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**Increased hormonal stress response of Apennine chamois induced by interspecific interactions and anthropogenic disturbance**

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**Abstract**

Responses of animals to environmental changes, and their interactions with other species play an important role in conservation. Sharing a common habitat may lead to interspecific competition for resources, but field assessment of these biological events is not always easily accomplished. By using a non-invasive method, we evaluated the physiological stress responses of Apennine chamois (*Rupicapra pyrenaica ornata*) to the presence of cattle, sheep and goat, red deer (*Cervus elaphus*), people (hikers) and predators to identify which factors may affect this endangered species. During September 2012, November 2012 and July 2013, a total of 318 faecal samples was collected in representative sites and analysed for faecal cortisol metabolites (FCM). FCM concentration was analysed through linear mixed effect models. A significant increase in FCM values in Apennine chamois sharing their habitat with domestic animals was recorded during all study periods. On the contrary, stress responses to red deer and people were limited in time and emerged only during summer months, when hikers are more frequent and red deer extend their altitudinal range reaching chamois' habitat. The observed effects of domestic animals, red deer and hikers should be considered in future Apennine chamois management plans, which should include the regulation of pastured domestic livestock, anthropogenic disturbances and possible interferences with other wild species within parks.

**Keywords**

Competition, *Rupicapra pyrenaica ornata*, *Cervus elaphus*, domestic ruminants, human activities, glucocorticoids

## Introduction

For all animal species, a thorough evaluation of factors influencing individual fitness is paramount to the implementation of wildlife management and conservation strategies. Some interspecific interactions, such as competition, predation and parasitism can be detrimental to one or more components of fitness (Begon et al. 2006). In wild animal populations, determining these impacts by quantifying reduction in survival or reproduction can be problematic and/or requires long-term population studies (McCallum 2000). However, all environmental pressures, including interspecific competition, lead to physiological changes at the individual level and these variations in physiological parameters can be assessed and quantified efficiently, offering a measure of impacts induced by these pressures. Under natural conditions, animals can cope with environmental challenges by stimulating their endocrine system to secrete glucocorticoids (stress responses; Huber et al. 2003; Palme et al. 2005; Rehnus et al. 2009; Palme 2012; Corlatti et al. 2014). In wild ungulates, different glucocorticoid responses have been associated with several intrinsic physiological traits, such as age (i.e., growth), sex and reproductive status, digestion, immunity or energy mobilisation (Huber et al. 2003; Corlatti et al. 2014). Social and behavioural dynamics, especially during the mating period, can also increase hormonal reactions (Mooring et al. 2006; Fichtel et al. 2007; Corlatti et al. 2012, 2014). Additionally, physiological stress responses of wild ungulates can be influenced by extrinsic, predictable environmental factors, such as seasonal changes in food availability and temperature (Huber et al. 2003; Konjević et al. 2011; Jachowski et al. 2015); or even by less predictable events such as flooding (Corlatti et al. 2011), human disturbance (Cederna and Lovari 1985; Zwijacz-Kozica et al. 2013; Jachowski et al. 2015) or hunting (Corlatti et al. 2014; Santos et al. 2018; Zbyryt et al. 2018). Glucocorticoids have an adaptive value since they can provoke rapid physiological and behavioural adjustments that allow animals to react more efficiently to adverse/new circumstances (Möstl and Palme 2002; Sheriff et al. 2011, Formenti et al. 2015). However, when a stressor persists in time, it may lead to a chronic stress state that can have a severe impact on individual health and thus play a role in populations' decline (Corlatti et al. 2014; Arlettaz et al. 2015).

In the field, glucocorticoid levels should be evaluated through non-invasive methods (Palme et al. 1999), because invasive procedures (i.e. blood sampling) induce a stress response as a consequence of handling the animal, thus altering the target physiological and behavioural parameters (Huber et al. 2003; Munerato et al. 2015). On the contrary, faeces can be easily obtained without manipulation, and provide an integrated measure of fluctuating blood concentrations approximately 10-15 hours before sampling (Palme et al. 1999; Touma and Palme 2005; Kleinsasser et al. 2010).

The Apennine chamois (*Rupicapra pyrenaica ornata*) is endemic to central Italy and listed as 'especially protected species' under the Italian law (n. 157/1992). This subspecies is also included in annexes II and IV of the European

Union Habitats and Species Directive, and in appendix II of Convention on International Trade in Endangered Species of Wild Fauna and Flora (Lovari et al. 2014). The population is currently fragmented and restricted to the central, innermost parts of the Apennine and, despite a great conservation and repopulation effort, during the last decade the number of Appennine chamois has decreased by at least 30% (Lovari et al. 2014). Thus, there is a compelling need to evaluate the health status of Apennine chamois populations, and identify which environmental factors may affect this endangered species. Under these delicate conservation constraints, research must take care to not interfere with populations. Consequently, non-invasive methods are preferable, and even opportunistic sampling can be highly informative, due to the scarcity of available information.

Here, we took advantage of field sampling carried out for health monitoring purposes within the LIFE project “Cornata” (LIFE Cornata Team, 2015). We used this opportunistic sampling to analyse faecal glucocorticoid metabolites of Apennine chamois to retrospectively evaluate whether their hormonal stress responses are affected by interactions with other animal species/stressors present in their habitat. Specifically, we explored the effect on hormonal responses induced by predators (Dalmau et al. 2010), (i.e. wolves (*Canis lupus*), bears (*Ursus arctos marsicanus*)), and red deer (*Cervus elaphus*), which have been suggested to have an impact on Apennine chamois population dynamics (Ferretti et al. 2015). In addition, we evaluated whether human activities, such as farming or tourism (Patterson 1988), elicited any stress responses in Apennine chamois.

## <u>Material and methods</u>

### ***Study area***

The study areas are located in the Italian Central Apennine (altitudinal range: 900-2912 m. a.s.l.) within the boundaries of three National parks: Abruzzo, Lazio e Molise National Park (41°48'31.70''N, 13°47'24.29''E, 49.680 ha); Majella National Park (42°23'35.20''N, 13°45'24.20''E, 74.095 ha; the Gran Sasso e Monti della Laga National Park (42°29'26.33''N, 13°29'50.73''E, 148.935 ha) (Fig. 1). The Apennine chamois is present in all the three parks with reported population sizes of 600 (www.camosciodabruzzo.it), 840 (www.camoscioappenninico.it) and 622 (www.gransassolagapark.it) individuals (Antonucci et al., 2010a; Di Domenico et al., 2015), respectively. All three parks are inhabited by red deer and top predators (wolf and bear).

Fig. 1 HERE

### ***Field Sampling***

In 2012 and 2013, a total of 318 fresh faecal samples from Apennine chamois were collected opportunistically within a broader health monitoring plan (Antonucci et al. 2010b). In detail, in all three parks sampling was carried out in

September 2012 and July 2013, with an additional sampling period in November 2012, but only in the "Abruzzo, Lazio and Molise" National park. These months cover the main physiological seasonal changes of chamois biology, and incorporate the diverse environmental conditions the field sites experience over the year. In particular, July follows chamois parturition and during this month presence of hikers is high, and livestock (cattle (*Bos taurus*), sheep (*Ovis aries*) and goats (*Capra hircus*)) are present on the pastures. September is the period of chamois' weaning and in this month livestock and hikers are almost absent. During November the mating period occurs, but no particular other stressors are present (Zubiani and Latini, unpublished data; Asprea 2009). Unlike blood samples, faecal steroid metabolite concentrations are less affected by episodic fluctuations or by the pulsatility of hormone secretion and might represent the endocrine profile of an animal more accurately than a single plasma sample (Palme et al. 1999; Touma and Palme 2005). As FCM concentrations allow us to evaluate longer-term chamois reactions, our sampling design obviates the need to consider the direct interactions of chamois with a given stressor and their concomitant presence at the time of sampling. Therefore, within each park, two to three sampling sites were selected known to be representative for the presence of deer/people (i.e., hikers)/livestock/predators. In detail, for each area we defined whether red deer were present or absent based on annual censuses and seasonal dynamics (Duprè et al. 2001). Similarly, data on livestock presence (cattle, sheep and goats) were obtained through the Italian Veterinary Informative System (Ministry of Health), which monitors the distribution of domestic animals within National parks. Due to the ranging behaviour of large predators (i.e. wolves and bears), their presence could not be excluded from any sampling area, hence sites were classified as high vs occasional predator occurrence, based on information supplied by the National parks. Finally, hiking is regulated by parks, with areas where human recreational activities are allowed and others where hikers are forbidden. A detailed description of the characteristics of each study site is provided in Table 1.

Table 1 HERE

Sampling was carried out by two experts that performed transects twice a day, both in the morning and afternoon (Lovari and Cosentino 1986; Richard-Hansen et al. 1992; McCullough et al. 1994; Largo et al. 2008). In order to collect exclusively fresh faecal samples and avoid repeated sampling from the same individual, animals were first localized visually and observed defecating (Caughley 1978; McCullough et al. 1993; Loison et al. 2006). Fresh samples were then collected only after the chamois had moved away to avoid any interference with the studied population. Samples were kept cold with silica-gel until storage at -20° C was possible.

***Analysis of glucocorticoid metabolites***

Faecal cortisol metabolites (FCM) were analysed with an 11-oxoaetiocholanolone enzyme immunoassay (EIA, Möstl et al., 2002), previously used in chamois (Corlatti et al. 2012). FCM concentrations reflect plasma hormone level and can therefore be used to monitor endocrine status (Touma and Palme 2005). Briefly, 0.5 g of each well-homogenized faecal sample was extracted with 5 ml of 80 % methanol (Palme et al. 2013). Next, an aliquot of the supernatant was further diluted with assay buffer and analysed with the above mentioned EIA.

***Statistical analyses***

FCM concentrations were log transformed and considered as the response variable in linear mixed effect models. Sampling month and the presence of red deer, cattle, sheep/goat, people (hikers) and predators were included as explanatory variables. To avoid pseudoreplication due to repeated sampling from the same study areas, each park was included in the model as a random factor. Since our opportunistic sampling did not provide a fully crossed and balanced design between explanatory variables, we first fitted a full model including those first order interactions which were both biologically meaningful and computationally possible. In detail these were: the interaction of sampling months with presence of red deer, hikers and sheep/goat, and the interaction between presence of hikers and red deer. Additionally, because sampling in November occurred only in one study area, which did not provide all the conditions of the other variables, we excluded the data sampled in this month. The full model was then simplified based on maximum likelihood ratio test and evaluation of AIC, by discarding those terms that did not contribute to fit the model (Bolker et al. 2009). Thus, we obtained a minimal adequate model which retained only those terms that contributed to describe FCM variability. To assess the goodness-of-fit of the minimal model, we estimated $R^2$ to provide the 'variance explained' by the model (Nakagawa and Schielzeth 2013). Post-hoc comparisons were based on pair-wise t-tests of Differences of Least Square Means (DLSM), applying Tukey correction for multiple comparisons. The analyses were performed using R 3.3.3 (R Foundation for Statistical Computing, 2017) fitting linear mixed-effect models with the 'lmer' function of the package lme4, "r.squaredGLMM" in the package MuMIn to estimate $R^2$, and "lsmean" in package lsmean for post-hoc comparisons; the significant threshold was $p < 0.05$.

## Results

Concentrations of faecal cortisol metabolites of chamois in the three parks are given in Figure 2.

Fig 2 HERE

The minimal adequate model (Table 2), with the lowest AIC, explained 61.3% of the FCM variation observed in our sample.

Table 2 HERE

FCM concentrations were affected by sheep/goats (Table 2), with a significant increase in FCM levels in areas where flocks were present (Fig. 3).

Fig 3 HERE

The effect of red deer and hikers on FCM concentrations depended on the sampling month (Table 2). The presence of red deer was associated with higher FCM levels during July (DLSM $p<0.0001$), while in September FCM levels were significantly lower in areas with red deer present compared to those where red deer were absent (DLSM $p<0.0001$ ) (Fig. 4).

Fig. 4 HERE

Similarly, the presence of hikers (Fig. 5) was associated with higher FCM levels during July (DLSM $p=0.0001$), while lower FCM levels were found during September (DLSM $p=0.0001$).

Fig. 5 HERE

## **Discussion**

The present study aimed to investigate physiological stress responses of Apennine chamois to interactions with red deer, predators, livestock and people. Higher FCM values were found in subjects sharing their habitat with flocks of sheep/goats during all the study period, while stress responses induced by red deer and hikers emerged only during specific sampling months, and predators had no effect on FCM concentrations.

Apennine chamois is an endangered species and poaching, habitat destruction, urbanization and epidemics are known to threaten its conservation (Crestanello et al. 2009). Some studies have highlighted that restocking/reintroduction of other ungulates can also have an impact on this species (Crestanello et al. 2009). Indeed, recent reintroductions and consequent progressive expansion of red deer in the study area have been suggested as an additional source of disturbance for Apennine chamois (Lovari et al. 2014; Ferretti et al. 2015). Similar results have been observed for the Alpine chamois (*R. r. rupicapra)* (Anderwald et al. 2015). Our results are consistent with previous studies as the higher FCM concentrations recorded in chamois sharing their habitat with red deer in July suggest that this species might be a source of disturbance. Indeed, during summer months the red deer extend their range to higher altitudes, thus overlapping with chamois' habitat (Clutton-Brock et al. 1982). The increase in FCM values could be due to direct and/or indirect interactions between the two species. On the one hand, there is an almost complete diet overlap between red deer and Apennine chamois (Lovari et al. 2014), on the other hand, large herds of red deer (up to 90 individuals) may affect vegetation quality and availability through grazing and/or trampling (Ferretti et al. 2015). However, while we found higher hormonal stress levels during summer when chamois share its habitat with red deer, during September

FCM concentrations were higher when red deer were absent. This suggests that other factors, not included in the present analyses, such as a differential distribution of food or seasonal changes in chamois behaviour, may act as additional and influential stressors. In particular, low quality food during autumn months (Bruno and Lovari 1989) together with the specific need of a protein rich diet following lactation and the need to restore energy reserves (Ferrari et al. 1988; Bruno and Lovari 1989) may influence glucocorticoid release. At the same time, changes in behaviour associated with the mating period could increase hormonal reactions (Mooring et al. 2006; Fichtel et al. 2007; Corlatti et al. 2012, 2014).

Moreover, our results revealed that hormonal stress responses are also influenced by tourist activities (i.e., hikers) and the presence of sheep and goats. While the former had a restricted temporal effect limited to the summer season when tourists' presence is higher (Patterson 1988; Zwijacz-Kozica et al. 2013), the effect induced by the presence of sheep/goats remained constant across the study period, highlighting a more continuous impact. These increases in cortisol responses could be ascribed either to spatial competition, with a potential segregation of the Apennine chamois (Chirichella et al. 2013), or to competition for food, as has been previously described in Alpine chamois (Fankhauser et al. 2008; La Morgia and Bassano 2009), Pyrenean chamois (*R. p. pyrenaica*) and Cantabrian chamois (*R. p. parva*) (Berducou 1984; Rebollo et al. 1993). Moreover, the presence of shepherds and their dogs, which are often free-ranging even during night, should be considered as further potential stressors for chamois (Stankowich 2008). Indeed, Chirichella et al. (2013) showed that Alpine chamois were more likely to be closer to rocks when large groups of domestic ruminants were close by, and especially when shepherd's dogs were present.

Our data suggest that Apennine chamois are influenced by the presence of other animal competitors and by human activities. Considering the adverse effect of glucocorticoids on individual metabolism and immunity (McEwen 1998), these increased hormonal responses could represent an additional threat for this species. This result highlights how, for endangered species, scientific investigations aimed at supporting their conservation should not be restricted to the analysis of population dynamics, but should be extended to include patho-physiological aspects. These studies would require thorough information on individual and environmental factors, such as quantitative data on the abundance of other competing species. Unfortunately, for endangered species information across multiple variables and scenarios is not always available, but scarcity of data in a given area should not inhibit investigation. It is clear that even limited data can provide important guidance for management strategies and/or indicate future research directions for endangered species. Our study, although partially affected by these limits, clearly suggests a negative influence of animal farming, and a time-limited negative effect of red deer and the presence of hikers, encouraging us to take these factors into account in future management plans.

In conclusion, our results indicate a relationship between increased stress hormonal levels of Apennine chamois and the presence of sheep/goats during the whole study period, and an effect of both red deer and hikers during the summer.

These higher FCM values might be detrimental to chamois as induced stress can alter an animals' body condition and reduce their resistance to diseases (Rehnus et al. 2014). Therefore, an increase in stress could harm these declining populations, and potential stressors must thus be considered as additional threats for this endangered species. The observed effects of livestock, red deer and people should encourage management plans to further investigate these effects, which should include the regulation of pastured domestic livestock, anthropogenic disturbances and possible interference of other wild species within the National parks.

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**Compliance with Ethical Standards**

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**Ethical approval:** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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## Figure Captions

**Fig. 1** Map of the three study areas and sampling locations (circles).

**Fig. 2** Mean (± SE) values of FCM (ng/g) recorded in the three parks and during the three sampling months

**Fig. 3** Model predictions of the effects of the presence/absence of sheep/goats on faecal cortisol metabolites (FCM) in Apennine chamois. FCM concentrations are presented in logarithmic values with 95% confidence limits given.

**Fig. 4** Model predictions of the effects of the presence/absence of red deer during the three sampling months on faecal cortisol metabolites (FCM) in Apennine chamois. FCM concentrations are presented in logarithmic values with the 95% confidence limits given.

**Fig. 5** Model predictions of the effects of the presence/absence of hikers during the three sampling months on faecal cortisol metabolites in Apennine chamois. FCM concentrations are presented in logarithmic values with the 95% confidence limits given.