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You're stressing me out! Effect of interspecific competition from red deer on roe deer physiological stress response

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Abstract

Cortisol is the main glucocorticoid hormone commonly measured to assess adreno-cortical activity in vertebrates in response to potential stressors. Competition and habitat quality may act as chronic stressors leading to an increase in cortisol accumulation. Moreover, chronic cortisol concentrations may seriously hamper the reproductive potential, health status and body condition of a species. Using hair collected from the rump of 88 adult roadkill roe deer *Capreolus capreolus* in the north-eastern Italy, we aimed to assess the effect of both ungulate competitor population densities and habitat suitability on the long-term physiological stress response of roe deer, using hair cortisol as a reference parameter. Our findings revealed that roe deer showed higher cortisol concentrations in response to higher red deer *Cervus elaphus* population density, most likely as a consequence of direct interspecific competition. No significant cortisol changes were however recorded in response to both wild boar *Sus scrofa* and roe deer population densities, probably as a result of reduced competition because of abundant feeding resources, different species population densities and spatial/temporal avoidance among species. Moreover, the absence of a significant effect as far as habitat suitability is concerned, suggest that roe deer are well adapted to live even in poor quality habitats most likely as long as they are close to better quality habitats which provide access to food resources and refuge sites. Our results highlight the importance of hair cortisol as a suitable parameter to assess physiological chronic stress response towards different environmental/ecological conditions.

Introduction

The acute stress response system allows individuals to perform better during potential challenging situations (Sapolsky et al., 2000; Wingfield et al., 2011). In fact, the activation of the hypothalamic–pituitary–adrenal (HPA) axis leads to the release of glucocorticoids with the aim to re-establish the normal activities (Sapolsky et al., 2000). As part of the acute stress response, glucocorticoids promote energy mobilization by stimulating catabolic actions and through the inhibition of long-term functions such as reproduction to redirect energy towards potential emergency situations (Sapolsky et al., 2000; Wingfield et al., 1998). Cortisol is frequently measured to assess the adrenocortical activity in vertebrates and is widely recognized as the main glucocorticoid hormone involved in allostasis (McEwen, 1998a). Nevertheless, chronic levels of cortisol accumulation (i.e. chronic stress) as a consequence of prolonged stimulation of the HPA axis may induce some serious negative effects in terms of, for example, immune system

alterations (Sapolsky et al., 2000; Romero, 2004), lower body condition and survival (McEwen, 1998b), impaired cognition (McEwen & Sapolsky, 1995) and reduced fertility (Sapolsky et al., 2000). Cortisol concentrations are mainly measured in blood (Gardiner & Hall, 1997) and faeces (Fattorini et al., 2018). However, compared to other matrices, measuring cortisol cumulated in hair (Filacorda et al., 2021; Franchini et al., 2019; Prandi et al., 2018) represents a valuable method to assess chronic stress in wild species, since it is incorporated into the hair shaft during the anagen phase (i.e. active growth; Montillo et al., 2014) and over an extended time period (Meyer & Novak, 2012). Understanding the mechanisms of adrenal activity is important to assess the effects of those natural and/or non-natural conditions which may lead to an increasing allostatic load in wild species. Here, we examined the relationship between hair cortisol concentrations (hereafter, HCCs) in roe deer *Capreolus capreolus* and both intra- and interspecific competition and habitat suitability, in order to assess how these variables impacted HCCs of roe deer.

The roe deer is an ecotonal ungulate species which in Friuli Venezia Giulia (hereafter, FVG) share its habitat with other ungulates such as the red deer *Cervus elaphus* and the wild boar *Sus scrofa* (Piano Faunistico Regionale, 2015). Low presence of natural predators including the Eurasian brown bear *Ursus arctos*, grey wolf *Canis lupus* and Eurasian lynx *Lynx lynx*, especially in the past, may have promoted increases in abundance of this species at a regional level. However, despite the more recent recovery, the roe deer population is now declining in various regional territories due to several factors including illegal poaching, incorrect hunting management plans, habitat transitions from young forests and scrublands to old forests, collisions with road vehicles and competition with the red deer (Piano Faunistico Regionale, 2015). Competition among individuals is expected to be the most common interaction among herbivores in the absence of large predators (Putman, 1996). Furthermore, it represents one of the main elements affecting population dynamics and distribution (Begon et al., 2006), along with metabolic responses of wild species (Fattorini et al., 2018; Filacorda et al., 2021; Santicchia et al., 2018). Resource competition between red and roe deer (Borkowski et al., 2021; Richard et al., 2010), as well as between wild boar and roe deer (Melberg, 2012), have already been documented and may significantly affect the roe deer long-term physiological stress response. Furthermore, even habitat quality may exert a key role in shaping the HPA axis response (Jaimez et al., 2011; Martínez-Mota et al., 2007; Rangel-Negrín et al., 2009). Physiological stress response in wild species towards habitats of poor quality has already been assessed in other species including primates (Jaimez et al., 2011; Martínez-Mota et al., 2007; Rangel-Negrín et al., 2009), felids (Filacorda et al., 2021), rodents (Cordeschi et al., 2021) and ungulates (Spong et al., 2020). Nevertheless, the results presented are contradictory and mostly refer to the impact of habitat fragmentation (e.g. Filacorda et al., 2021; Martínez-Mota et al., 2007) and/or anthropogenic disturbance (e.g. Cordeschi et al., 2021; Spong et al., 2020). Physiological stress responses in roe deer towards landscape modifications (Klich et al., 2020) and habitat quality (Horcajada-Sánchez et al., 2019) are still poorly evaluated. Furthermore, to the best of our knowledge, research dealing with the physiological stress response of the roe deer in response to the presence of larger potential competitors are still absent, thus claiming the need of further investigation.

The purpose of this research was hence to assess the effect of ungulate population density and habitat suitability on the HPA axis response of roe deer, by measuring HCCs of individuals killed by road vehicles in different areas of the FVG region (north-eastern Italy). We assumed that (1) the presence of conspecifics and/or other ungulates, such as red deer and wild boar, would produce chronic levels of cortisol accumulations in the hair of roe deer, and therefore we predicted to observe higher HCCs in the areas characterized by higher ungulate population densities; (2) habitat quality may significantly affect the physiological stress response of roe deer because of the abundance and/or quality of the available resources. Therefore, we predicted to observe higher cortisol concentrations in response to low-suitability habitats and/or lower cortisol concentrations in relation to high-suitability habitats.

Materials and methods

Study area

The FVG is the most north-eastern Italian region (Fig. 1a) and is characterized by a mosaic of different habitats in relation to the altitude considered. Alpine areas, primarily composed of woodlands and forests habitats, include both the Carnic and Julian Alps, in which the highest peaks exceed 2700 m.a.s.l. (i.e. Coglians 2780 m). Both Alpine and pre-Alpine areas are characterized by forested and shrubland habitats while lowland areas present less vegetation cover mainly due to logging and agriculture.

Within the region, red deer, wild boar and roe deer live in sympatry and their abundance varies at different elevations (Piano Faunistico Regionale, 2015). The red deer are mainly distributed in the Alpine and pre-Alpine areas (Piano Faunistico Regionale, 2015). In 2018, 10 939 individuals were estimated to be present (Official Website of the Friuli Venezia Giulia Region, 2021). As far as the wild boar is concerned, the species occupies Alpine, pre-Alpine and lowland areas at different densities (the highest ones reached in Alpine and pre-Alpine territories; Piano Faunistico Regionale, 2015), with 4062 specimens counted in the same year across the region (Official Website of the Friuli Venezia Giulia Region, 2021). As with the red deer, the roe deer is mainly distributed in the Alpine and pre-Alpine territories, while lowlands represent neo-colonization areas (Piano Faunistico Regionale, 2015). The species estimated the population at 27 323 individuals (at the very least) in 2018 (Official Website of the Friuli Venezia Giulia Region, 2021). Data on red deer, wild boar and roe deer population density estimates were obtained from censuses performed from 2016 to 2018 at local hunting reserves (Official Website of the Friuli Venezia Giulia region, 2021; Romani et al., 2018) and considering the locations of roadkill events.

Data collection and analysis

Hair samples were opportunistically collected from the rump of 88 adult roe deer (40 males, 42 females and six sex-unidentified individuals) as roadkill in Alpine (*n* = 37), pre-Alpine (*n* = 29) and lowland areas (*n* = 22) of the FVG region (Fig. 1a) in the period spanning from 2016 to 2018. Because roe deer do not typically show long seasonal migration patterns (Mysterud, 1999; Ramanzin et al., 2007; Wahlström & Liberg, 1995), have small home ranges (Morellet et al., 2013; Mysterud, 1999; Ramanzin et al., 2007) and are typically philopatric and territorial (especially males; Wahlström & Liberg, 1995), we may affirm that HCCs measured in roadkilled specimens were the product of physiological stress response activated by the individuals to better counteract the potential challenging situations imposed by the environmental conditions faced at these elevations. The choice to collect hair from the rump was made because this represents an area with a lower chance of contamination by external agents (e.g. soil elements). All individuals were classified as adults based on tooth eruption patterns and wear rate in the jaws (Høye, 2006).
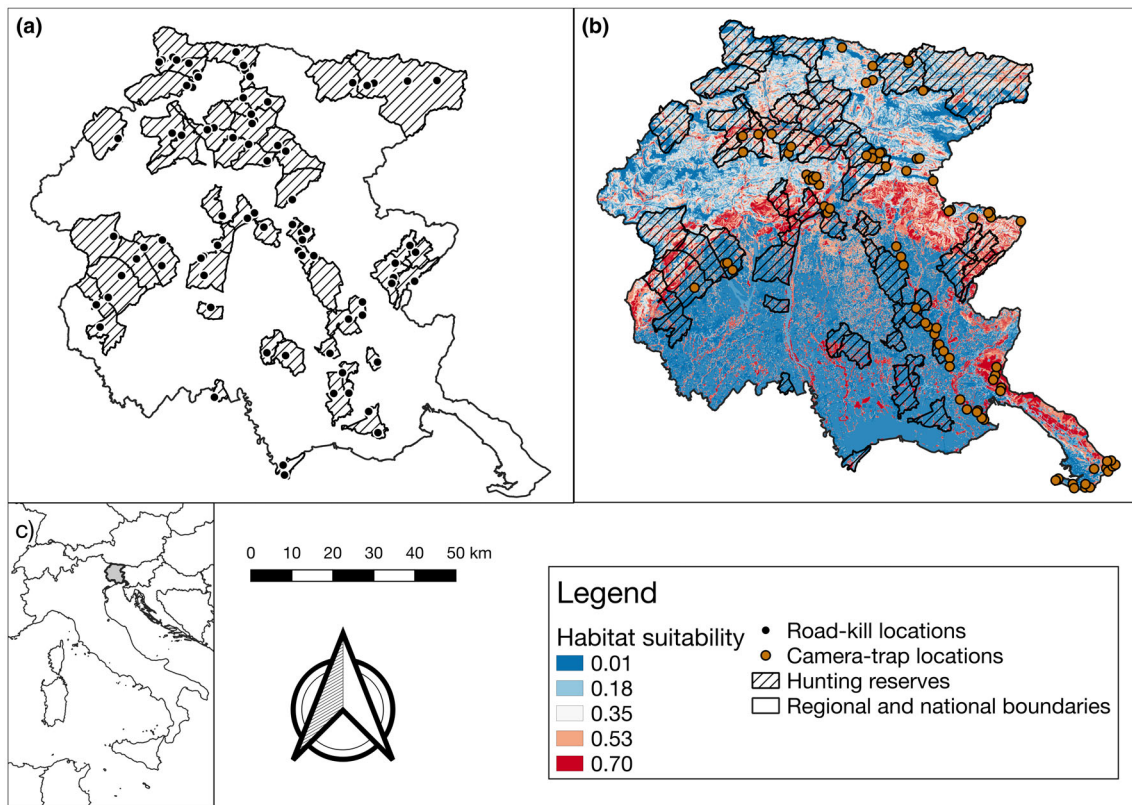
Figure 1 Location of the study area (Friuli Venezia Giulia region) along with (a) roadkill locations, and (b) habitat suitability map realized for the roe deer using camera trap locations. Panel (c) indicates the location of the Friuli Venezia Giulia region in Italy. Hunting reserves where roadkilled roe deer were found are indicated by black borders.

Since there is a time delay between cortisol incorporation into the hair and the time spent by this hair section arriving at the skin surface (Montillo et al., 2014; Stalder & Kirschbaum, 2012), we assume that the fatal vehicle collision has not affected cortisol accumulation in the hair in the short-term, as glucocorticoids accumulated in such a matrix are laid down over an extended time frame, varying from weeks to years (Meyer & Novak, 2012). Because the roe deer undergo two moultings, one in the autumn and another in the spring (approximately in April/May; Johnson & Hornby, 1975), we used hair samples collected from November up to May to recreate the HPA axis activity during the entire period of hair growth. Furthermore, we did not obtain appreciable differences in terms of HCCs between months (see Results), which led us to exclude potential periodic effects linked to the species' behavioural ecology.

Hair cortisol analysis

Strands of hair were placed in a glass tube and washed in 3 mL isopropanol (3 min at RT for two times) and dried. We then extracted cortisol from 50 mg of un-minced hair in a glass vial with 3 mL of methanol and incubated at 37°C for 16 h. After centrifugation, the methanol was decanted into a separate vial and evaporated to dryness at 37°C under an airstream suction hood. The remaining residue was dissolved in 0.35 mL of 0.05 M phosphate-buffered saline (PBS), pH 7.5 (RIA buffer). HCCs were measured using a solid-phase microtiter radioimmunoassay (RIA) procedure. In brief, a 96-well microtiter plate (OptiPlate, Perkin-Elmer Life Science, Boston, MA, USA) was coated with goat anti-rabbit γ-globulin serum, diluted 1:1000 in 0.15 mM sodium acetate buffer, pH 9 and incubated overnight at 4°C. The plate was washed twice with RIA buffer, pH 7.5 and incubated overnight at 4°C with 200 µL of the anti-cortisol serum diluted 1:20 000. The rabbit anti-cortisol antibody used was obtained from Biogenesis (Poole, UK). The cross-reactivities of this antibody with other steroids are as follows: cortisol 100%, corticosterone 1.8% and aldosterone <0.02%. After washing the plate with RIA buffer, standards (5–200 pg/well), a quality control extract, the test extracts and tracer (Hydrocortisone (Cortisol, [1,2,6,7-3H (N)]-), Perkin-Elmer Life Sciences, Boston, MA, USA) were added, and the plate was incubated overnight at 4°C. The bound hormone was separated from the free hormone by decanting the extract and washing the wells in RIA buffer. After the addition of 200 µL scintillation cocktail, the plate was counted on a beta-counter (Top-Count, Perkin-Elmer Life Sciences, Boston, MA, USA). The intra-assay and inter-assay coefficients of

variation were 3.6% and 9.8% respectively. The assay sensitivity (defined as the hormone concentration resulting in a displacement of the labelled hormone at least 2 standard deviations from maximal binding) was 1.23 pg/well. To determine the comparability between cortisol standards and endogenous cortisol in roe deer, hair samples containing high concentrations of endogenous cortisol were serially diluted in 0.05 M PBS, pH 7.5. The relationship between HCCs and the standard cortisol curve was determined through linear regression ($y = 0.9796x + 1.68$; $r = 0.99$).

Habitat suitability and spatial analyses

We used roe deer presence data ($n = 84$) obtained from opportunistic camera trapping to calibrate a habitat suitability model (HSM) through the implementation of the maximum entropy algorithm in MAXENT Software (v. 3.4; Phillips et al., 2006). The data refer to locations where the species was detected using 61 camera traps during 2019–2021 period. MAXENT is a popular machine learning method for predictions of species distributions using presence-only data (Elith et al., 2006; Phillips et al., 2006) and it has low sensitivity to small sample sizes (Wisz et al., 2008). Predictor values are compared at species locations with a sample of random background points and a map of occurrence probability is created (Phillips et al., 2006). However, HSMs may be affected by sampling bias (i.e. unequal survey effort) (Bystriakova et al., 2012; Leitão et al., 2011), which may artificially increase spatial auto-correlation of locations, potentially leading to model overfitting (Boria et al., 2014). Among the various ways to account for sampling bias, Fourcade et al. (2014) found that spatial filtering (i.e. subsampling regularly in the geographical space of the species' locations) performed well among different types of sample bias. Therefore, we used the 'spThin' R package (Aiello-Lammens et al., 2015) to spatially thin our initial dataset while retaining the maximum number of locations that were at least 500 m apart. Such a cut-off was chosen because of the high habitat diversity in a relatively small area (FVG). Furthermore, we tested different MAXENT implementations using the 'ENMeval' R package (Muscarella et al., 2014) because MAXENT outputs are sensitive to initial modelling settings (Merow et al., 2013). This package helps to find the best settings configuration which optimizes the trade-off between the goodness-of-fit and overfitting (Muscarella et al., 2014). We tested for different regularization values and feature classes using default settings, resulting in 48 combinations of the latter. The model which showed the lowest Akaike's information criterion corrected (AICc; Burnham & Anderson, 2002) was considered as the best model. To furtherly reduce the degree of overfitting, we used the *checkerboard2* partitioning method (Muscarella et al., 2014) which represents a variation of the 'masked geographically structured' data partitioning proposed by Radosavljevic and Anderson (2014). We randomly sampled 10 000 locations across our study area as background points. The predictive performance of the model was assessed by measuring the area under the receiver operating characteristic curve (AUC; Hanley & McNeil, 1982) and the difference between calibration and evaluation AUCs (AUCdiff; Warren & Seifert, 2011).

We selected five meaningful environmental variables (Fourcade et al., 2017) according to roe deer habitat requirements (Evcin et al., 2019; Mahmoodi, Shabani, Zeinalabedini, Ashrafi, et al., 2020; Mahmoodi, Shabani, Zeinalabedini, Khalilipour, et al., 2020) to calibrate the HSM: land use/land cover (LULC), tree cover density, imperviousness, elevation and slope. The roe deer has been defined as a wood-dependent species (Putman, 1996; Lovari & San Josè, 1997; Morellet et al., 2011) moving through densely vegetated areas (Hansson, 1994), even though they may locally colonize a variety of habitat types because of its ecological plasticity (Cibien et al., 1989; Kaluzinski, 1974; Morellet et al., 2011). Therefore, as habitat variables, we investigated landcover and tree density cover. As the landcover variable, we used the 2018 Corine Land Cover raster file which was reclassified into 11 new land-cover types (urban areas, arable lands, permanent crops, pastures and grasslands, complex cultivations, cultivations with natural elements, broad-leaved woodlands and shrublands, coniferous forests, mixed forests, areas without vegetation and wetlands). Moreover, we analysed topographic variables (i.e. elevation and slope) since it has been shown that they potentially affect roe deer habitat suitability (Evcin et al., 2019; Mahmoodi, Shabani, Zeinalabedini, Ashrafi, et al., 2020; Mahmoodi, Shabani, Zeinalabedini, Khalilipour, et al., 2020). Finally, because roe deer spend much of their daytime in woodlands, near human settlements and infrastructures (Bonnot et al., 2012), we investigated the degree of human-modified landscapes throughout the imperviousness variable, which represents the percentage of soil sealing. All the environmental variables were obtained from the Copernicus service (© European Union, Copernicus Land Monitoring Service 2020, European Environment Agency) and represented as raster layers of 100 m cells. Multicollinearity was tested using the variance inflation factor (VIF) which measures to what extent the variance of a regression coefficient is inflated due to multicollinearity in the model (Zuur et al., 2010). Because the VIF was <5 (Akinwande et al., 2015), all variables were included in the analysis.

In order to investigate the influence of habitat suitability on HCCs, we generated a 610 m radius buffer around the roadkill locations, and we calculated the mean of the habitat suitability values within each buffer. Such a radius was chosen to reproduce the average home-range size of a male roe deer within the alpine environment (Ramanzin et al., 2007). Thereby, we calculated the mean habitat suitability value within each buffer.

However, comparing data from different periods (i.e. roadkills and camera trapping) may cause misleading inferences due to changes in species distribution and environmental conditions. We are confident that the habitat suitability map can be meaningful for the 2016–18 roadkill data because no substantial changes occurred from 2016 to 2021 in roe deer numbers and distribution (Official Website of the Friuli Venezia Giulia region, 2021), or in environmental conditions (© European Union, Copernicus Land Monitoring Service 2020, European Environment Agency).

Because the habitat suitability index varies from 0 to 1, to assess the proportions of roe deer inhabiting habitats of different quality, we arbitrarily divided the latter into three classes:

'lower' (index range = 0.00–0.33), 'intermediate' (index range = 0.33–0.66) and 'higher' (index range = 0.66–1).

Statistical analyses

The difference in terms of HCCs among months was tested using the Kruskal–Wallis non-parametric test (Kruskal & Wallis, 1952). Data normality distribution was assessed through the Shapiro–Wilk normality test implemented in R, while the homogeneity of variance was checked using the non-parametric Levene's test using the 'car' R package (Fox & Weisberg, 2019).

Because of the relatively small number of samples, statistical analyses were divided into two steps: in the first step, the single effect of sex, competitors (i.e. red deer, wild boar and roe deer population densities, expressed as n. ind./100 ha) and habitat suitability was tested using generalized linear models (GLMs; Zuur et al., 2009) with residuals normally distributed and an *inverse* link function. The choice of the link function to use was taken after implementing three most complex models with three different link functions (i.e. *inverse*, *identity* and *log*), and then we selected the one showing the lowest Akaike's information criterion (AIC—Akaike, 1974). In the second step, the interactive effect between both sex and habitat quality with the significant independent variable obtained from the first step (see Results) on HCCs, was tested using further sets of GLMs (Zuur et al., 2009) with residuals normally distributed and an *identity* link function. The decision to use GLMs was taken because the assumptions of linear regression models, tested using the 'gvlma' R package (Pena & Slate, 2006), were not met. Furthermore, in the whole analysis, HCCs (pg cortisol/mg hair) were log-10 transformed to reduce data variation, minimize the effect of outliers and encourage data normality distribution.

Multicollinearity among covariates was tested using the VIF and considering VIF ≥ 5 as a threshold value to define high correlation among variables (Akinwande et al., 2015). Model simplification was done using the *principle of parsimony* based on which a maximal model was fitted and then simplified through the removal of non-significant explanatory variables. Model ranking was done based on the AIC (Akaike, 1974) and ΔAIC (Burnham & Anderson, 2002). In the presence of models showing ΔAIC < 2 (hence, considered as competitors of the best model; Burnham & Anderson, 2004), we performed model averaging by calculating the Akaike's weight (ωi), which express the relative amount of variation explained by each model compared with all other models (Burnham & Anderson, 2002).

Statistical analyses were conducted using the R Software (v. 4.0; R Development Core Team, 2021) and setting the *alpha* value (i.e. level of significance) at 0.05.

Results

Habitat suitability calculation

After applying spatial filtering, we kept a final dataset of 76 roe deer camera trap locations (see Fig. 1b). Among the 48

combinations of model settings, the one with the lowest ΔAICc had a regularization multiplier set to three and included linear + quadratic + hinge + product feature classes. The model showed very good predictive performance based on the AUC (0.83, var = 0.003) and the difference between calibration and evaluation AUCs was low (0.04, var = 0.004).

Tree cover density and slope mostly affected the HSM. Specifically, higher suitability values were depicted within higher tree cover densities and less steep slopes. Moreover, imperviousness and landcover slightly affected the HSM, whereas elevation had the lowest contribution. Indeed, pastures, broadleaved forests and areas without vegetation were the mostly influential landcover types, while HSM values slightly decreased when imperviousness increased. In conclusion, HSM values did not change when elevation increased.

The results obtained revealed that $n = 40$ (45.45%) roe deer inhabited 'lower' quality habitats, while $n = 48$ (54.55%) 'intermediate' quality habitats (Appendix S1).

Factors affecting hair cortisol concentrations (HCCs)

The Kruskal–Wallis test revealed no significant difference ($\chi^2 = 2.11$, $P = 0.15$) in terms of HCCs between months.

From the first set of GLMs, it turned out that the best model was the one in which the single effect of the independent variables sex and red deer population density was considered (Table 1). This model revealed no significant differences (GLM, $resD = 7.99$, $P = 0.12$) in terms of HCCs between sexes (males: mean ± SD = 0.48 ± 0.31; females: mean ± SD = 0.37 ± 0.33) and a significant effect (GLM, $resD = 7.99$, $P = 0.04$) of red deer population density, the HCCs being higher in response to increasing red deer population density (Fig. 2).

Also, the second set of GLMs revealed that the best model was the one in which the single effect of the independent variables sex and red deer population density was considered (Table 2). This model revealed no significant differences (GLM, $resD = 7.96$, $P = 0.12$) in terms of HCCs between sexes and a significant effect (GLM, $resD = 7.96$, $P = 0.04$) of red deer population density, the HCCs being higher in response to increasing red deer population density (Fig. 2).

Discussion

Hair cortisol concentrations between months and sexes

Our findings revealed the absence of significant variations in HCCs between months and are in line with other studies in which the effect of season/period have produced mixed results. For instance, in Eastern chipmunks *Tamias striatus*, HCCs were higher in samples collected during summer than in spring (Martin & Réale, 2008). In brown bears, HCCs tended to be higher in hair samples collected in late summer and autumn than in those harvested during spring (Cattet et al., 2014). The rise in HCCs from spring to summer has seemed to be more intense in cold temperate regions than in warm temperate ones

Table 1 Generalized linear models (GLMs) ranking with the best model marked in *italic*

Model ID	Independent variable/s	K	−2loglik	AIC	ΔAIC	ωi
1	*Sex + red*	*3*	*44.29*	*50.29*	*0.00*	*0.30*
2	Sex + boar + red	4	42.34	50.34	0.05	0.29
3	Red	2	47.16	51.16	0.87	0.19
4	Sex + habitat + boar + red	5	41.78	51.78	1.49	0.14
5	Sex + habitat + roe + boar + red	6	41.69	53.69	3.39	0.05

AIC, Akaike's information criterion; boar, wild boar density (n. ind./100 ha); habitat, habitat suitability; K, number of parameters; logLik, log-likelihood; red, red deer density (n. ind./100 ha); roe, roe deer density (n. ind./100 ha); ΔAIC, difference with the AIC value of the best model; ωi, Akaike's weight.
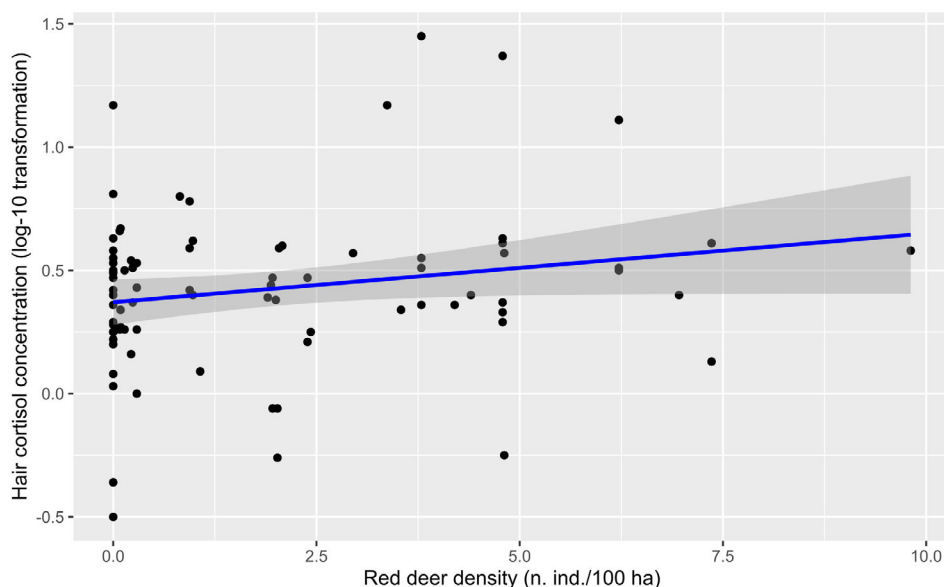
Figure 2 Relationship between roe deer hair cortisol concentration (log-10 transformation) and red deer density (n. ind./100 ha).

Table 2 Generalized linear models (GLMs) ranking with the best model marked in *italic*

Model ID	Independent variable/s	K	−2loglik	AIC	ΔAIC	ωi
1	*Sex + red*	*3*	*44.00*	*50.00*	*0.00*	*0.38*
2	Sex	2	46.77	50.77	0.77	0.26
3	Sex + habitat + red	4	43.37	51.37	1.37	0.19
4	Habitat:red + sex + habitat + red	5	42.95	52.95	2.95	0.09
5	Sex:red + habitat:red + sex + habitat + red	6	42.02	54.02	4.02	0.05
6	Sex:habitat + sex:red + habitat:red + sex + habitat + red	7	41.04	55.04	5.04	0.03

Interaction between independent variables was denoted by ':'.

AIC, Akaike's information criterion; habitat, habitat suitability (average value); K, number of parameters; logLik, log-likelihood; red, red deer density (n. ind./100 ha); ΔAIC, difference with the AIC value of the best model; ωi, Akaike's weight.

(Uetake et al., 2018). Furthermore, higher stress may be associated with seasonal variations in sexual and territorial behaviours, in turn leading to higher HCCs (Ventrella et al., 2018). Therefore, the possible occurring monthly variations in hormone concentrations not reflected in HCCs in roe deer suggest that multiple factors may be involved in obtaining appreciable differences.

The lack of influence of sex on HCCs in our results is in accordance with other studies conducted on red deer (e.g. Caslini et al., 2016; Huber et al., 2003) and Alaskan caribou *Rangifer tarandus* (Ashley et al., 2011). Diverging or opposite results were obtained also in studies on other species. For instance, Lafferty et al. (2015) observed significantly higher HCCs in male American black bears *Ursus americanus* compared to females,

which are likely a consequence of multiple interacting factors (e.g. food availability, social interactions). Conversely, Cattet et al. (2014) found significantly higher HCCs in female brown bears compared to males, which may be partly explained by sex differences in the body condition index. In addition, studies conducted on Asiatic black bears *Ursus thibetanus* (Malcolm et al., 2013) and/or Canada lynx *Lynx canadensis* (Terwissen et al., 2013) failed to find appreciable cortisol variations between the sexes. Therefore, conflicting results between different studies suggest that a diverging cortisol secretion between males and females is hard to explain since it may be affected by several factors such as different behaviour, body condition and metabolism of gonadal steroids (Heimbürge et al., 2019).

Influence of direct competition

Our prediction regarding the potential effect of either intra- or interspecific competition on the long-term physiological stress response of roe deer was partially confirmed, as significant cortisol variations were observed only in response to red deer population density. Our findings are consistent with other studies in which negative effects exerted by red deer on roe deer have been highlighted. Richard et al. (2010) showed that the body mass of roe deer fawns decreased consistently in areas characterized by higher red deer population density, most likely as a consequence of the negative impact of red deer on vegetation which reduces the amount of high-quality food for roe deer. A similar result was obtained by Borkowski et al. (2021) when they found that roe deer population density was notably lower within the Słowiński National Park (where higher red deer population densities were recorded) compared to the neighbouring non-protected areas where hunting is allowed. They assumed that red deer at higher population density most likely exert a negative impact on the vegetation, thus reducing feeding resources for roe deer. According to Storms et al. (2008), red deer have a broader and more diverse diet than roe deer, especially during winter. Graminoids predominate in red deer diet (Gebert & Verheyden-Tixier, 2001), while *Rubus spp*. and forbs constitute the staple of the diet of roe deer (Obidziński et al., 2013). Dwarf shrubs are consumed by both species (Obidziński et al., 2013). Therefore, since red deer use almost all food items eaten by roe deer (Storms et al., 2008), we may expect that higher red deer population densities may exert a substantial effect on roe deer forage availability, thus leading to chronic HCCs in roe deer in relation to the potential challenging situation imposed by scarce food resources. In FVG, red and roe deer share the same habitats being distributed mainly in Alpine and pre-Alpine areas (Piano Faunistico Regionale, 2015). Living in sympatry can not only induce a negative effect on vegetation as a consequence of red deer overgrazing, but it may even involve direct interactions between the species. Ferretti et al. (2011) showed that roe deer tended to avoid fallow deer *Dama dama*, especially at feeding sites, as a consequence of fallow deer aggression. Therefore, we cannot exclude that potential physical interactions with red deer may lead to increasing HPA axis activity in roe deer.

Regarding wild boar, the absence of a significant increase in terms of HCCs in roe deer may be attributable to (1) the low

wild boar population density across the region (Piano Faunistico Regionale, 2015), and/or (2) behavioural changes adopted by roe deer to reduce the likelihood of encounters with wild boar (Melberg, 2012). Concerning the latter, it has been shown that roe deer fawns tend to be spatially segregated in relation to wild boar activity, suggesting that boars may have a potential impact on cervid fawns (Sadrak et al., 2015). Moreover, roe deer displayed a non-random movement pattern towards wild boar rooting behaviour. Furthermore, lower roe deer presence at rubbing trees (i.e. grooming sites) was found, suggesting that adult individuals avoided sites often frequented by wild boar. Finally, a pattern of temporal avoidance was found between the two species in which both roe deer and wild boar were active during the same hours but with different frequency.

Concerning the absence of chronic levels of HCCs in roe deer in relation to increased roe deer population density, beneficial interactions between intraspecific ungulates have been to date poorly described. In general, population density affects group size of large herbivores as reduced groups lead to important implications in terms of benefits (e.g. alleviating predation risk) and costs of social behaviours (e.g. competition for resources and mates, intraspecific pathogen transmission; Barja & Rosellini, 2008; Wal et al., 2013). However, antipredator benefits promote group formation in ungulates, and an inverse relationship between vigilance effort and group size has been frequently reported and thought to reflect the consequent decrease in perceived predation risk (Marino, 2010). Nevertheless, concerning the FVG, we cannot affirm that the aggregation of individuals may be the result of an antipredatory strategy since predators and especially wolves, which notoriously prey on roe deer in Italy (e.g. Mattioli et al., 2004), have only recently started to re-colonize the region, and therefore their population densities are still too low to consider this hypothesis plausible. Thereby, we assume that the absence of significant hair cortisol variations in relation to increased roe deer population density may be attributable to reduced competition between individuals due to the abundance of resources.

Effect of habitat suitability

Physiological responses of wild ungulates may vary depending on human disturbance (e.g. Zwijacz-Kozica et al., 2013), presence of anthropogenic structures (e.g. Klich et al., 2020) and habitat quality (Horcajada-Sánchez et al., 2019). Our prediction regarding the effect of habitat suitability on the HPA axis activity of roe deer was not realized, as we did not observe significant cortisol variation in response to this variable. Horcajada-Sánchez et al. (2019) found higher faecal cortisol concentrations in roe deer inhabiting pine compared to oak forests, thus confirming that the habitats preferred by roe deer are those mainly composed of open deciduous forest and rich undergrowth (e.g. Virgós & Tellería, 1998) which are considered relevant in influencing roe deer abundance and body condition (Padaiga, 1998; Pettorelli et al., 2001). In our research, habitat suitability was evaluated on a wider scale, considering multiple landscape features which contributed to defining the

overall level of habitat quality for the species. The results revealed that the proportion of roe deer inhabiting areas characterized by 'lower' and 'intermediate' quality habitat is almost 1:1. In this sense, similar levels of HCCs may be affected by the absence of a remarkable difference among individuals inhabiting habitats of different quality. However, the absence of chronic HCCs in roe deer may also suggest that the species is well adapted to live even in poor or non-optimal quality habitats. This result may find explanation in the remarkable ecological plasticity of the roe deer which allows the species to occupy different habitats ranging from closed deciduous forests to human-modified landscapes (Boisaubert & Boutin, 1993; Mateos-Quesada, 2005). Moreover, several studies have reported that roe deer show behavioural changes according to the habitat they occupy and modifications they endure (Jepsen & Topping, 2004; Maublanc et al., 1987).

This study represents one of the first important contributions in the exploration of the ecological factors potentially affecting the long-term physiological stress response in roe deer. Based on the results obtained, we speculate that chronic HCCs in roe deer in response to higher red deer population densities may be due to negative red–roe deer interactions in terms of food depletion and direct aggression exerted by red deer on roe deer. However, we did not analyse red deer impact on the biomass of the main vegetation groups (including those which represent the staple of the diet of roe deer), and we did not conduct any behavioural analysis to assess aggressive interactions involving both species. Moreover, we recognize that census data collected by hunters may be prone to bias and require data validation through integration with information directly collected in the field (i.e. pellet counts). Lastly, even climatic condition may have a prominent role in shaping the HPA axis activity of wild ungulates (e.g. Spong et al., 2020). In our case, carcasses of roe deer were collected during the entire wintering period and at different elevations. Consequently, the physiological stress response of roe deer might also have been influenced by the harsh climate at higher altitudes. Further research is therefore strongly needed to cover these aspects in detail.

The use of cortisol as a biomarker to evaluate the physiological stress response of wild species towards challenging situations has acquired remarkable attention, especially in recent years. However, the impacts of environmental and/or ecological factors on the physiological stress response of wild ungulates using hair cortisol as a reference parameter are still poorly understood. Our findings confirmed the importance of hair cortisol as a suitable indicator to assess physiological adaptations of a species towards different natural/non-natural conditions. Despite these considerations, the examination on whether these changes are adaptive will require further research to better understand the impact of these stress-related changes on ungulate populations.

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Conflict of interests

No potential conflict of interests is reported by the authors.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Mean habitat suitability value calculated within each buffer and for each road-kill location. Because the habitat suitability index varies from 0 to 1, the habitat quality was arbitrarily divided into three classes: 'lower' (index range = 0.00–0.33), 'intermediate' (index range = 0.33–0.66), 'higher' (index range = 0.66–1).