal of Animal Ecology*, 73, 26–  
533 35. doi:10.1111/j.1365-2656.2004.00791.x

534 Gurnell, J., Lurz, P. W. W. & Wauters, L. A. (2015). Years of interactions and conflict in Europe: competition  
535 between Eurasian red squirrels and North American grey squirrel. In: *Red squirrels: Ecology, Conservation &*  
536 *Management in Europe*. (eds Shuttleworth, C. M., Lurz, P. W. W. & Hayward, M. W.), pp 19–37,  
537 Woodbridge, UK: European Squirrel Initiative.

538 Haigh, A., Butler, F., O’Riordan, R. & Palme R. (2017). Managed parks as a refuge for the threatened red  
539 squirrel (*Sciurus vulgaris*) in light of human disturbance. *Biological Conservation*, 211, 29–36.  
540 doi:10.1016/j.biocon.2017.05.008

541 Herman J. P., Adams, D. & Prewitt, C. (1995). Regulatory changes in neuroendocrine stress-integrative  
542 circuitry produced by a variable stress paradigm. *Endocrinology*, 61, 180–190. doi:10.1159/000126839

543 Jessop, T. S., Anson, J. R., Narayan, E. & Lockwood, T. (2015). An introduced competitor elevates  
544 corticosterone responses of a native lizard (*Varanus varius*). *Physiological and Biochemical Zoology*, 88, 237–  
545 245. doi:10.1086/680689

546 Keller, R. P., Geist, J., Jeschke, J. M. & Kühn, I. (2011). Invasive species in Europe: ecology, status, and policy.  
547 *Environmental Sciences Europe*. 23, 23. doi:10.1186/2190-4715-23-23

548 Kuznetsova, A., Brockhoff, P. B. & Christensen, R. H. B. (2016). lmerTest: Tests in linear mixed effects models.  
549 R package version 2.0-33. <https://CRAN.R-project.org/package=lmerTest>

550 Leary, S., Underwood, W., Anthony, R., Cartner, S., Corey, D., Grandin, T., ... Yanong, R. (2013). AVMA  
551 guidelines for the euthanasia of animals: 2013 edition.

552 Martin, L. B. (2009). Stress and immunity in wild vertebrates: Timing is everything. *General and Comparati-*  
553 *ve Endocrinology*, 163, 70–76. doi:10.1016/j.ygcen.2009.03.008

554 Mazzamuto, M. V., Bisi, F., Wauters, L. A., Preatoni, D. G., Martinoli, A. (2017). Interspecific competition  
555 between alien Pallas's squirrels and Eurasian red squirrels reduces density of the native species. *Biological*  
556 *Invasions*, 19, 723–735. doi:10.1007/s10530-016-1310-3

557 McInnes, C. J., Coulter, L., Dagleish, M. P., Deane, D., Gilray, J., Percival, A., ... Sainsbury, A. W. (2013). The  
558 emergence of squirrelpox in Ireland: Squirrelpox in Ireland. *Animal Conservation*, 16, 51–59.  
559 doi:10.1111/j.1469-1795.2012.00570.x

560 Millspaugh, J. J., Washburn, B. E. (2004). Use of fecal glucocorticoid metabolite measures in conservation  
561 biology research: considerations for application and interpretation. *General and Comparative*  
562 *Endocrinology*, 138, 189–199. doi:10.1016/j.ygcen.2004.07.002

563 Mooney, H. A. & Cleland, E. E. (2001). The evolutionary impact of invasive species. *Proceedings of the*  
564 *National Academy of Sciences USA*, 98, 5446–5451. doi:10.1073/pnas.091093398

565 Narayan, E. J., Cockrem, J. F. & Hero, J-M. (2013). Sight of a predator induces a corticosterone stress  
566 response and generates fear in an amphibian. *PLoS ONE*, 8, e73564. doi:10.1371/journal.pone.0073564

567 Narayan, E. J., Jessop, T. S. & Hero, J-M. (2015). Invasive cane toad triggers chronic physiological stress and  
568 decreased reproductive success in an island endemic. *Functional Ecology*, 29, 1435–1444.  
569 doi:10.1111/1365-2435.12446

570 R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical  
571 Computing, Vienna, Austria. URL <https://www.R-project.org/>

572 Riege, D. A. (1991). Habitat specialization and social factors in distribution of red and gray squirrels. *Journal*  
573 *of Mammalogy*, 72, 152–162. doi: 10.2307/1381990

574 Rodrigues, D., Wauters, L. A., Romeo, C., Mari, V., Preatoni, D., Mathias, M. L., ... Martinoli, A. (2010). Living  
575 on the edge: Can Eurasian red squirrels (*Sciurus vulgaris*) persist in extreme high-elevation habitats? Arctic,  
576 Antarctic and Alpine Research, 42, 106–112. doi:10.1657/1938-4246-42.1.106

577 Romeo, C., Ferrari, N., Lanfranchi, P., Saino, N., Santicchia, F., Martinoli, A. & Wauters, L. A. (2015).  
578 Biodiversity threats from outside to inside: effects of alien grey squirrel (*Sciurus carolinensis*) on helminth  
579 community of native red squirrel (*Sciurus vulgaris*). Parasitological Research, 114, 2621–2628.  
580 doi:10.1007/s00436-015-4466-3

581 Romeo, C., McInnes C. J., Dale T. D., Shuttleworth C. M., Bertolino S., Wauters L. A. & Ferrari N.  
582 (2018). Disease, invasions and conservation: no evidence of squirrelpox virus in grey squirrels introduced to  
583 Italy. Animal Conservation. In press.

584 Romero, L. M. (2004). Physiological stress in ecology: lessons from biomedical research. Trends in Ecology &  
585 Evolution, 19, 249-255. doi:10.1016/j.tree.2004.03.008

586 Santicchia, F., Dantzer, B., van Kesteren, F., Palme, R., Martinoli, A., Ferrari, N. & Wauters, L. A. (2018). Data  
587 from: Stress in biological invasions: introduced invasive grey squirrels increase physiological stress in native  
588 Eurasian red squirrels. Dryad Digital Repository. <https://doi.org/10.5061/dryad.bp8jf84>

589 Sapolsky, R. M., Romero, L. M. & Munck, A. U. (2000). How do glucocorticoids influence stress responses?  
590 Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews, 21, 55–89.  
591 doi:10.1210/edrv.21.1.0389

592 Schwarz, G. E. (1978). Estimating the dimension of a model. Annals of Statistics, 6, 461-464.  
593 doi:10.1214/aos/1176344136

594 Sheriff, M. J., Dantzer, B., Delehanty, B., Palme, R. & Boonstra, R. (2011). Measuring stress in wildlife:  
595 techniques for quantifying glucocorticoids. Oecologia, 166, 869–887. doi:10.1007/s00442-011-1943-y



596 Sheriff, M. J., Krebs, C. J. & Boonstra, R. (2009). The sensitive hare: sublethal effects of predator stress on  
597 reproduction in snowshoe hares. *Journal of Animal Ecology*, 78, 1249–1258. doi:10.1111/j.1365-  
598 2656.2009.01552.x

599 Sikes, R. S. & Gannon, W. L. (2011) Guidelines of the American Society of Mammalogists for the use of wild  
600 mammals in research. *Journal of Mammalogy*, 92, 235–253. doi:10.1644/10-MAMM-F-355.1

601 Simberloff, D., Martin, J-L., Genovesi, P., Maris, V., Wardle, D. A., Aronson, J., ... Vilà, M. (2013). Impacts of  
602 biological invasions: what's what and the way forward. *Trends in Ecology & Evolution*, 28, 58–66.  
603 doi:10.1016/j.tree.2012.07.013

604 St. Juliana, J. R., Khokhlova, I. S., Wielebnowski, N., Kotler, B. P. & Krasnov, B. R. (2014). Ectoparasitism and  
605 stress hormones: strategy of host exploitation, common host-parasite history and energetics matter.  
606 *Journal of Animal Ecology*, 83, 1113–1123. doi:10.1111/1365-2656.12217

607 Strayer, D. L. (2012). Eight questions about invasions and ecosystem functioning. *Ecology Letters*, 15, 1199–  
608 1210. doi:10.1111/j.1461-0248.2012.01817.x

609 Touma, C., Sachser, N., Möstl, E. & Palme, R. (2003). Effects of sex and time of day on metabolism and  
610 excretion of corticosterone in urine and feces of mice. *General and Comparative Endocrinology*, 130, 267–  
611 278. doi:10.1016/S0016-6480(02)00620-2

612 Wauters, L. A., Githiru, M., Bertolino, S., Molinari, A., Tosi, G. & Lens, L. (2008). Demography of alpine red  
613 squirrel populations in relation to fluctuations in seed crop size. *Ecography*, 31, 104–114.  
614 doi:10.1111/j.2007.0906-7590.05251.x

615 Wauters, L. A. & Gurnell, J. (1999). The mechanism of replacement of red squirrels by grey squirrels: a test  
616 of the interference competition hypothesis. *Ethology*, 105, 1053–1071. doi:10.1046/j.1439-  
617 0310.1999.10512488.x

618 Wauters, L., Gurnell, J., Martinoli, A. & Tosi, G. (2001). Does interspecific competition with introduced grey  
619 squirrels affect foraging and food choice of Eurasian red squirrels? *Animal Behaviour*, 61, 1079–1091. doi:  
620 10.1006/anbe.2001.1703

621 Wauters, L., Gurnell, J., Martinoli, A. & Tosi, G. (2002b). Interspecific competition between native Eurasian  
622 red squirrels and alien grey squirrels: does resource partitioning occur? *Behavioral Ecology and*  
623 *Sociobiology*, 52, 332–341. doi:10.1007/s00265-002-0516-9

624 Wauters, L., Tosi, G. & Gurnell, J. (2002a). Interspecific competition in tree squirrels: do introduced grey  
625 squirrels (*Sciurus carolinensis*) deplete tree seeds hoarded by red squirrels (*S. vulgaris*)? *Behavioral Ecology*  
626 *and Sociobiology*, 51, 360–367. doi:10.1007/s00265-001-0446-y

627 Wauters, L. A., Vermeulen, M., Van Dongen, S., Bertolino, S., Molinari, A., Tosi, G. & Matthysen, E. (2007).  
628 Effects of spatio-temporal variation in food supply on red squirrel *Sciurus vulgaris* body size and body mass  
629 and its consequences for some fitness components. *Ecography*, 30, 51–65. doi:10.1111/j.2006.0906-  
630 7590.04646.x

631 White, A., Lurz, P., Bryce, J., Tonkin, M., Ramoo, K., Bamforth, L., ... Boots, M. (2016). Modelling disease  
632 spread in real landscapes: Squirrelpox spread in Southern Scotland as a case study. *Hystrix, the Italian*  
633 *Journal of Mammalogy*, 27, 75–82. doi:10.4404/hystrix-27.1-11657

634 Wingfield, J. C., Maney, D. L., Breuner, C. W., Jacobs, J. D., Lynn, S., Ramenofsky, M., Richardson, R. D.  
635 (1998). Ecological bases of hormone-behavior interactions: the “emergency life history stage”. *Integrative*  
636 *and Comparative Biology*, 38, 191–206. doi:10.1093/icb/38.1.191

637 Woodruff, J. A., Lacey, E. A., Bentley, G. E. & Kriegsfeld, L. J. (2013). Effects of social environment on baseline  
638 glucocorticoid levels in a communally breeding rodent, the colonial tuco-tuco (*Ctenomys sociabilis*).  
639 *Hormones and Behavior*, 64, 566–572. doi:10.1016/j.yhbeh.2013.07.008

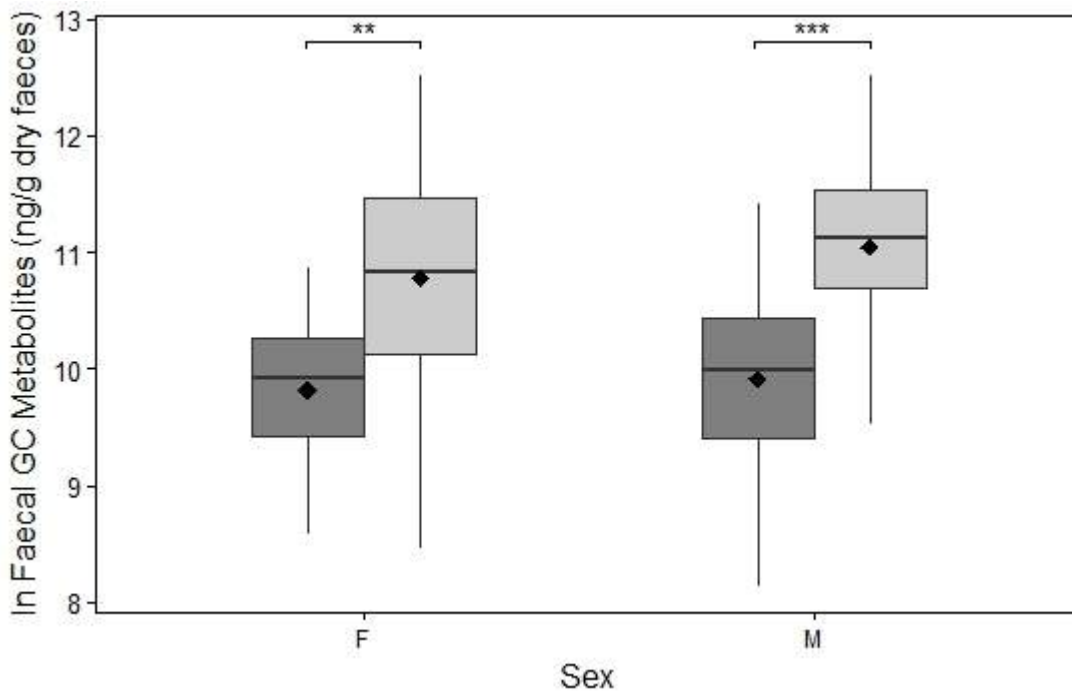
640 Zuur, A. F., Ieno E. N. & Elphick, C. S. (2010). A protocol for data exploration to avoid common statistical  
641 problems. *Methods in Ecology and Evolution*, 1, 3–14. doi:10.1111/j.2041-210X.2009.00001.x

642 **Table 1.** Data of the grey squirrel removal experiment in four study sites with both squirrel species. Period =  
643 capture period with removal (see methods); Mean Number Alive (MNA) of red squirrels (density/ha  
644 between brackets); Estimated number of grey squirrels present at the start of each removal period  
645 (density/ha between brackets); Number of grey squirrels removed during each capture period,  $m_i$ , (the  
646 cumulative % grey squirrels removed at the start of the capture period, hence this is 0% for Period 1). Ln  
647 FGM (ng/g dry faeces) = changes in the concentration of FGM (ln FGM, mean  $\pm$  SD, sample size between  
648 brackets) over time (period) in red squirrels, following removal of invasive grey squirrels.

Period	Red squirrels MNA (N/ha)	Grey squirrels present (N/ha)	Grey squirrels removed ( $m_i$ ) (%)	ln FGM (ng/g dry faeces) red squirrels (n)
<b>Vanzago (74.73 ha)</b>				
Period 1 (January 2016)	16 (0.21)	18 (0.24)	12 (0)	(13) 10.890 $\pm$ 0.996
Period 2 (March 2016)	19 (0.25)	6 (0.08)	5 (67)	(19) 10.284 $\pm$ 1.051
Period 3 (May 2016)	16 (0.21)	1 (0.01)	1 (94)	(12) 10.372 $\pm$ 1.176
<b>Lambro (18.43 ha)</b>				
Period 1 (November 2015)	6 (0.33)	54 (2.93)	24 (0)	(5) 10.441 $\pm$ 0.467
Period 2 (December 2015)	5 (0.27)	30 (1.63)	11 (44)	(5) 10.170 $\pm$ 0.942
Period 3 (March 2016)	6 (0.33)	19 (1.03)	19 (65)	(6) 9.321 $\pm$ 0.887
<b>Passatempo (18.33 ha)</b>				
Period 1 (November 2015)	9 (0.49)	22 (1.20)	9 (0)	(8) 10.958 $\pm$ 1.234
Period 2 (March 2016)	10 (0.55)	13 (0.71)	12 (41)	(9) 10.642 $\pm$ 1.417
Period 3 (May 2016)	11 (0.60)	1 (0.05)	1 (95)	(11) 10.352 $\pm$ 0.918
<b>Castelbarco (65.86 ha)</b>				
Period 1 (December 2015)	21 (0.32)	44 (0.67)	10 (0)	(12) 10.361 $\pm$ 0.715
Period 2 (January 2016)	19 (0.29)	34 (0.52)	17 (23)	(14) 9.922 $\pm$ 0.964
Period 3 (March 2016)	25 (0.38)	17 (0.26)	17 (61)	(17) 10.392 $\pm$ 0.919

649

650



651

652 **Figure 1.** Comparison of faecal glucocorticoid (GC) metabolite concentrations in Eurasian red squirrels  
 653 between red-only (dark-grey colour; 125 samples, 57 females, 68 males) and red-grey (light-grey colour; 135  
 654 samples, 57 females, 78 males) study sites per sex (F = females; M = males). Boxplots show median (solid  
 655 horizontal line), mean (black diamond) and 1st (25%) and 3rd (75%) quartiles. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

656

657

658

659

660

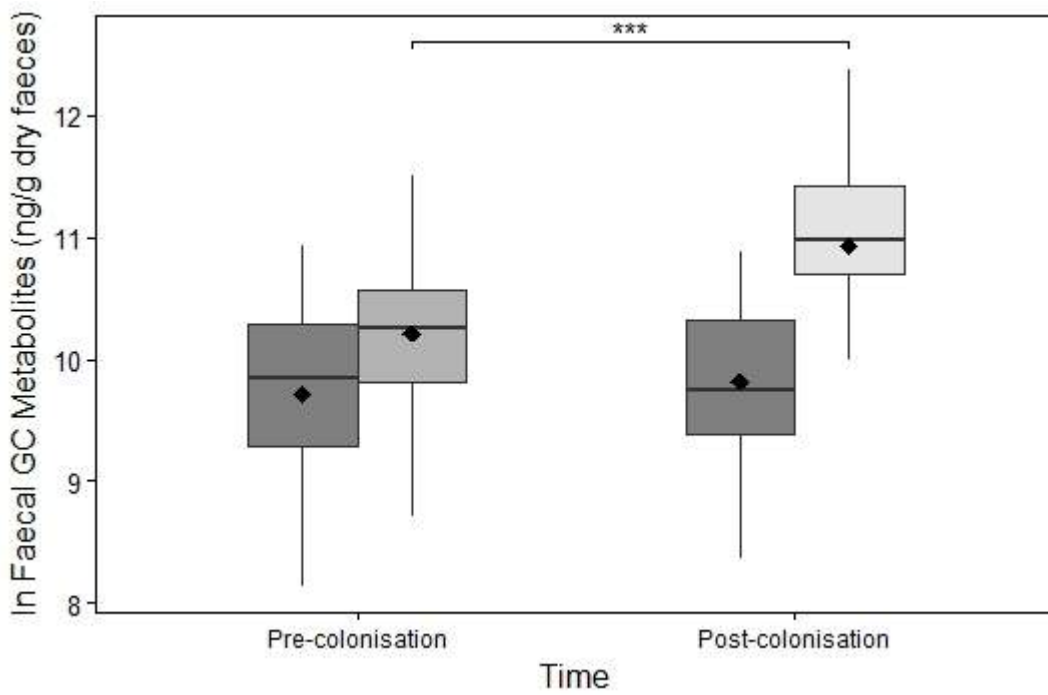
661

662

663

664

665



666

667

668 **Figure 2.** Comparison of faecal glucocorticoid (GC) metabolite concentrations in Eurasian red squirrels  
 669 between pre-colonisation time and post-colonisation time of sites not colonised by the invasive species  
 670 (dark-grey colour: pre- colonisation time, 34 samples; post- colonisation time, 62 samples) and of sites  
 671 colonised by the grey squirrels over the same time (grey colour: pre- colonisation time, 29 samples; light-  
 672 grey colour: post- colonisation time, 58 samples). Boxplots show median (solid horizontal line), mean (black  
 673 diamond) and 1st (25%) and 3rd (75%) quartiles. \*\*\*  $p < 0.001$ .

674

675

676

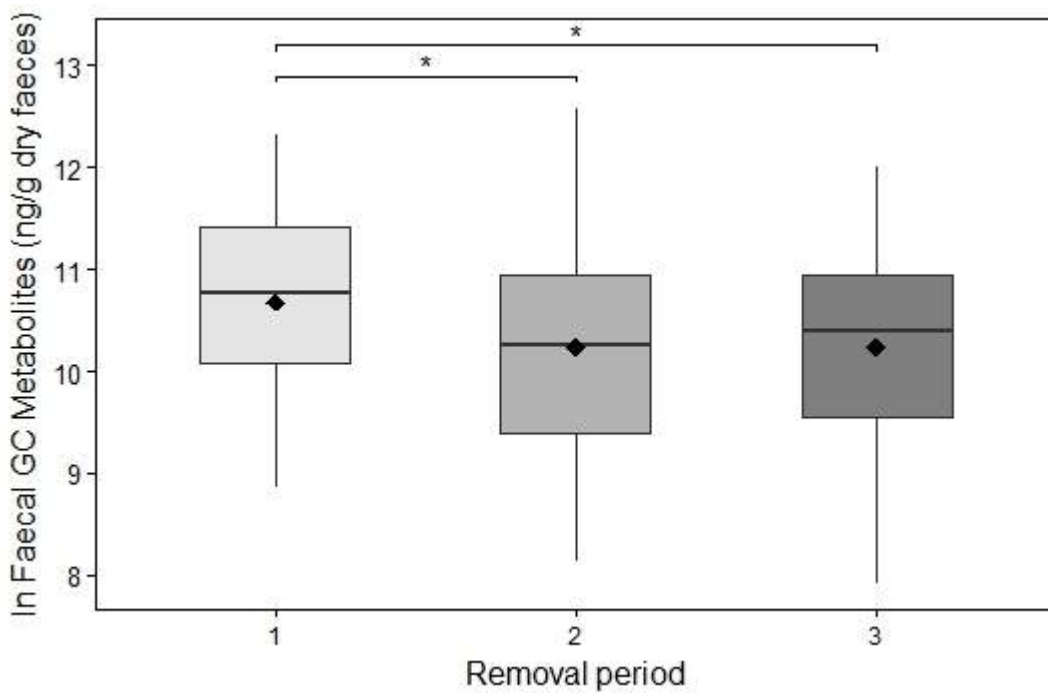
677

678

679

680

681



682

683

684 **Figure 3.** Comparison of faecal glucocorticoid (GC) metabolite concentrations in Eurasian red squirrels in  
 685 trapping period 1 (high grey squirrel density, light-grey colour), period 2 (with lower numbers of grey  
 686 squirrels, grey colour) and period 3 (low grey squirrel density, dark-grey colour). During the removal  
 687 experiment 131 samples of 67 different red squirrels were collected: 38 at the start of removal (period 1),  
 688 47 during period 2 and 46 samples during period 3. Boxplots show median (solid horizontal line), mean  
 689 (black diamond) and 1st (25%) and 3rd (75%) quartiles. \*  $p < 0.05$ .