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

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

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

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24 **Abstract**

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

44

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

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50 **1 Introduction**

51 The introduction of non-native species that subsequently become invasive (invasive alien species,
52 IAS) can cause large economic losses and affect human activities and health (Simberloff et al., 2013).
53 Moreover, IAS represent a threat to native species worldwide through different ecological processes (Keller,
54 Geist, Jeschke & Kühn 2011), such as interspecific competition (e.g. Cadi & Joly, 2003; Gurnell, Wauters, Lurz
55 & Tosi 2004), predation (Berger, Wikelski, Romero, Kalko & Rödl 2007; Banks & Dickman, 2007),
56 transmission of infectious diseases (Daszak, Cunningham & Hyatt 2000; Strauss, White & Boots 2012), and
57 even changes in ecosystem functioning (Ehrenfeld, 2010; Strayer, 2012). Such detrimental effects can lead
58 to extinction of native species and consequent loss of biodiversity (Clavero & García-Berthou, 2005; Keller
59 et al., 2011). For example, the introduction of alien species in a new environment can produce high levels of
60 niche overlap with ecologically similar native species, and competition for key resources (Glen & Dickman,
61 2008; Mazzamuto, Bisi, Wauters, Preatoni & Martinoli 2017), that can ultimately result in exclusion
62 competition and extinction of the native species (Mooney & Cleland, 2001; Broennimann et al., 2007).

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

76 of growth and/or reproduction (Cabezas, Blas, Marchant & Moreno 2007; Sheriff, Krebs & Boonstra 2009),
77 increase in blood glucose levels, suppression of digestion (Caso, Leza & Menchen 2008) and suppression of
78 immunity and the inflammatory response (Romero, 2004; Raouf, Smith, Brown, Wingfield, Brown 2006; St.
79 Juliana, Khokhlova, Wielebnowski, Kotler & Krasnov 2014) can be observed. Competitive food resource
80 exploitation (Chase, Flynn & Todgham 2016), parasite-mediated competition (Raouf et al., 2006; St. Juliana
81 et al., 2014), introduction of (alien) predators (Berger et al., 2007; Anson et al., 2013), and/or invasive
82 competitors (Boronow & Langkilde 2010; Narayan et al., 2015) are all documented cases of direct and/or
83 indirect interspecific interactions, that show, through different mechanisms, how negative stimuli lead to
84 increased physiological stress in at least one of the species involved.

85 In many vertebrate species, the amplitude and duration of the chronic stress response correlates
86 with the overall health of an animal (Boonstra et al. 1998; Martin, 2009). Consequently, the so-called
87 baseline GC concentrations, which in wild mammals are measured primarily using concentrations of faecal
88 glucocorticoid metabolites (FGM levels; e.g. Möstl & Palme, 2002; Millspaugh & Washburn, 2004; Sheriff,
89 Dantzer, Delehanty, Palme & Boonstra 2011) are increasingly used in ecological and conservation studies as
90 indices of animal well-being. Hence, measuring these hormones (stress response) in individuals of a native
91 species in situations with and without the alien species (the environmental stressor) can help us understand
92 if and how the stressor affects animals in native species' populations (Dantzer, Fletcher, Boonstra & Sheriff
93 2014).

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

99 Here, we examined whether the presence of an invasive species causes an increase in physiological
100 stress in individuals of a native species. We use the well-known case of competitive replacement of the
101 Eurasian red squirrel (*Sciurus vulgaris*) by the introduced invasive Eastern grey squirrel (*Sciurus carolinensis*)

102 in Europe to explore effects of stress at the individual level using faecal glucocorticoid metabolites (FGM;
103 Sheriff et al., 2011; Dantzer et al., 2014; Dantzer et al., 2016; Haigh, Butler, O’Riordan & Palme 2017). Tree
104 squirrels are often successful invaders (Bertolino, 2009; Di Febbraro, Martinoli, Russo, Preatoni & Bertolino
105 2016), and the rapid spread of the invasive grey squirrel and subsequent decline and widespread extinction
106 of the native red squirrel on the British Isles and in parts of Italy has been documented in many studies. The
107 two species have similar space use and activity patterns, and compete for food resources, resulting in
108 smaller body size, and reduced female reproduction and juvenile recruitment in red squirrels (Wauters, Tosi
109 & Gurnell 2002a; Wauters, Gurnell, Martinoli & Tosi 2002b; Gurnell et al., 2004), ultimately causing a
110 decrease in population size and (local) extinction (Bertolino, Montezemolo, Preatoni, Wauters & Martinoli
111 2014). On the British Isles, a squirrel pox virus determines disease-mediated competition, with grey
112 squirrels acting as a reservoir and transmitting the virus to red squirrels, for which, in most cases, the
113 infection is lethal (Mc Innes et al., 2013; White et al., 2016).

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

128 colonised than before the colonisation by the alien species (experiment 1); and FGM concentrations in
129 syntopic red squirrels (red-grey sites) will decrease after the removal of the grey squirrels (experiment 2).

130

131 **2 Materials and methods**

132 *2.1 Study sites.* —We trapped Eurasian red squirrels in five study sites without grey squirrels
133 (historically red-only sites) in Lombardy, North Italy (Supporting Information, Table S1). Two of these sites
134 (Vanzago, Castelbarco) were colonised by the alien species during the study; hence they were used as red-
135 only sites before colonisation (respectively before March 2015 and November 2014), and as red-grey sites
136 afterwards. We trapped both squirrel species (red-grey sites) in seven study sites in Lombardy and
137 Piedmont, Northern Italy (Supporting Information, Table S1). All red-grey sites and the two red-only sites
138 mentioned above (Vanzago, Castelbarco) are lowland mixed deciduous woodlands in the Po plain, while the
139 other three red-only sites (Bormio, Cancano, Valfurva) are subalpine conifer forests in the Central Italian
140 Alps (1620-2150 m elevation).

141 *2.2 Live-trapping, handling and faecal sample collection.* —We trapped squirrels in all sites during
142 trapping sessions that lasted four or five days each (sites and trapping dates listed in Supporting
143 Information, Table S1). We used Tomahawk traps (model 202, Tomahawk Live Trap, WI, USA) with a fine
144 mesh added underneath traps to prevent contamination between urine and faeces. We checked traps two
145 to three times a day. Each trapped squirrel was individually marked using numbered metal ear-tags (type
146 1003 S, National Band and Tag, Newport, KY, USA). We weighed squirrels to the nearest 5 g using a spring-
147 balance (Pesola AG, Baar, Switzerland) and measured the length of the right hind foot (without nail, 0.5
148 mm) with a ruler (Wauters et al., 2007). A female's reproductive status was defined as non-breeding
149 (anoestrous, vulva small, no longitudinal opening, not lactating), post-oestrous, and pregnant (vulva partly
150 or strongly swollen with longitudinal opening, enlarged belly during late pregnancy), or lactating (nipples
151 large, milk excretion can be stimulated). We recorded reproductive condition of males (testes size and
152 position) as non-breeding (testes abdominal or semi-scrotal and scrotum small) or breeding (testes scrotal

153 and scrotum large). We used the minimum number of animals known to be alive from trapping and
154 observations (MNA, see also Wauters et al., 2008) during each trapping session as an estimate of
155 population size and squirrel density. Previous studies showed that MNA estimates were strongly correlated
156 with estimates from capture-mark-recapture models (e.g. MARK, Wauters et al., 2008).

157 To test effects of removal of the alien species on FGM concentrations in co-occurring native red
158 squirrels we analysed samples from four study sites where all grey squirrels that were trapped over three
159 subsequent periods (one period every six to ten weeks between November 2015 and May 2016) were
160 removed. If the number of grey squirrels removed in period i is m_i then $m_1 + m_2 + m_3 = m_{\text{tot}}$ the total number
161 of grey squirrels removed over the entire experiment. Red squirrel faecal samples collected in period 1
162 corresponded with high grey squirrel densities (m_{tot}), those collected in period 2 with intermediate densities
163 of grey squirrels ($m_2 + m_3$), and those collected in period 3 with the lowest grey squirrel densities (m_3) (see
164 Table 1). We used m_{tot} to estimate the minimum number of animals known to be alive (MNA) of the alien
165 species, considering that no immigration or recruitment of juveniles occurred during the removal period.
166 Removal of grey squirrels was part of the LIFE09 NAT/IT/000095 EC-SQUARE project: animals were
167 euthanized by CO₂ inhalation, following the EC and AVMA guidelines (Close et al., 1996, 1997; Leary et al.,
168 2013).

169 After capture and handling, faecal samples were collected from underneath the traps using forceps,
170 and placed individually into 1.5 mL vials (Dantzer et al., 2010) and the fine mesh and ground under the traps
171 were cleaned to remove any remaining faecal material. We only used faecal samples from squirrels that had
172 not previously been trapped or handled within 72 h prior to capture to minimize effects of capture stress on
173 FGM concentrations (Dantzer et al., 2016). We obtained multiple samples in different seasons/periods from
174 most squirrels, but not all captured individuals produced faeces within a given trapping session. Each faecal
175 sample was classified as being taken in the morning (10.00 – 13.00h) or in the afternoon (15.00 – 18.00h) to
176 account for potential variation in FGM concentration over the 24 h cycle (Millspaugh & Washburn, 2004).
177 We placed faecal samples into an insulated bag with ice packs while in the field, and samples were stored at
178 -20°C within 3-4 h after collection, which should not cause temperature-induced changes in faecal hormone

179 metabolite concentrations (Dantzer et al., 2010). Trapping and handling of squirrels complied with the
180 current laws on animal research in Italy, and was carried out with permission of the Region of Lombardy
181 (Decree n. 11190 of 29/11/2013). All of these procedures abided by ASM guidelines (Sikes & Gannon,
182 2011).

183 *2.3 Extraction of hormone metabolites and enzyme immunoassay.* —Methods of extraction of FGM
184 and enzyme immunoassay validation for red squirrels and grey squirrels are described in detail elsewhere
185 (Bosson, Palme & Boonstra 2013; Dantzer et al., 2016). Briefly, samples were lyophilized overnight, ground
186 up under liquid nitrogen and weighed to 0.05 g (\pm 0.004 g), and extracted using 80% methanol, shaking at
187 1500 r.p.m on a multivortex for 30 min, centrifuging at 2500 g for 15 min, and aspirating the supernatant.
188 Supernatants were diluted in assay buffer and assayed using a 5 α -pregnane-3 β , 11 β , 21-triol-20-one
189 enzyme-immunoassay (EIA) which detects glucocorticoid metabolites with a 5 α -3 β , 11 β -diol structure (for
190 cross-reactivity see Touma, Sachser, Möstl & Palme 2003) to measure FGM concentrations (ng/g dry faeces,
191 Dantzer et al., 2010, 2016). Samples were analysed in duplicate. We used data already published by Dantzer
192 et al. (2016) (n = 125), and 193 new faecal samples were assayed on a total of 22 EIA plates. Pools of grey
193 squirrel faecal extracts were used as intra-assay controls at dilutions of 1:50 (~30% binding) and 1:400
194 (~70% binding). Average intra-assay coefficients of variation (CVs) were 9.5% and 9.4% respectively for pools
195 diluted 1:50 and 1:400. Inter-assay CVs were estimated from standards of known concentration with a high
196 (n = 22 plates, 12.3% binding) and low (n = 22 plates, 81.6% binding) concentration that had inter-assay CVs
197 of 16.1% and 9.3%, respectively.

198 *2.4 Statistical analyses.* —All analyses were performed in R version 3.3.3 (R Development Core
199 Team, 2017) using the lme4 package (version 1.1-12, Bates, Mächler, Bolker & Walker 2015). For each of the
200 models described, we conducted linear mixed-effects models with FGM concentrations (transformed using
201 the natural logarithm, ln of ng/g dry faeces) as the dependent variable and squirrel identity (ID) nested in
202 study site as a random intercept term to account for repeated samples on the same individuals. Sex and
203 reproductive condition nested in sex were added as fixed effects to account for potential changes in FGM
204 concentrations with reproductive activity in males and females (Goymann, 2012; Dantzer et al., 2016).

205 Residuals were visually inspected to verify the assumptions of normality and homoscedasticity (Zuur, Ieno &
206 Elphick 2010). Where necessary, we assessed significance of pair-wise comparisons using differences of
207 least square means (DLSM) with Satterthwaite approximations to degrees of freedom in R package lmerTest
208 (version 2.0-33, Kuznetsova, Brockhoff & Christensen 2016). All full models (see below) contained
209 interactions. When interactions were not significant and including them did not improve model fit (Δ BIC
210 between model with and without interactions > 2.0 ; Schwarz & Gideon, 1978), they were removed from the
211 models to obtain reliable parameter estimates for the fixed effects.

212 *2.4.1 Comparisons between red-only vs. red-grey sites.* —We first assessed the direct effects of
213 invasion status (sites with only red squirrels vs. sites with both red and grey squirrels) including also season
214 (winter [December to March], spring-summer [April to August], or autumn [September to November]) and
215 daytime (animal sampled in morning or afternoon) as factors, and body mass as a continuous variable. We
216 also tested the interaction between sex and invasion status to explore whether the effect of the presence of
217 grey squirrels on FGM concentrations in red squirrels differed between the sexes. Study site nested in
218 invasion status was added as random intercept to account for potential differences in FGM concentrations
219 between sites. Since sites with only red squirrels occurred in coniferous as well as in deciduous forests, we
220 also explored the effect of habitat type on FGM concentrations (models and results in Supporting
221 Information).

222 *2.4.2 Effect of colonisation by grey squirrels.* —We had two study sites (45 km apart) that were
223 colonised by the alien species during the study, and we explored whether FGM concentrations in red
224 squirrels changed after the colonisation (samples collected between 6-10 months after the pre-grey sample
225 collection, see also Table S1). We also used data of three non-colonised study sites (Valfurva, Bormio and
226 Cancano, see Table S1) that were monitored over the same time-period as control data to test if differences
227 in FGM were simply time-related. We investigated the effects of colonisation (yes or no), time-period
228 (before vs. after, the dates of colonisation), and their interaction, and included sex, reproductive condition
229 nested in sex, daytime and body mass in the full model. Study site nested in colonisation status was added
230 as a random intercept to account for potential differences in FGM concentrations between sites.

231 *2.4.3 Removal experiment.* —We assessed the effects of grey squirrel removal considering
232 treatment period (initial sampling when no grey squirrels had been removed yet = period 1; intermediate
233 sampling, with a low proportion of grey squirrels removed = period 2; final sampling with a higher
234 proportion of grey squirrels removed = period 3; see methods and Table 1), study site, sex, reproductive
235 condition nested in sex and daytime as factors, and body mass as an explanatory variable, and two factor
236 interactions between the period by study site, and period by sex, to explore whether the removal of grey
237 squirrels would result in a significant decrease in FGM concentrations in sympatric red squirrels. In order to
238 account for the fact that the observed differences in FGM concentrations between removal periods were
239 influenced by seasonal variation, we also explored a model in which we used season as a fixed effect
240 instead of removal period (both factors could not be fitted in a single model because of problems with
241 collinearity, Zuur et al., 2010). We used the difference in BIC value to test which model best fitted the data
242 (Schwarz & Gideon, 1978).

243

244 **3 Results**

245 *3.1 FGM concentrations of red squirrels in red-only vs. red-grey sites.* — FGM concentrations in
246 native red squirrels (260 samples from 166 different animals) were three times higher in sites that
247 contained invasive grey squirrels (n samples = 135, mean \pm SD = 78133 \pm 61074 ng/g dry faeces) than in
248 sites that only contained native red squirrels (n = 125, 24890 \pm 20566 ng/g dry faeces; estimate of the
249 invasion status effect on ln FGM 0.97 \pm 0.20; $t_8 = 4.85$; $p = 0.0012$). There was no evidence that the presence
250 of invasive grey squirrels impacted the FGM concentrations of the two sexes differently (sex by invasion
251 status interaction $t_{165} = 0.98$; $p = 0.33$; Fig. 1). FGM concentrations did not differ between the sexes
252 (estimate males against females on ln FGM -0.04 \pm 0.25; $t_{210} = 0.18$; $p = 0.86$), or with reproductive
253 condition (males: breeding compared to non-breeding 0.06 \pm 0.16; $t_{247} = 0.40$; $p = 0.69$; females pregnant
254 compared to lactating -0.12 \pm 0.23; $t_{249} = 0.52$; $p = 0.60$; pregnant compared to non-breeding -0.34 \pm 0.23;
255 $t_{226} = 1.50$; $p = 0.14$).. Native red squirrels had lower FGM concentrations in spring-summer than in autumn
256 (spring-summer against autumn estimate -0.33 \pm 0.14; $t_{220} = 2.39$; $p = 0.018$; all other comparisons $p >$

257 0.05). FGM concentrations did not vary significantly with daytime (hour of faecal sample collection, $t_{223} =$
258 0.76; $p = 0.45$) or squirrel body mass ($t_{226} = 0.30$; $p = 0.76$).

259 *3.2 Effect of colonisation by grey squirrels.* — FGM concentrations in red squirrels increased with
260 time-period and with colonisation status, and there was a significant time by colonisation status interaction
261 (Supporting Information, Table S2). Red squirrels in the two sites colonised by the alien species during our
262 study (29 samples before, 58 samples after colonisation) had a significant increase in FGM from the pre- to
263 post-colonisation period (difference in ln FGM estimate 0.74 ± 0.18 ; $t_{173} = 3.93$; $p = 0.0004$; Fig. 2). In
264 contrast, red squirrels from the three sites without colonisation did not show a significant increase in FGM
265 concentrations over the same time-period (34 samples in first period, 62 samples in second period,
266 difference in ln FGM estimate 0.21 ± 0.15 ; $t_{152} = 1.27$; $p = 0.21$; Fig. 2). In this dataset there was no
267 statistically significant effect of sex, reproductive condition nested in sex, daytime, or body mass on FGM
268 concentrations (Supporting Information, Table S2).

269 *3.3 Removal experiment.* — After the removal of alien squirrels, FGM concentrations in co-occurring
270 red squirrels decreased significantly in periods 2 and 3 compared to FGM concentrations at the start of the
271 experiment (period 1, Fig. 3 and Table 1). Interactions between period and study site and between period
272 and sex were not significant, and were removed from the model (Supporting Information, Table S3).
273 Daytime and body mass also did not influence FGM concentrations and were removed during stepwise
274 model selection (Supporting Information, Table S3). FGM concentrations in red squirrels decreased
275 significantly after the first removal session, but there was no further, significant, decrease after the second
276 removal session (period 2–period 1 estimate -0.58 ± 0.23 , $t_{121} = 2.53$; $p = 0.012$; period 3 – period 1 estimate
277 -0.50 ± 0.23 , $t_{121} = 2.18$; $p = 0.031$; period 2 – period 3 estimate 0.08 ± 0.21 ; $t_{121} = 0.35$; $p = 0.73$).

278 There was no difference between males and females in FGM levels, but in this dataset breeding
279 males had higher FGM levels than non-breeding males (Supporting Information, Table S3). Variation
280 between study sites was nearly significant only for two sites, with overall lower FGM levels in Lambro than
281 in Passatempo (Table 1; difference in ln FGM estimate -0.63 ± 0.32 ; $t_{121} = 1.89$; $p = 0.06$).

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

285

286 **4 Discussion**

287 We explored whether the presence of an invasive alien competitor affected faecal glucocorticoid
288 metabolite concentrations in individuals of a native species. We predicted that the threat posed by invasive
289 grey squirrels would increase FGM concentrations in co-occurring native red squirrels. FGM concentrations
290 were significantly higher in sites invaded by grey squirrels than in red-only sites, and in the two areas
291 colonised by grey squirrels during our study, FGM concentrations in the native red squirrels increased with
292 respect to pre-colonisation concentrations. Both results support our hypothesis and suggest that the
293 invader is the causal factor driving observed differences in FGM concentrations (see also Graham et al.,
294 2017). This was further supported by our grey squirrel removal experiment. Removal of grey squirrels
295 resulted in a significant decrease in glucocorticoids in co-occurring red squirrels over a two month interval,
296 taking into account seasonal variation in glucocorticoid concentrations and changes in reproductive
297 condition in both males and females (see also Boonstra et al., 2001; Dantzer et al., 2010). The reduction in
298 glucocorticoids was more pronounced in two sites: these were the two smallest woodlands that, at the start
299 of the experiment, had the highest grey squirrel densities (Passatempo and Lambro, Table 1) and where red
300 squirrel home ranges were more strongly overlapped by individuals of the alien species (Wauters L.A.
301 unpublished data 2016).

302 *4.1 Comparisons between red-only vs. red-grey sites.* — Native red squirrels had higher FGM
303 concentrations in areas where they co-occurred with the alien grey squirrels than did those in areas without
304 the introduced competitor. This pattern is supported by previous studies. Stress levels of native lizards
305 (*Sceloporus undulatus*) were higher in sites with a long history of co-occurrence with invasive fire ants
306 (*Solenopsis invicta*) than in sites without the invader (Graham et al., 2012). Similar results were found in a
307 system of a native lizard (the lace monitor, *Varanus varius*) and an introduced alien predator (red fox, *Vulpes*

308 *vulpes*) in Australia. In habitats with high fox densities, lizards produced a significantly greater basal and
309 capture stress-induced corticosterone response compared to individuals in low-fox density habitat,
310 suggesting competition with red foxes, perhaps via nutritional stress and increased hypersensitivity of the
311 adrenocortical axis in lizards (Jessop et al., 2015). So far, it is not clear whether such responses mediate
312 lizard fitness. Another study examined whether introduced foxes caused elevated glucocorticoid (GC)
313 hormone concentrations (predator stress hypothesis) on a native predator (*Varanus varius*) and a prey
314 species (ringtail possum, *Pseudocheirus peregrinus*). No differences were found in glucocorticoid
315 concentrations or in haemoparasite loads between areas with and without fox control in either of the two
316 native species (Anson et al., 2013). The different effects of foxes found by these two studies suggest that
317 interspecific interactions have a greater role in eliciting a corticosterone response than a fear effect caused
318 by the predator's presence. These types of observational studies reveal the need for experimental
319 manipulations to investigate the potential causality of the relationship between the presence of invasive
320 species and increased stress in native species.

321 4.2 Pre – post colonisation. —The colonisation by grey squirrels in two of our study sites can be
322 considered as a natural experiment of introducing an alien competitor. The strong increase in FGM
323 concentrations we detected following colonisation of grey squirrels suggests that grey squirrels are causing
324 the higher levels of physiological stress detected in field samples from the native species. Extra support to
325 this interpretation is given by the fact that no such increase occurred in red squirrels monitored over the
326 same time-period in sites that were not colonised. A similar pattern was documented in the only study we
327 found which used experimental approaches to investigate changes in stress levels in the native and
328 endangered Fijian ground frog (*Platymantis vitiana*) caused by the presence of introduced cane toads
329 (*Rhinella marina*) that can prey on the native frogs (Narayan et al., 2013, 2015). Artificial exposure or
330 introduction of the alien predator resulted in an increase in urinary corticosterone metabolites (Narayan et
331 al., 2013), and subsequently reduced reproductive success (fewer eggs laid in enclosures which also
332 contained alien predators than in no-predator enclosures) in the endemic species (Narayan et al., 2015).

333 4.3 *The removal experiment.* —A next step is manipulation of the presence/density of the alien
334 species that acts as stressor. Previous studies used an approach where they compared areas without
335 predator manipulation to areas with predator manipulation (e.g. Anson et al., 2013; Jessop et al., 2015). We
336 used a new approach: removal over time comparing the observed changes in faecal glucocorticoid
337 metabolites within populations using sites as independent replicates. In other words the FGM
338 concentrations in individual red squirrels within a given population/study site are measured under high
339 pressure of grey squirrels (no removal) and compared with lower pressure of the alien species (after
340 removal). This experiment may be confounded by temporal effects on glucocorticoids, such as the season
341 effect found in the data comparing red-only with red-grey sites, and the time-span from the beginning of
342 the removal and the measurements. However, our models showed that seasonal variation in FGM measures
343 was not statistically significant in this dataset and that models with removal period better fitted the data
344 than models including a season effect. Moreover, the strongest decrease in FGM concentrations of red
345 squirrels after removal of grey squirrels occurred between period 1 and period 2. FGM data for period 1
346 were gathered in autumn and winter, and all data for period 2 were taken in winter, and in the large dataset
347 comparing red-only with red-grey sites there was no significant difference between winter and autumn
348 levels of glucocorticoids. In a dataset of red squirrels in areas without the invasive species, glucocorticoids
349 were even higher in winter than in autumn (Dantzer et al., 2016), hence the opposite trend. Hence, these
350 data support our conclusion that the observed decrease in faecal glucocorticoid metabolites in red squirrels
351 was indeed caused by a reduction of the density of alien grey squirrels.

352 We found that FGM concentrations in native red squirrels decreased significantly (between the first
353 and second period) in relation to invasive species removal. FGM levels also remained lower in the third
354 period (see Fig. 3), but a further decrease with respect to period 2 was observed only in the two smallest
355 study sites, where, as mentioned above, grey squirrels occurred at the highest densities when the
356 experiment started (see Table 1). This suggests that the impact of the alien competitor on individual red
357 squirrels' FGM concentrations is probably related to the degree of home range (or core-area) overlap, and
358 the resulting differences in food competition and pilfering of red squirrel caches by overlapping greys
359 (Wauters & Gurnell, 1999; Wauters, Tosi & Gurnell 2002a).

360

361 4.4 *Physiological relevance and fitness consequences.* —In wild animals, it is difficult to assess the
362 direct physiological relevance of increased FGM. However, we documented a 3-fold increase in FGMs in red
363 squirrels in red-grey sites compared to red-only sites, a 2-fold increase in sites that were colonised by the
364 alien species and a 1.5-fold decrease when grey squirrels were removed. Placing these results in the context
365 of other studies from the literature is difficult, because changes in FGMs reported in our and other studies
366 are highly influenced by the assay used to quantify the change in GCs (e.g. Fanson et al., 2017).
367 Nonetheless, a few studies suggest that the changes in FGMs that we observed are comparable to chronic
368 stress paradigms in the laboratory, and that they may have other effects on life history traits. For example,
369 in laboratory rats, exposure to a variable chronic stress paradigm caused a ~1.5 fold increase in plasma
370 corticosterone levels (Herman, Adams & Prewitt 1995). In a communally breeding rodent (*Ctenomys*
371 *sociabilis*) the difference in baseline glucocorticoid levels between animals kept in less or more stressful
372 situations was about 1.5 to 2-fold (Woodruff, Lacey, Bentley, Kriegsfeld 2013). A previous study in North
373 American red squirrels (*Tamiasciurus hudsonicus*) showed that pregnant females have 30% higher FGMs
374 when high population density conditions were simulated, and that this was associated with a change in
375 offspring postnatal growth rates (Dantzer et al., 2013). Therefore, we conclude that alien grey squirrels
376 increase FGM concentrations in native red squirrels, supporting the physiological impact of elevated levels
377 of glucocorticoids.

378 To date, few studies have been able to demonstrate the consequences of prolonged elevations of
379 glucocorticoids caused by IAS on native species' fitness components (Narayan et al., 2015). Our study was
380 too short to reveal fitness consequences of the increase in glucocorticoids levels in red squirrels in areas
381 invaded by the congener. However, our previous studies clearly demonstrated negative effects of grey
382 squirrels, exacerbated at high densities of the alien species, on red squirrel body size and reproductive rate
383 (Wauters, Gurnell, Preatoni & Tosi 2001; Gurnell, Wauters, Lurz & Tosi 2004). Both these effects are in
384 agreement with alterations of the hormonal functions of glucocorticoids in regulating of body growth and
385 reproduction (Cabezas et al., 2007; Sheriff et al., 2009). Moreover, studies on animal models indicate that
386 chronic stress can reduce immune-efficiency (Sapolsky et al., 2000), and further research will explore

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

389 The interaction between invasive and native species is a phenomenon that occurs through many
390 different mechanisms (e.g. direct competition, parasite-mediated competition, disease transmission,
391 predation, habitat destruction or alteration) in a wide variety of ecosystems. This study shows that direct
392 and/or indirect effects of increases in physiological stress in native species caused by the presence of an
393 invasive alien species should be considered as one of these mechanisms. We suggest that future studies
394 should assess if and how changes in stress levels are involved in species interactions and invasion processes
395 and to what extent it has direct or indirect (e.g. by increasing susceptibility to parasite infections) fitness
396 consequences for the native species.

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404 **Authors' contributions**

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

411

412 **Data accessibility**

413 Data will be available from the Dryad Digital Repository.

414 **References**

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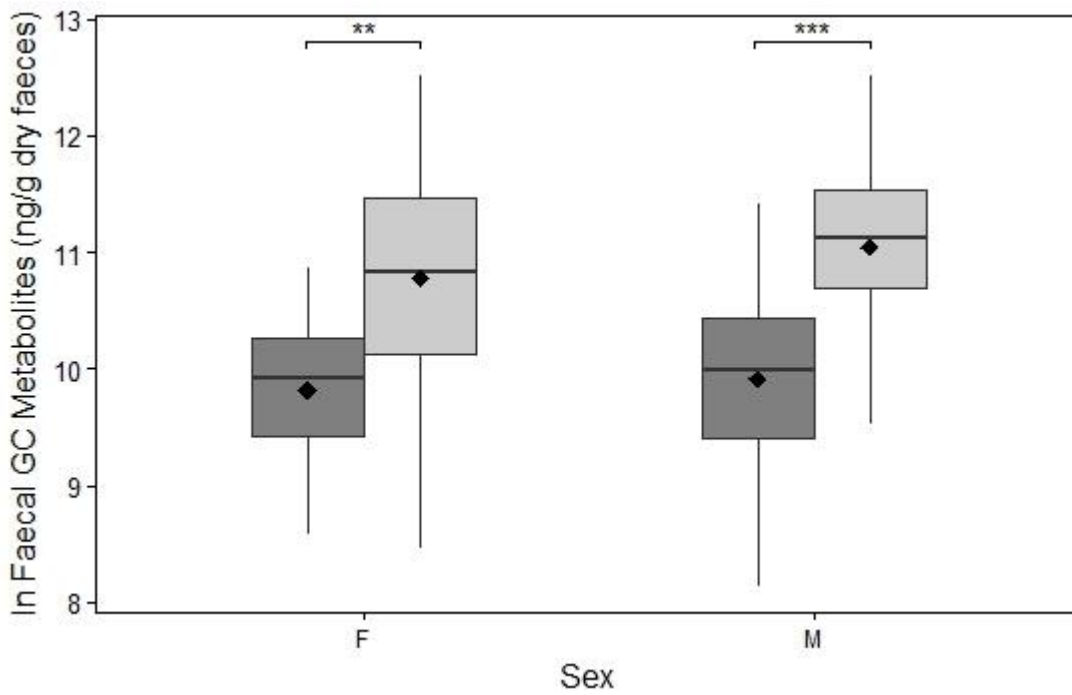
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603 **Table 1.** Data of the grey squirrel removal experiment in four study sites with both squirrel species. Period =
604 capture period with removal (see methods); Mean Number Alive (MNA) of red squirrels (density/ha
605 between brackets); Estimated number of grey squirrels present at the start of each removal period
606 (density/ha between brackets); Number of grey squirrels removed during each capture period, m_i , (the
607 cumulative % grey squirrels removed at the start of the capture period, hence this is 0% for Period 1). Ln
608 FGM (ng/g dry faeces) = changes in the concentration of FGM (Ln FGM, mean \pm SD, sample size between
609 brackets) over time (period) in red squirrels, following removal of alien 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)
Vanzago (74.73 ha)				
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
Lambro (18.43 ha)				
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
Passatempo (18.33 ha)				
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
Castelbarco (65.86 ha)				
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

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613 **Figure 1.** Comparison of faecal glucocorticoid (GC) metabolite concentrations in Eurasian red squirrels
 614 between red-only (dark-grey colour; 125 samples, 57 females, 68 males) and red-grey (light-grey colour;
 615 135 samples, 57 females, 78 males) study sites per sex (F = females; M = males). Boxplots show median
 616 (solid horizontal line), mean (black diamond) and 1st (25%) and 3rd (75%) quartiles. ** $p < 0.01$, *** $p <$
 617 0.001.

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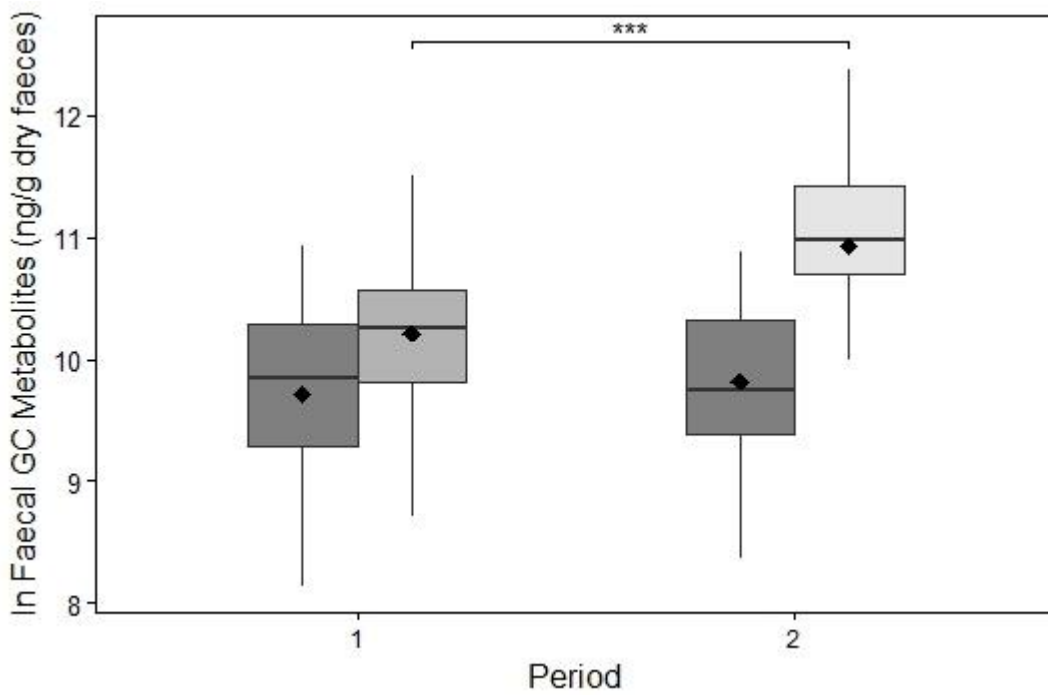
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630 **Figure 2.** Comparison of faecal glucocorticoid (GC) metabolite concentrations in Eurasian red squirrels

631 between pre- colonisation (period 1, grey colour, 29 samples) and post- colonisation (period 2, light-grey

632 colour, 58 samples) of sites invaded by the grey squirrels, and over the same time-period in areas not

633 colonised by the invasive species (period 1, dark-grey colour, 34 samples; period 2, dark-grey colour, 62

634 samples). Boxplots show median (solid horizontal line), mean (black diamond) and 1st (25%) and 3rd (75%)

635 quartiles. *** $p < 0.001$.

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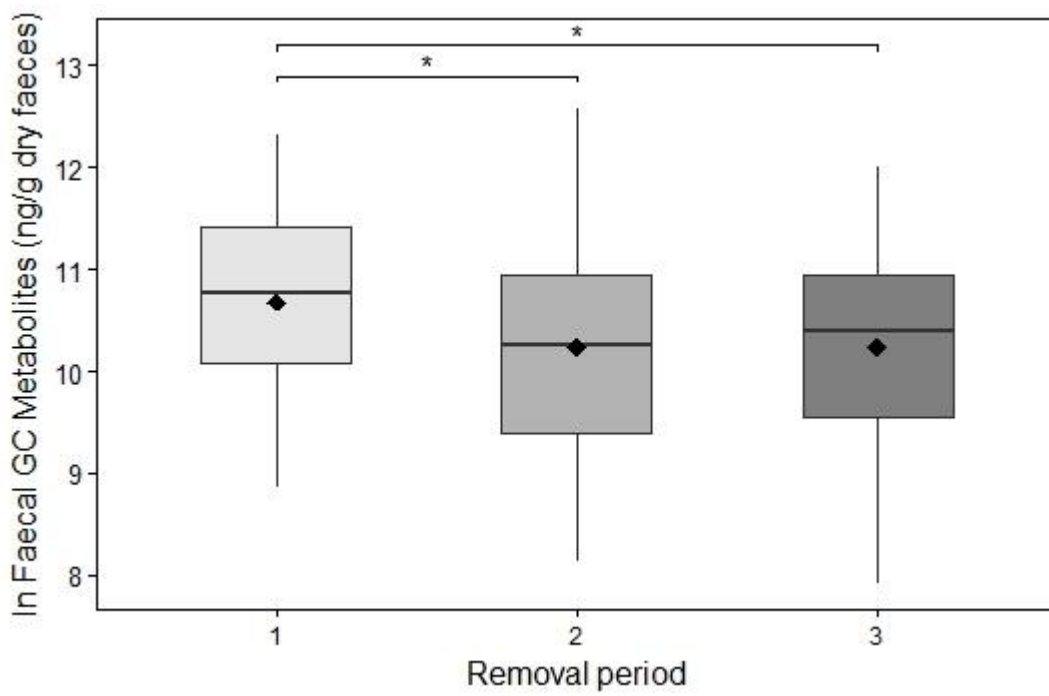
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646 **Figure 3.** Comparison of faecal glucocorticoid (GC) metabolite concentrations in Eurasian red squirrels in
 647 trapping period 1 (high grey squirrel density, light-grey colour), period 2 (with lower numbers of grey
 648 squirrels, grey colour) and period 3 (low grey squirrel density, dark-grey colour). During the removal
 649 experiment 131 samples of 67 different red squirrels were collected: 38 at the start of removal (period 1),
 650 47 during period 2 and 46 samples during period 3. Boxplots show median (solid horizontal line), mean
 651 (black diamond) and 1st (25%) and 3rd (75%) quartiles. * $p < 0.05$.