University of Sassari
Ph.D. School in Natural Sciences
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DEALING WITH STRESS:
RESPONSES FOR A GOOD FITNESS ON ALPINE IBEX

PH.D. CANDIDATE: ***Nicoletta Sica***

DIRECTOR OF THE SCHOOL: ***Prof. Marco Curini Galletti***

SUPERVISOR: ***Prof. Marco Apollonio***

CO-SUPERVISOR: ***Dr. Stefano Grignolio***

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Summary

[ENGLISH] A disturbance that elicits allostatic individual responses in addition to that imposed by life cycle, is defined as stress. This thesis integrated behaviour and endocrinology (using fecal cortisol and testosterone metabolites) to analyse how Alpine ibex (*Capra ibex*) deals with environmental pressure. The level of cortisol was directly correlated with maximum temperature in summer, showing the sensitiveness of ibex to thermal stress. I demonstrated that the selective pressures have favoured a definition of the social status in advance respect to the mating season. It remained stable and affected male reproductive success. Testosterone and cortisol are correlated and they jointly reinforced the initial impulse to compete, potentiated pre-existing pattern of aggression, but they have no effects on dominance.

I then evaluated the behavioural response of ibex to changes in the landscape of fear. Ibex responded adjusting group behaviour and in particular adult males moved closer to safer areas at the expenses of a suboptimal food intake. I demonstrated, thanks to behavioural and endocrinological data, that the chemical immobilization employed to capture ibex has a reduce impact on ibex biology and it is suitable for conservation projects.

The findings of this thesis highlight the preeminent role of the environment in shaping ibex physiology and behaviour. Moreover shown the ability of the specie to efficiently respond and recover both to predictable and to unpredictable disturbance.

Key words: cortisol, dominance, individual fitness, stress, testosterone.

Riassunto

[ITALIAN] Lo stress è definito come una minaccia, percepita dall'individuo, che esula dai disturbi generalmente connessi con il ciclo vitale. Questa tesi, sviluppando tecniche di analisi comportamentale ed endocrina (grazie all'impiego dei metaboliti fecali di testosterone e cortisolo), analizza le strategie adottate dallo stambecco alpino (*Capra ibex*) in risposta alle pressioni ambientali. Nella stagione estiva è stata rilevata una correlazione positiva tra i livelli dei metaboliti fecali del cortisolo e la temperatura massima. Questo risultato sottolinea come lo stambecco sia particolarmente sensibile ad innalzamenti di temperatura.

Ho dimostrato come queste abbiano favorito una definizione precoce della scala gerarchica (nella stagione estiva). Questa rimante stabile fino alla stagione riproduttiva (in inverno) ed influenza direttamente il successo riproduttivo maschile. Testosterone e cortisolo sono correlati e agendo in sincronia rinforzano l'impulso iniziale alla competizione e potenziano *patter* di aggressione già intrinsechi nell'individuo, ma non sono invece direttamente correlati con la posizione gerarchica individuale.

In seconda analisi ho valutato la riposta dello stambecco a modificazioni della *lanscape of fear*. Lo stambecco risponde modificando i *patter* di aggregazione e in particolare i maschi adulti si avvicinano alle aree rifugio alle spese di un qualità di foraggiamento sub-ottimale.

Grazie all'integrazione di tecniche di analisi comportamentale ed endocrina, ho dimostrato come la tecnica di cattura utilizzata nell'area di studio (immobilizzazione chimica) abbia un impatto ridotto sulla biologia dello stambecco e sia quindi adatta a essere impiegata in progetti di cattura a scopi conservazionisti.

Questa tesi rileva come i pattern comportamentali e fisiologici caratteristici dello stambecco alpino si siano evoluti in risposta ad una forte pressione ambientale. Inoltre è stato possibile mettere inevidenza la grande plasticità della specie, che ha mostrato una riposta efficace ed un veloce recupero sia in seguito a disturbi prevedibili che non.

Parole chiave: cortisolo, dominanza, fitness individuale, stress, testosterone.

Introduction

Daily routine activities are centred in obtaining nutrition intake to maintain normal activities and to anticipate additional requirements e.g. breeding and acclimating to cold and heat (McEwen and Wingfield 2003). Despite fluctuating environmental conditions, individuals achieved physiological and behavioural stability through changes (allostasis). Allostasis comprises responses both to predictable (e.g. reproduction, seasonal changes) and to unpredictable (conflict in social hierarchy, competition for resources, predation or human disturbance) events.

Historically, every noxious stimuli as well as the coping response to which an individual is exposed were referred as “stress” (Romero 2004). McEwen and Wingfield (2003) clarify the use of this term defining it as threatening event for an individual, which elicits allostatic physiological and behavioural responses in addition to that imposed by daily life cycle.

Modifications of population demographic, behavioural and physiological patterns could reflect the impact of disturbance events, like human activities (Bleich et al. 1994), the presence of a predator (Creel et al. 2005) or unfavourable climatic conditions (van Beest et al. 2012). In most of the cases the consequences of stress disturbance are firstly expressed and observed at individuals’ physiology and secondly perceived at population level (Wingfield et al. 1998; Ellis et al. 2012). Physiological measures (mainly hormone levels) have greater predictive capacity than traditional demographic methods to detect in advance the effects of disturbance events (Wikelsky and Cooke 2006; Madliger and Love OP 2014).

Environmental endocrinology, a branch of physiology, analyses hormonal variation as indicators of reproductive function, nutritional or social stress. In this regards two classes of steroid hormones of particular interest are glucocorticoid and androgens. The first ones (cortisol in large mammals) are the front-line endocrine mechanisms to defend organism against stressful conditions (Möstl and Palme 2002) and are often referred as stress hormones. In the short-term their release redirect resources to mobilize energy to meet metabolic demands associates with behavioural challenging displays like courtship and copulation (Broom and Johnson 1993). Chronic exposure to stressors, and consequent high level of cortisol for more than a few days, can lead to detrimental effects that include muscle wasting, suppression of growth, inhibition of reproduction and suppression on the immune function (Sapolsky et al. 2000).

Another class of steroids hormones strictly connected with fitness are androgens, among which the main important is the testosterone. In males it is associated with morphological, physiological and behavioural changes resulting in an increase of aggressiveness and courtship activities (Lincoln et al. 1972; Wingfield et al. 1990; Oliviera 2004; Pereira et al. 2005). Elevated testosterone levels impose also several costs including increased metabolic rate and reduced immune functions (Ketterson and Nolan 1992).

In the past blood sampling has been the most common method to assess animal endocrine status. In free-ranging wildlife this methodology implies the capture of animals and the demanding risks and logistic costs. Moreover, it is not necessary the most informative method (Möstl and Palme 2002; Mormede et al. 2007): it could be misleading if not taken in consideration that blood samples represent concentrations at only single point and in many species many hormones has a circadian rhythm (Irvine and Alexander 1994; de Jong et al. 2000). Moreover, capture and handling of animals are high stressful events for the involved animals and can biased the cortisol results (Cook et al. 2000). Alternatively, the development of non-invasive technique that determined hormone metabolites in fecal samples, offer a solution to overcome these disadvantages, but to have reliable results a careful validation for each species and sex investigated is mandatory (Touma and Palme 2005).

In polygynous species male fitness is strictly connected with reproductive success, frequently driven by intrasexual competition for mate (Andresson 1994). Among ungulates this often results in the establishment of a hierarchical order where dominance is usually correlated with phenotypic quality that enhance fighting ability (McElligott et al. 2001; Pelletier et al. 2003). Specific behavioural strategies are also regulated by steroid hormones, including androgens and glucocorticoids (Wingfield et al. 1990; Creel 2001). In turn, social environment (i.e. network of interacting individual; Wingfield et al. 1990) and individual characteristics (hierarchic rank; Creel 2001) could have a positive or negative feedback control on hormonal levels. Among polygynous ungulates, Alpine ibex (*Capra ibex*) is one of the most dimorphic species. Females reach their maximum weight at the age of 4-5, while males show a slow and progressive increase of body mass until the age of 9-10 and weighted about twice than of adult females. As a consequence of this prolonged weight gain, males present a remarkable dimorphism among different age classes (Bassano et al. 2003). Ibex life cycle is characterized by accentuate seasonality, which is reflected in behavioural patterns, as reported for other species living in a temperate environment. In spring male ibex aggregate at the lowest altitude meadow, characterised by first green up of the vegetation, engaging frequent fights to define social status. During the reproductive season (December- half January) males actively roves (roving strategies) looking for oestrous females, which are widely and unpredictable distributed. In male's ibex has been identified two alternatives mating tactics: tending and coursing (Willish and Neuhaus 2009; Apollonio et al. 2013). Older and larger males classically perform the first tactic: they persistently follow a female, defending her from the approach of other males. The alternative mating tactics, the so-called coursing tactic, is more dynamic. Younger individuals try to mount running females bypassing the tending males. Individuals of all age classes switch between the two mating tactics, but older male are more successful in tending (Willisch and Neuhaus 2009; Apollonio et al. 2013).

Here I proposed a study on the behavioural and physiological responses to routine and threatening events in Alpine ibex. We choose this species for its several peculiarities, but mainly for two reasons: i) high detectability of the ibex; ii) pronounced dimorphism between sexes and among males of different age classes. The possibility to easily observe animals throughout the year provided me the opportunity to define a detailed male hierarchy, to record mating behaviour and assess the weight of ibex without capture them. The difference in body mass of individuals of different sex-age classes gave me the chance to point out the behavioural and physiological pattern in response of disturbance events highlighting the differences, and consequently the key role, of the body and armament (horns) sizes.

In the first and second chapters I explored the allostatic processes regarding the natural life cycle of the species. I analysed how male's ibex improve their fitness exploring the correlation between reproductive success, social status, age, body mass, and fecal hormone metabolites. Living in a mountain environment, ibex must cope with strong seasonal changes (Boonstra 2004). Reproductive season occurs in winter, so individuals have to balance energy expenditure to increase their chance to survive and reproduce. The pre-reproductive season of the specie is long (from late spring to late autumn). Individuals have to cope with high degree of intra-male interaction and exploit the greening time of the vegetation. The followed chapters are focused on the species change in response to a natural and anthropogenic threat. I evaluated the behavioural plasticity of the specie coping with a changing of landscape of fear. Then I integrated behavioural, environmental and physiological data to explore stress response to capture and manipulation. Thereby, I evaluated the progressive behavioural and to endocrine patterns of adjustments of the species.

The main goal of the **first chapter** is to analyse the determinants of male reproductive success. In particular I first tested if the outcome of the fights engaged during the pre-reproductive season results in a linear and stable hierarchical order. I looked for the main determinants of rank, evaluating the role of individual characteristics such as age, body mass, and endocrine level of fecal testosterone and cortisol metabolites (FTM and FCM respectively). Moreover, I assessed the ability of male of this specie to regulate energy allocation to respond to the strong seasonality of environment. During the pre-reproductive season male ibex engage frequent fights to define social status (Bergeron et al. 2010). Generally, male polygynous ungulates concentrate the high amount of males' competitions during the reproductive season (Clutton-Brock et al. 1979; Apollonio et al. 1990; McElligott et al. 1998); on the contrary in ibex it was observed rare conflicts between male during the rut (Willisch and Neuhaus 2010). The subsequent hypothesis states that the species define the hierarchical order in the season with best quality and quantity of food resources (summer), so to face the wintry rut with social status already defined.

In the **annex** I looked for the possible trades off in reproductive strategy or roe deer (*Capreolus capreolus*). I examined the differences in use of space and aggregation with the aim to test the presence of strategies to improve not only individual, but also relative's fitness.

The main goal of the **second chapter** is to describe the seasonal and yearly variations in the level of FCM and FTM to define a correlation with the behavioural patterns. Since in literature only the measurement of fecal cortisol metabolites (FCM) has been validated (Möstl et al. 2002; Posautz 2010) a first step of our analyses, is to validate a still missing enzyme immunoassay for the measurement of fecal testosterone metabolites (FTM) in male of Alpine ibex. Then I integrated in the analysis environmental characteristics (weather condition and diet quality) and individual traits (age, body mass, social status) to comprehend allostatic responses to seasonal patterns of variation of FCM and FTM.

In the **third chapter**, I evaluated the behavioural responses of ibex to changes in the landscape of fear taking into account the differences among age classes, sex, and group size. The main predator of the species, wolf (*Canis lupus*) was absent from the study area (Gran Paradiso National Park) form one century until 2006, when the presence of a resident pack was confirmed. This gave the rare opportunity to monitor behavioural responses of a prey in face of predation risk. Moreover, in the same years the ibex density decreased in the studied population. I assessed if these modifications in the landscape of fear induced very quick behavioural responses and, in the case, I tried to disentangle which factors led the modifications.

The aim of the **fourth chapter** is to assess the impact of capture methodologies integrating ibex behavioural and endocrine responses. The capture is necessary, among the others, for marking individuals (make them individually recognizable) and to collect information such as morphometric measurements or serum biochemistry for population sanitary controls (Powell and Proulx 2003; Garshelis 2006), but is one of the most stressful events of wild ungulates life (Wesson et al. 1979a,b; Spraker 1993). I took in consideration ibex movements, total activity and male hormonal levels to evaluate the impact and the duration of this stress. I also compared female productivity of marked females in the years in which they have undergone the capture and in the subsequent years to assess if the drug or the stress induced by the captures affected the probability to give birth.

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Chapter 1

Make it in advance and slowly: social rank and reproductive success constrains in Alpine ibex.

Nicoletta Sica [a], Achaz von Hardenberg [b], Francesca Brivio [a], Rupert Palme[c], Marco Apollonio [a], Stefano Grignolio [a]

[a] University of Sassari, Department of Science for Nature and Environmental Resources, via Muroni 25, I-07100, Sassari, Italy

[b] Alpine Wildlife Research Centre, Gran Paradiso National Park, Via della Rocca 47, I-10123 Torino, Italy

[c] Institute of Physiology, Pathophysiology and Biophysic, Department of Biomedical Sciences, University of Veterinary Medicine, Veterinärplatz , 11210 Vienna, Austria.

Abstract

In polygynous mammals, male dominance is correlated with individual qualities that enhance fighting ability and it is essential to regulate the access to oestrus females. Ungulates living in demanding habitat like the Alps have to cope with harsh environmental conditions, regulating energy allocation especially during the rut. Male Alpine ibex (*Capra ibex*), during the pre-reproductive period engage frequent fights to established individual social status. We explored the link between age, body mass and social status and tested if the hierarchical order is enough stable to last till winter, allowing males to reduce intra male competition during the breeding season. Since behavioural strategies, interactions associated with dominance and reproductive success are regulated by steroid hormones like androgens and glucocorticoids, we evaluated if they play a role in the attainment of high rank position or reproductive success. Our research revealed that males' ibex built a stable hierarchical order during summer and, thanks to the social status gained, they could reduce male-male interaction for the access to the females during the rut. Social status was independent from the level of testosterone and cortisol; its main determinant was the interaction between age and body mass. The level of testosterone was directly determined by the interaction between age and body mass and by the level of fecal cortisol metabolites. The preliminary interpretation is consistent with a permissive role of testosterone that potentiates pre-existing pattern of aggression. In respect to cortisol we did not find any sign of chronic stress in our ibex population. On the other hand, we hypothesised that a modest elevation of the level of this hormone could have a preparatory function specific for agonistic encounters.

Key words: *Capra ibex*, cortisol, dominance, testosterone, path analysis, reproductive success.

Introduction

Polygynous mating system suggests that male reproductive success is frequently driven by intrasexual competition for mate (Andresson 1994). Among ungulates this often results in the establishment of a hierarchical order where dominance is usually correlated with phenotypic quality that enhance fighting ability (McElligott et al. 2001; Pelletier et al. 2003; Willish and Nehaus 2010). As advantages in male competition, sexual selection will favour the evolution of large body and armaments (Gosling and Petrie 1990; Andersson 1994). Generally, as a male aged, it becomes heavier, with larger armament and more experienced (Hass and Jenni 1991; Pelletier et al. 2003). Specific behavioural strategies and interactions associated with dominance and reproductive success are also regulated by steroid hormones, including androgens and glucocorticoids (Wingfield et al. 1990; Creel 2001). In turn, social environment (i.e. network of interacting individual; Wingfield et al. 1990) and individual characteristics (hierarchic rank; Creel 2001) could have a feedback control on hormonal levels. Among androgens, testosterone has been related to aggressive encounters with pairs (Wingfield et al. 1990) and male-female sexual encounters (Gleason et al. 2009). Conversely, it can have an effect on the immune system (Zuk 1996; Stoehr and Kokko 2006). Although in many species has been found a direct correlation with social dominance (Pelletier et al. 2003; Shargal et al. 2008;), several studies failed to demonstrate significant correlation between aggressiveness or dominance rank and testosterone levels (Barrett et al. 2002; Lynch et al. 2002; Decristophoris et al. 2007).

Glucocorticoids (cortisol, in large mammals) are also important in mediating the relationship between agonistic interactions, rank, reproduction and immunocompetence. It is recognized that a temporary increase in the level of glucocorticoids regulates the metabolism and facilitate physical activities and basic stress responses. Nevertheless, a chronic high level of these steroids could trigger potential dangerous responses with negative effects on reproduction, immune defence, body condition and thus, compromise fitness (McEwen 1998; Sapolsky et al. 2000; Muller and Wrangham 2004; Reeder and Kramer 2005; Narayan et al. 2014). Testosterone and cortisol are part of a biological balance that influences primary reaction to threat and mediates important social behaviours. Therefore, it is important to include the effects of both these hormones in the analyses on wildlife's reproductive success to have a more precise picture of the many factors driving them.

In species with a wide and unpredictable distribution of females during the rut, the more profitable male reproductive strategy is the roving strategy (Clutton-Brock 1989): males actively search for receptive females, defending individual females for a part or for their full oestrus cycle. Alpine ibex (*Capra ibex*) is a polygynous, highly dimorphic, mountain ungulate and males adopt this reproductive strategy. In ibex has been identified two alternatives mating tactics: tending and coursing (Willish and Neuhaus 2009; Apollonio et al. 2013). Older and larger male classically performs the first tactic: they persistently follow a female, defending her from the approach of other males. The alternative mating tactic, the so-called coursing tactic, is more dynamic. Ibex, often a young individual, try to mount running females bypassing the tending males. During the tending courtship, males try to defend the oestrus females from other competitors, whereas younger males attempt to exploit sudden mating opportunities or to tend non-defended females. As a consequence the latters will court a higher number of females with respect to the older ones. Individual of all age classes switched between the two mating tactics, but older and bigger males are more successful in the tending one. Younger males, with body mass and horn length not enough to compete with fully growth males, are more successful adopting the coursing tactic (Apollonio et al. 2013; Willisch and Neuhaus 2009).

Previous works showed that male Alpine ibex defined a linear social hierarchy during summer (Bergeron et al. 2010) and that intra male conflicts were rare during the breeding season (Willisch and Neuhaus 2010). On the contrary, what is typically observed in polygynous ungulates is that males’ fights usually peak during the mating season (Clutton-Brock et al. 1979; Apollonio et al. 1990; McElligott et al. 1998). The consequent hypothesis states that male ibex define their hierarchical order in late spring and in summer, when the food resources are more abundant, and they maintain it valid till the mating season. We supposed that ibex evolved this conservative strategy because their mating period occurs from the late fall to the onset of winter in a temperate clime. In this season the energy expenditure reaches highest level as a consequence of sever decrease in ambient temperature, scarce food availability and the presence of snow that generally persist till spring. This hypothesis is also partially supported by other behavioural patterns selected to save energy during the rut: males maximized energy intake when not actively engaged in mating activities and the magnitude of the rut-induced hypophagia is reduced and related to age and, consequently to possibility to mate (Brivio et al. 2010). Moreover, males seemed to be able to regulate their energy allocation when environmental conditions became hard by reducing the time spent in mating activities and increasing the energy allocated to survival (Apollonio et al. 2013). We tested this theory, analysing the correlation between social status achieved in summer and winter reproductive success in a two years study on the only natural population of Alpine ibex in Gran Paradiso National Park (GPNP). Taking into account the results of Bergeron et al. (2010), we predicted that the most of the variance in rank order would be explained by age and body mass and that if the hierarchy was sufficiently stable it would last during the reproductive season. We explored the casual relation between pre-rut fecal testosterone metabolites (FTM) and individual characteristic (age and body mass). As it has been shown that high level of testosterone entail important costs including immunosuppression (Decristophoris et al. 2007), we focused on the research of the benefits that could balance the negative effect. Thus, we tested the direct correlation of FTM on males' rank position or reproductive success. To improve our understanding of the relationship between ibex social behaviour and endocrinology, we added, for the first time in this species, fecal cortisol metabolites (FCM) to this picture, evaluating the possibility that FTM and FCM could jointly regulate dominance and reproductive success.

Materials and Methods

Study area and population

Data were collected in Levionaz basin in Valsavaranche, one of the main valley of GPNP (45°25' N, 7°34' E), Western Italian Alps. Hunting it's not allowed in the Park. More than 80% of the study area is constituted of rocks, glacier, stone ravines and Alpine meadows (*Carex curvula* and *Festuca* spp.), the forests, mainly coniferous woods (*Pices abies, Larix decidua* and *Pinus cembra*) are represented for less than 10%. The climate is cold temperate continental, rather rigid and dry. Ibex use elevation from about 1400m above the sea level to beyond the upper limit of vegetation at about 3000m (Parrini et al. 2003). During winter and early spring they feed on pasture at lower altitude; as the snow melts, animals move to highest meadow, reaching the highest elevation in summer. The population it's intensively studied in the Levionas valley since 1999. Animals were captured by chemical immobilization and marked. They were accurately aged by counting the annual horn growth rings (annuli) at capture (Ratti and Habermehl 1977; von Hardenberg et al. 2004). More details about capture and marking procedure of ibex in

GPNP can be found in *Chapter* 4. Almost the 90% of males from 3 to 17 years old it's marked with plastic ear tag with a unique combination of colour.

Data collection and data analysis

The study was carried on from the 1st of May to the end of September in 2012 and 2013, covering the pre-reproductive season of the species and from the 1st of December 2012 to the 15th of January 2013 and in the same period of the next year covering the rut.

Dominance

During the pre-reproductive seasons we registered all agonistic interactions involving marked male ibex following *ab libitum sampling* methodology (Altman 1974). For each interaction, we recorded date, time, identity of the contenders and outcome of the fights. The distinction between dominant and submissive males was made thanks to the identification of specific displays described by Bergeron et al. (2010) and reported in *Chapter* 2. We assessed the linearity of dominance order estimating *h'* index (de Vries 1995; 1998) with the software Matman 1.1 (Noldus Information Technology 2003) and tested its significance based on 10,000 randomizations. We also calculated a directional consistency (DC) index (van Hooff and Wensing 1987), that measured how consistently one individual win against another individual. The hierarchical order was estimated by means of Elo-rating (Albers and de Vries 2001; Neumann et al. 2011). It based on the sequence in which the interactions occurred and continuously updates ratings by looking at interactions sequentially. The stability of hierarchy order was estimated by Neumann stability index and by Stability index (S) proposed by McDonals and Shizuka (2013), which unlike Neumann index it's independent from group size. Elo-ratings and Neumann stability index were calculated using scripts included in Neumann et al. (2011) and S index was estimated as described in McDonals and Shizuka (2013) by R software. A more detailed description of the methodology can be found in *chapter* 2.

Body mass estimation

During both pre-reproductive seasons we collected weights of marked ibex following the methodology suggested by Bassano et al. (2003). We obtain comparable data adjusting ibex body weight to the 1st of August (von Hardenberg 2005) using linear mixed effect model (Pinheiro and Bates 2000) with restricted maximum likelihood method and setting individual identity as a random factor (Pelletier et al. 2007). We collected 403 observations of weight (190 in 2012 and 213 in 2013) at the scale and we estimated the weight at the 1st of August for 44 marked males in 2012 and 41 in 2013 observed at least twice during the data collection.

Hormonal samples

Considering that we estimated the dominance order on the basis of the interactions recorded from May till September, we chose to consider the hormonal level estimated in September in our analysis (see *chapter* 2 for details on sampling methodologies). The samples were collected immediately after the deposition, with a plastic bag, registering: date, time and male's identity. They were stored in a refrigerator at -20°C within 12h, until the extraction procedure.

In the laboratory, we extract 0.5g of each fresh fecal sample, adding 5ml of 80% methanol (Palme et al. 2005) and shaking 1-2 min with a handvortex (Vortex mixer, company Velp). After centrifugation (Allegra X-12R, company Beckmann / GS-6KR) for 15 min at 2,500g, 300 µl of the supernatant was dilute (1:10) with assay buffer (ph 7.5). The amount of cortisol and testosterone metabolites were determined respectively by 11-oxoaetiocholanolone EIA (5 β–androstano-11,17-dione structure for FCM; Posautz, 2010 and Möstl et al. 2002) and 17-oxo-androgens (Epiandronsterone, for FTM; Palme and Möstl 1994 and *Chapter 2*).

Reproductive success

During the reproductive seasons we collected data on behavioural patterns of marked males following *focal animal sampling* over continuous period (Altmann 1974) applying the methodology used by Apollonio et al. (2013) in the same study area. The focal individual was monitored with spotting scopes for at least 1 hour. It was chosen randomly among males of which we knew the hierarchical position, paying attention to homogeneously distribute observations on males of each age (from 3 to 16 years old). Identity of the focal animal, group size and composition (females, kid, yearling, other males) were recorded. The observer punctually described individual behaviour, distinguishing between non-courtship (feeding, standing, moving, lying down, agonistic interactions between males) and courtship display. The latter included, following Willish and Nehaus (2009), low stretch (neck straightened and head held in-plane with the back with the muzzle pointed toward a female), tongue-flick (flicking tongue toward a female), sniff (sniffing a female), touch (touching a female), self-stimulation (touching penis with snout while standing next to a female), mount attempt (raising front half body to mount a female), copulation (subset of mounts in which the abdomen of the male is firmly pressed against the vulva of the female, resulting in a supposed intromission of the penis). Moreover, for each male observed the number of courted females was recorded.

Definition and analysis of the statistical models

We used confirmatory path analysis (Shipley 2000a) to analysed direct and indirect effects of fecal hormones metabolites on rank and reproductive success. Path analysis tests for casual links between variables based on the conditional independencies implied by a hypothetical causal model represented by a directed acyclic graph (DAG). To perform the confirmatory path analysis, we used Shipley's directional separation test (d-sep test; Shipley 2000b, 20003), which allows to take into account the hierarchical structure (repeated measures on the same individual) of our data, using linear mixed-effects models (GLMM; Shipley 2009). To perform the d-sep test, the first step is to specify the list of all, and only those, pairs of variables (independence claims) that are statistically independent conditioning on a set of other variables (Gonzalez-Voyer and von Hardenberg 2014). Every independence claim must be true if the path model is correct. The null probability from each claim is used to calculate Fischer’s C statistic:

$$C=-2\sum \ln(P) \qquad (1)$$

where C follows a χ^2 distribution with 2*K degrees of freedom. K is the number of independence claims tested. A Significant P-value for this test (α level =0.05) suggests that the structure of the path model is not consistent whit empirical data (Shipley 2000, 2009). The selection of the best path analysis model among the ones supported by the dataset can be done with an information criterion, similar to the Aikaike Information Criterion (AIC), based on Fisher's C Statistic (Cardon et al. 2011). Shipley (2013) has shown that the information criterion based on the C statistic is mathematically equivalent to the AIC. von Hardenberg and Gonzalez-Voyer (2013) have suggested to call the C-Statistic based Information Criterion, CIC to distinguish it from AIC and thus, its adaption for small sample sizes, CICc. Following model selection procedure (Johnson and Omland 2004), the model that showed the lowest value of CICc was considered as the “best model”. Following Symonds and Moussalli (2011), we selected a single best model if ΔCICc (CIC_i-

CIC_{best}) value <2. Finally, we calculated the path coefficients of the best model by fitting linear mixed effects models in which each dependent variable was a function of its direct causes as specified by the causal graph.

We tested path analytic models of the causal relationships among fecal hormonal metabolites (FTM and FCM; ηg/g), age, body mass (kg) and rank position (starting from 1, top ranking individual). Considering the difficulty to observe mounting attempts, as proxy of male reproductive success (MRS) we use the mean number of females/courtship time. Individual identity and year were set as random factors in all linear mixed models. MRS was transformed with an arcsin square root transformation and FCM and FTM values were log transformed before all analysis to meet the assumption of normality for the residuals. We constructed 2 casual models (fig. 1) with the main aim to test the relationship between testosterone and rank order: the model *A* tested if individual rank was directly influenced by the level of testosterone and if the hierarchical order directly influenced male reproductive success.

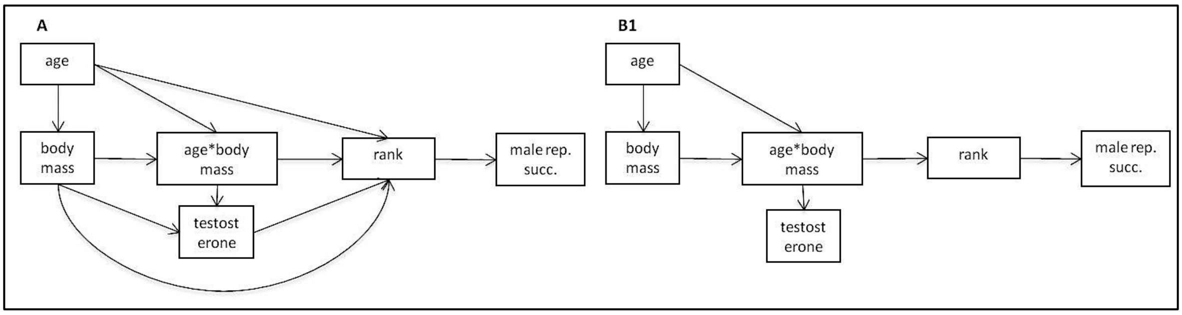

Figure 1. Path models constructed to evaluate the casual effects between rank position achieved in the pre-reproductive season (rank), male reproductive success (male rep. succ.), fecal testosterone metabolites (testosterone), age, body mass and the interaction term between age and body mass. See the text for the hypotheses considered to define the path.

On the basis of the selected structure (B_1 model, see result section for the details) in B_2 path we evaluated the contribution of FCM to the path, testing the independence of cortisol from the other variables. We then constructed other 6 causal models (fig. 2): in the *C*, *D* and *E* models the term age*bm directly influenced FCM and FTM and FTM was the direct cause of FCM, but in the first (*C*) the hormones metabolites were independent from rank position; in the second (*D*) FCM was the direct cause of rank position; while in the third (*E*) rank position was the direct cause of FCM. In the *F* path FCM was again directly influenced by rank and age*body mass, but the two hormones metabolites were independent. In the last two paths (*G*, *H*) the term age*body mass directed affected FTM, both hormones metabolites were independent from rank order, but in one case (*G*) it was the direct cause of FTM, while in the other (*H*) it was directly determined by FTM.

Results

We recorded 574 male interactions (referred to 53 marked males) in 2012 and 564 (referred to 45 marked males) in 2013 from which we estimated the hierarchical order.
Alpine ibex established a hierarchy significantly linear with high DC values both in 2012 and 2013 (see tab.1 for descriptive statistic). A stable dominance relationship is estimated by Elo-rating (S index 2012: 0.992; 2013: 0.996).

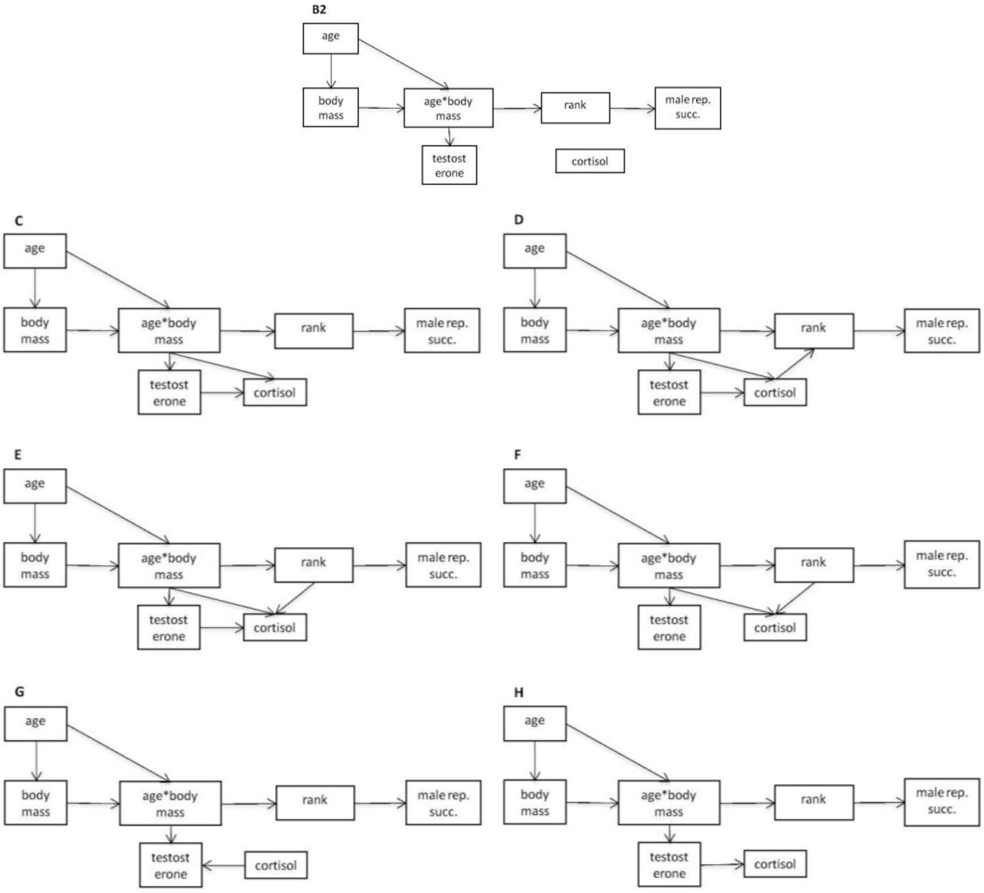

Figure 2. Path models constructed to evaluate the casual effects between rank position achieved in the pre-reproductive season (rank), male reproductive success (male rep. succ.), fecal testosterone metabolites (testosterone), fecal cortisol metabolites (cortisol), age, body mass and the interaction term between age and body mass. See the text for the hypotheses considered to define each path.

Table 1. Dominance matrices of Alpine ibex males in 2012 and 2013 in the Gran Paradiso National Park, Italy

Year	*h'*	P-value	DC	S
2012	0.125	0.001	0.950	0.992
2013	0.136	<0.001	0.930	0.996

Concerning the relationship between age, body mass, FTM, rank and MRS we found that both path *A* and path B_1 well supported the data, because their *C*-values did not statistically differ from a chi-square distribution with 2*k* degree of freedom. Among them, the B_1 casual model gave the lowest CICc value (tab. 2). In this model the hierarchy build in the pre-reproductive season directly affected MRS. In turn, rank position was independent from the direct single effect of age and body mass, but directly affected by their interaction (age*body mass). The only variable that directly influenced FTM in the pre-reproductive season is the interaction term between age and body mass.

Table 2. Result of the model selection among A and B_1 casual models (fig.1) constructed to test the relationships among, age, body mass, fecal testosterone metabolites, rank and reproductive success. The best model is highlighted in grey.

Casual model	df	C	P-value	CICc	ΔCICc	W_i
B_1	46	15.649	0.617	141.543	0	0.999
A	46	11.158	0.674	195.158	54.005	<0.001

Starting from this casual model, we explored the role of FCM. We tested the independence of FCM from the model previously selected (fig. 2; mod. B_2). This model was not consistent with the data (C=43.314; p-value=0.029; appendix 1), consequently we added FCM to the casual model. At the base of this result, first of all there is the non-respected independence between FTM and FCM (appendix 1, mod B_2). We then constructed and tested other six casual models (fig. 2). Among these, the F_c model was also not consistent with the data (C=40.777; p-value=0.037; appendix 1). The other models are all statistically supported, but, looking at the CICc, ΔCICc and Wi value, the best casual model fitting the data is the path *G* (tab. 3). Accordingly to the model (fig. 3), MRS is directed affected by hierarchical order established in summer (0.338±0.142). Rank position is directly influenced by the interaction term age*body mass (-0.808±0.104): older and bigger males are at the top of the hierarchy. During the pre-reproductive season, fecal metabolites are independent from social status. FTM is directly affected by the interaction term age*body mass (0.369±0.098), but its variation is mainly driven by variation in FCM (0.610±0.098).

Table 3. Result of the model selection among five casual models (fig. 2) constructed to test the relationships among, age, body mass, fecal testosterone and cortisol metabolites, rank and reproductive success. The best model is highlighted in grey.

Casual model	df	C	P-value	CICc	ΔCICc	W_i
G	46	21.747	0.793	159.746	0	0.999
H	46	21.928	0.785	225.642	65.896	<0.001
C	46	18.476	0.858	244.933	85.187	<0.001
E	46	16.808	0.850	269.808	110.062	<0.001
D	46	17.611	0.821	270.611	110.865	<0.001

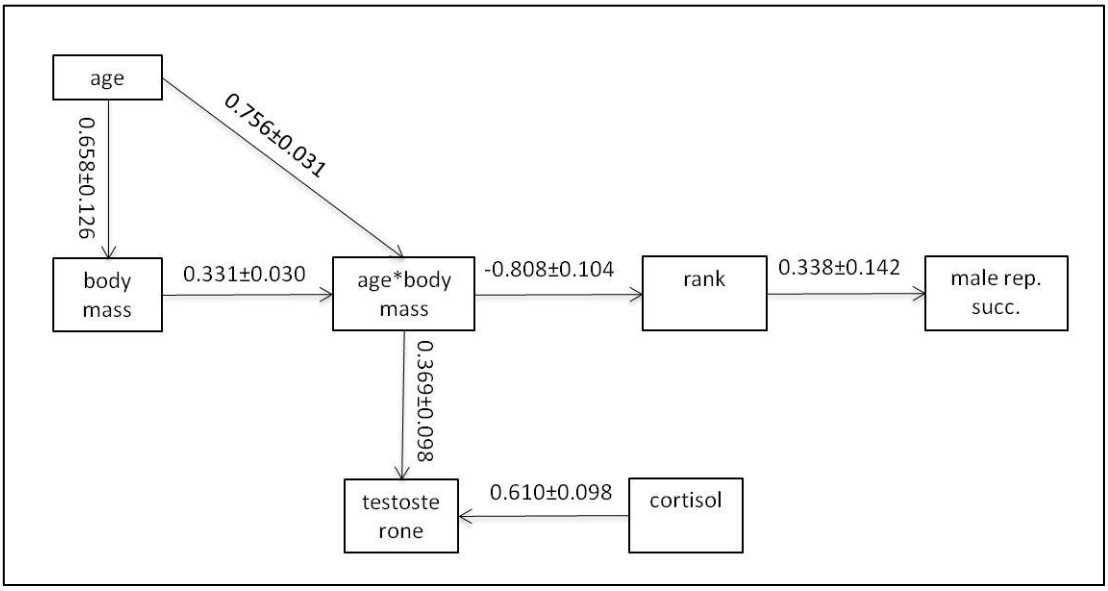

Figure 3. Best casual model for the relationships among fecal testosterone and cortisol metabolites, rank position, male reproductive success, age, body mass and age*body mass. Value along each path reports the standardized path coefficients and standard errors.

Discussion

Male Alpine ibex exhibited a linear and stable dominance hierarchy throughout summer. The gained social status directly influenced individual reproductive success in the next wintry reproductive season. Social rank was directly affected by the interaction term of age and body mass, but was independent from fecal testosterone metabolites. Androgens levels were directly influenced by the interaction term between body mass and age and by fecal cortisol metabolites.

In polygynous species males maximise their fitness by producing as many offspring as possible (Elmen and Oring 1977); consequently males compete intensely for the access to females (Andersson 1994). Our study showed that the hierarchical order established among male's ibex in the pre-reproductive season was linear and it remained stable through the summer, as shown by the high consistency of the outcome expressed by the DC index. This is consistent with findings on other ungulates species like per example fallow deer (*Dama dama*: McElligott et al. 1998), bighorn sheep (*Ovis canadensis*: Pelletier and Festa-Bianchet 2006), and red deer (*Cervus elaphus*: Appleby 1982), in which males typically form a stable relationship of dominance. In these species body mass is generally considered to be the main determinant of social rank or territorial acquisition (e.g., *Kobus kob thomasi*, Balmford et al. 1992; *Dama dama* Clutton-Brock et al. 1988; *Ovis Canadensis,* Pelletier and Festa-Bianchet 2006; *Bison bison*, Bowyer et al. 2007). For male ibex the probability for a male to hold a position of dominance was not directly determined only by body mass, rather by the interaction between body mass and age. This result is likely the consequence of the high level of intrasexual dimorphism that characterises this specie: male ibex growth slowly in size and in horn length, reaching asymptotic body mass late (around 9-11 y. o., Bassano et al. 2003). This singular life history trait influence several behavioural patterns (Brivio 2013; Brivio et al. 2014) and partially affects the male hierarchy: young males (3-5 y.o.) are half the weight of the full-grown ones and subsequently only the fully growth males are able to compete for the top of the hierarchy. However, it is not exclusively the age that determines the rank position, but the combined effects of individual mass, age and horn size (Bergeron et al. 2010). The high difference in body mass and horn length between the males of about 9 years of age and the younger ones make disadvantageous for the latter to engage agonistic interactions to establish the state of dominance. Indeed, the probability of escalation decreases with increasing difference in horn length and body mass, as previously showed by Bergeron et al. (2010). This situation led to a high number of dyad of male that did not interact, leading to a high proportion of unknown relationships that results in the low value of the *h'* index found by our analyses.

Our findings showed that social rank determined during the pre-reproductive period directly affected male reproductive success, as shown by the direct correlation between of rank and reproductive success. This supports our hypothesis and is in agreement with studies on polygynous ungulates, which typically report strong positive correlations between male social rank and breeding success (Hogg and Forbes 1997; McElligott et al. 2001; Preston et al. 2003). Unlikely other ungulates, male ibex reduce aggressive interactions during the rut (Willish and Neuhaus 2010). As highlighted by Willish and Neuhaus (2010), this is a rare phenomenon contradicting the common assumption that the level of aggression in polygynous species should increase during the mating season (Parker 1974). During winter ibex survival decreased (Jacobson et al. 2004), so in order to cope with hard environmental conditions males have to carefully regulate their energy allocation by adopting different conservative strategies, such as the reduction of the time spent in mating activities (Apollonio et al. 2013), the maximisation of the time spent foraging (Brivio et al. 2010) and the reduction of the time spent in intra-male competition (Willish and Neuhaus 2010). As a consequence, in order to establish the access to mate, male ibex have to built up the dominance order during summer, when they can find high-quality and abundant food resources. Our analyses pointed out that the state of dominance regulated the access to the receptive females during the reproductive season. The positive coefficient of the relationship between rank position and number of courted females/courted time suggested that dominant males were able to monopolise the access to the oestrous females defending them for long period from the approach of other individual, so that to increase their mate possibilities. As a result they courted less female per courting time. Conversely, subordinate males, relying on unexpected mating opportunity or tending non-defended females, presumably more distant to the oestrus, courted a major number of females but with lower chances to mate.

Direct associations between testosterone, rates of aggression, and dominance rank have been identified in several species (Pelletier et al. 2003; Shargal et al. 2008; Muehlenbein and Watts 2010). Our result assessed the direct correlation between level of testosterone and the relative dimension of male in respect to their age, but it is independent from social status, as previously reported by Decristophoris et al. (2007). These findings are consistent with the establishment of a stable hierarchical order between male ibex; in fact the positive correlation between testosterone and dominance is often been related to situation of social instability, to the establishment of territorial boundaries or to the presence of receptive females (Wingfield et al. 1990; Corlatti et al. 2014). Moreover when level of testosterone was experimentally manipulated in groups of male of Japanese quail (*Coturnix japonica*), this did not bring to rank order changes of the bottom- ranked individuals (Tsutsui and

Ishii 1981; Wingfield and Ramenofsky 1985). In accordance, studies on primate show little evidence of the correlation between short-term changes in testosterone levels and increased level of aggression (Mazur and Booth 1998) and rather hypnotized the permissive effect of testosterone that will potentiate pre-existing patterns of aggression (Dixson and Herbert 1977). According to this theory, normal level of aggression require basal level of testosterone and little changes in testosterone level in healthy individuals over time do not necessary predict changes in level of aggression within individuals (Mazur and Booth 1998; Archer 2006).

Despite potential facilitation, there are a number of costs intrinsically tight to an elevation in testosterone level, including increase metabolic rates (Ketterson and Nolan 1992) and immunosuppression (Muehlenbein and Bribiescas 2005) that could negatively affect individual survival. In this regards, cortisol, released in response to stress events, enhance energetic metabolism, so it is important in mediating the relationships between agonistic interactions, reproductive function and immunocompetence. We found a direct positive correlation between the levels of the two hormones analysed, with a direct effect of cortisol on testosterone. These results are in contrast to a vast number of studies that confirm the inhibiting role of stress in hormonal function of testicles, decreasing testosterone level in blood of different species (Sapolsky 1986; Elman et al. 2001; Manna et al. 2004). On the other hand, our fndings are consistent to other studies that report an initial increase in the level of testosterone at initial stages of acute stress, i.e. triggered by an increase in cortisol (Wingfield and Sapolsky 2003; Chichinadze and Chichinadze 2008). The possible factors that can contribute to the temporary increase of testosterone during acute stress are: absence of chronic stress, dominance status, more or less stable social relations in a population, individual ability to manage stress situation (Chichinadze and Chichinadze 2008). Our results showed that during the pre-reproductive season FCM has a direct and positive influence on FTM. In the period where male ibex interact to define social status (acute stress) an initial increase in the level on testosterone could have a preparatory function, specific for agonistic encounters (Booth et al. 1989).

In conclusion, our results confirm that the hierarchical order established during the pre-reproductive season is stable so that, during the rut, male ibex had a pre-defined dominance status. Thanks to this knowledge the access to oestrus females is regulated with a reduced number of male-male interactions, so that males could save energy to court females and to increase their probability of survival. Rank is directly dependent to the relative dimension (interaction between body mass and age) of each individual, but it is independent from fecal hormones metabolites. We did not find a positive effect that could balance the cost of a high level of testosterone, instead our results are more consistent with a permissive effect of testosterone. In respect to cortisol we did not find any sign of chronic stress in our ibex population. On the other hand, we hypothesised that a modest elevation of the level of this hormone could be preliminary for agonistic encounter.

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Appendix.1. Test of conditional independence in the basis sets implied by the path models in Fig. 1 Variables: Age (A), Body size (B), Rank (R), fecal testosterone metabolites (T), fecal cortisol metabolites (C), I (Age*Body Size), M (n° female/courtship time). X_||_Y$\{Z_{i},..\}$ means that X and Y are independent conditional on the combined set of $\{Z_{i},..\}$, parent variables.

Model	Basis set	Var	Partial slopes	SE	*t*-value (df)	Null Probability	C	P-value
B2	C_||_T {I}	T	0.494	0.076	6.435(15)	<0.001	43.314	0.029
	C_||_B {A}	B	0.006	0.008	0.787(15)	0.443		
	C_||_A {Ø}	A	0.038	0.026	1.443(16)	0.168		
	C_||_R {I}	R	-0.002	0.009	-0.252(15)	0.805		
	C_||_M {R}	M	0.813	1.433	0.567(15)	0.579		
	C_||_I {A, B}	I	-0.002	0.002	-0.839(14)	0.416		
	A_||_T {I}	A	0.092	0.148	0.619(15)	0.545		
	A_||_R {I}	A	1.343	1.934	0.693(15)	0.499		
	A_||_M {R}	A	-0.004	0.004	-0.969(15)	0.347		
	B_||_T {I, A}	B	0.029	0.020	1.435(14)	0.173		
	B_||_R {I, A}	B	-0.135	0.281	-0.481(14)	0.638		
	B_||_M {R, A}	B	0.002	0.001	1.909(14)	0.077		
	I_||_M {R, A, B}	I	$3.6*10^{-6}$	$2.0*10^{-4}$	0.018(13)	0.986		
	R_||_T {I}	R	0.005	0.012	0.425(15)	0.677		
	M_||_T {R, I}	M	0.678	2.035	0.333(14)	0.744		
C	A_||_T {I}	A	0.092	0.148	0.619(15)	0.545	18.476	0.858
	A_||_C {I, T}	A	-0.029	0.081	-0.360(14)	0.724		
	A_||_R {I, C}	A	1.343	1.939	0.693(15)	0.499		
	A_||_M {R}	A	-0.004	0.004	-0.969(15)	0.348		
	B_||_T {I, A}	B	0.288	0.020	1.435(14)	0.173		
	B_||_C {A, T, I}	B	0.004	0.012	0.363(13)	0.723		
	B_||_R {A, I}	B	-0.135	0.281	-0.481(14)	0.638		
	B_||_M {A, R}	B	0.002	0.001	1.909(14)	0.077		
	I_||_M {R, A, B}	I	$3.6*10^{-6}$	$2.0*10^{-4}$	0.018(13)	0.986		
	T_||_R {I}	T	0.108	1.500	0.072(15)	0.944		
	T_||_M {R, I}	T	0.004	0.012	0.333(14)	0.744		
	C_||_R {I, T}	R	-0.004	0.006	-0.635(14)	0.536		
	C_||_M {R, I, T}	M	0.527	1.049	0.502(13)	0.624		
D	A_||_T {I}	A	0.092	0.148	0.619(15)	0.545	17.611	0.821
	A_||_C {I, T}	A	-0.029	0.081	-0.360(14)	0.724		
	A_||_R {I, C}	A	1.343	1.939	0.693(15)	0.499		
	A_||_M {R}	A	-0.004	0.004	-0.969(15)	0.348		
	B_||_T {I, A}	B	0.288	0.020	1.435(14)	0.173		
	B_||_C {I, T, A}	B	0.004	0.012	0.363(13)	0.723		
	B_||_R {I, A}	B	-0.135	0.281	-0.481(14)	0.638		
	B_||_M {A, R}	B	0.002	0.001	1.909(14)	0.077		
	I_||_M {A, B, R}	I	$3.6*10^{-6}$	$2.0*10^{-4}$	0.018(13)	0.986		
	T_||_R {I, C}	T	-0.573	2.232	-0.257(14)	0.801		
	T_||_M {R, I}	T	0.004	0.012	0.333(13)	0.744		
	C_||_M {R, I, T}	M	0.527	1.049	0.502(13)	0.624		

Model	Basis set	Var	Partial slopes	SE	*t*-value (df)	Null Probability	C	P-value
E	A_||_T {I}	A	0.092	0.148	0.619(15)	0.545	16.808	0.850
	A_||_C {I, T, R}	A	-0.019	0.083	-0.229(13)	0.823		
	A_||_R {I}	A	1.343	1.939	0.693(15)	0.499		
	A_||_M {R}	A	-0.004	0.004	-0.969(15)	0.348		
	B_||_T {I, A}	B	0.288	0.020	1.435(14)	0.173		
	B_||_C {I, T, R, A}	B	0.003	0.012	0.283(12)	0.782		
	B_||_R {A, I}	B	-0.135	0.281	-0.481(14)	0.638		
	B_||_M {A, R}	B	0.002	0.001	1.909(14)	0.077		
	I_||_M {A, B, R}	I	$3.6*10^{-6}$	$2.0*10^{-4}$	0.018(13)	0.986		
	T_||_R {I}	T	0.108	1.500	0.072(15)	0.944		
	T_||_M {R, I}	T	0.004	0.012	0.333(13)	0.744		
	C_||_M {I, R, T}	C	0.527	1.049	0.502(13)	0.624		
F	A_||_T {I}	A	0.092	0.148	0.619(15)	0.545	40.777	0.037
	A_||_C {I, T, R}	A	-0.019	0.083	-0.229(13)	0.823		
	A_||_R {I}	A	1.343	1.939	0.693(15)	0.499		
	A_||_M {R}	A	-0.004	0.004	-0.969(15)	0.348		
	B_||_T {I, A}	B	0.288	0.020	1.435(14)	0.173		
	B_||_C {A, I, R}	B	0.016	0.015	1.094(13)	0.294		
	B_||_R {A, I}	B	-0.135	0.281	-0.481(14)	0.638		
	B_||_M {A, R}	B	0.002	0.001	1.909(14)	0.077		
	I_||_M {A, B, R}	I	$3.6*10^{-6}$	$2.0*10^{-4}$	0.018(13)	0.986		
	T_||_R {I}	T	0.108	1.500	0.072(15)	0.944		
	T_||_M {R, I}	T	0.004	0.012	0.333(13)	0.744		
	C_||_M {I, R}	M	0.867	1.445	0.600(14)	0.558		
	T_||_C {I, R}	T	0.495	0.078	6.335(14)	<0.001		
G	A_||_T {I}	A	0.092	0.148	0.619(15)	0.545	21.747	0.793
	C_||_A {Ø}	A	0.038	0.026	1.443(16)	0.168		
	A_||_R {I}	A	1.343	1.939	0.693(15)	0.499		
	A_||_M {R}	A	-0.004	0.004	-0.969(15)	0.348		
	B_||_T {I, A, C}	B	0.013	0.015	0.887(13)	0.391		
	B_||_C {A}	B	0.006	0.008	0.787(15)	0.444		
	B_||_R {A, I}	B	-0.135	0.281	-0.481(14)	0.638		
	B_||_M {A, R}	B	0.002	0.001	1.909(14)	0.077		
	I_||_M {A, B, R}	I	$3.6*10^{-6}$	$2.0*10^{-4}$	0.018(13)	0.986		
	I_||_C {A, B}	I	-0.002	0.002	-0.839(14)	0.416		
	T_||_R {I, C}	T	-0.573	2.232	-0.267(14)	0.801		
	T_||_M {R, I, C}	T	-0.002	0.016	-0.111(13)	0.913		
	C_||_R {I}	R	-0.002	0.009	-0.252(15)	0.805		
	C_||_M {R}	M	0.813	1.433	0.567(15)	0.579		
H	A_||_T {I}	A	0.092	0.148	0.619(15)	0.545	21.928	0.785
	A_||_C {T}	A	-0.0344	0.023	-1.514(15)	0.151		
	A_||_R {I}	A	1.343	1.939	0.693(15)	0.499		
	A_||_M {R}	A	-0.004	0.004	-0.969(15)	0.348		
	B_||_T {I, A}	I	0.029	0.020	1.435(14)	0.173		
	B_||_C {A, T}	A	0.001	0.006	0.112(14)	0.912		
	B_||_R {A, I}	B	-0.135	0.281	-0.481(14)	0.638		
	B_||_M {A, R}	B	0.002	0.001	1.909(14)	0.077		
	T_||_R {I}	T	0.108	1.500	0.072(15)	0.944		
	T_||_M {R, I}	B	0.004	0.012	0.333(14)	0.744		
	I_||_M {A, B, R}	I	$3.6*10^{-6}$	$2.0*10^{-4}$	0.018(13)	0.986		
	I_||_C {A, B, T}	I	-0.001	0.001	-0.350(13)	0.732		
	C_||_R {I, T}	R	-0.004	0.006	-0.635(14)	0.536		
	C_||_M {R, T}	M	0.600	1.047	0.573(14)	0.576		

Chapter 2

Yearly variations of fecal steroid metabolites: a response to environmental stressors?

Nicoletta Sica [a], Achaz von Hardenberg [b], Walter Arnold [d], Erich Möstl [c],
Rupert Palme [c], Marco Apollonio [a], Stefano Grignolio [a]

[a] University of Sassari, Department of Science for Nature and Environmental Resources, via Muroni 25, I-07100, Sassari, Italy.

[b] Alpine Wildlife Research Centre, Gran Paradiso National Park, Via della Rocca 47, I-10123 Torino, Italy.

[c] Institute of Physiology, Pathophysiology and Biophysic, Department of Biomedical Sciences University of Veterinary Medicine, Veterinärplatz , 11210 Vienna, Austria.

[d] Research Institute of Wildlife Ecology, University of Veterinary Medicine, Savoyenstr. 1, A-1160 Vienna, Austria.

Abstract

Androgens (testosterone) and glucocorticoids (cortisol) monitoring of wildlife is a well established tool helping biologist to deal with animal conservation. Over the last decades, investigating the endocrine status in free-ranging animals has become more affordable due to the development of non-invase technique involving the use of fecal hormone metabolites. Anyway, to implement accurate analyses a careful validation is needed for each species and sex under investigation. For this reasons, the first aim of this study was to validate an EIA allowing a reliable measure of fecal testosterone metabolites in male Alpine ibex (*Capra ibex*). This species is characterised by pre-rut with frequent fights occurring several month before the rut. Hence, we investigated the influence of seasonal environmental conditions during the pre-rut periods. We showed different seasonal trends of the two metabolites in the two years of the study. This result highlights the need of long time series of data in order to accurately assess endocrine variations before to make reliable inferences on natural or human-induced effects on wildlife populations. We found that fecal cortisol metabolites responded to environmental stress like high temperature. Moreover its level increased with age till 10-12 years old, i.e. the age when a male actively compete for the high rank position, suggesting a correlation with social dominance. Fecal testosterone metabolite is positive correlated with body mass, it peaks in the time of most intense fights between male, possibly providing the impulse to compete to gain a high-ranking position. Our findings assessed the seasonal basal level of fecal cortisol and testosterone metabolites and contribute to understand the role of environmental stress and sociality on hormone levels and *vice-versa,* highlighting the costs (immunosuppression) and benefits (current reproduction) related to maintaining high social rank positions.

Keywords: Alpine ibex, cortisol, fecal hormone metabolites, testosterone, cortisol, pre-rut, seasonality.

Introduction

Studies on wildlife endocrine activities are a well-established tool to improve our knowledge on animal biology. Hormones act as facilitators of behaviour and affect all the tissue in the body. Consequently, understanding mechanism that modulates their circulating level is important to deal with animal conservation. In the past repeated collection of blood samples was necessary to evaluate the endocrine status of an individual. In the last decades the development of non-invasive techniques involving the use of fecal hormone metabolites, allows the researcher to accurately assess the endocrine status in free-ranging animals (Möstl and Palme 2002; Sheriff et al. 2011; Schwarzenberger and Brown 2013). This technique offers several advantages (first of all, to avoid the capture and manipulation of animal), but to have reliable results a careful validation for each investigated species and sex is mandatory (Touma and Palme 2005). The focus of most endocrine studies has been on the hypothalamo–pituitary adrenocortical (HPA) and on the hypothalamo–pituitary gonadal (HPG) axes. The comprehension of these axes gave the possibilities to better understand topics as stress and reproduction (Pukazhenthi and Wildt 2004; Kersey and Dehnhard 2014). The physiological mechanism of stress responses has been wildly described (Sapolsky 1993). The HPA axis mediates the cascade of events part of the neuroendocrine stress response. Under normal conditions the hippocampus inhibits the HPA axis; as glucocorticoids levels increase, the hormone itself acts as a negative feedback (Wingfield and Sapolsky 2003; Sheriff et al. 2011). If the stressor stimulus is prolonged (chronic stress), the persistent exposure to glucocorticoids (cortisol in large mammals) can trigger potential dangerous response with effects on the reproductive hormones, immune systems, performance and behaviour (Muller and Wrangham 2004; Reeder and Kramer 2005; Narayan et al. 2014). Researchers supposed that predictability and duration of the stressor stimulus are most likely to cause pathology (Sapolsky 1992; Boonstra 2013). This is why many studies reported this risk associated to climate changing (Wingfield 2008), food availability or quality (Saltz and White 1991; Foley et al. 2001) and human harassment (Creel et al. 2002; Zwijacz-Kozica et al. 2012; Shutt et al. 2014), pointing out the importance of planning non-invasive glucocorticoid monitoring to establish the impact on animal populations.

The HPG axis is comprise, at its fundamental element, of gonadotropine releasing hormone (GnRH) expressing neurons. The release of GnRH immediately acts to stimulate, among others, the release of luteinizing hormone; the latter, reaching the receptors situated in the gonads, stimulate the synthesis of testosterone (Handa and Weiser al. 2014). In wild animals, seasonal rising of testosterone is related to environmental stimuli such as day light, aggressive encounters with pairs (Wingfield et al. 1990), male-female sexual encounters (Gleason et al. 2009) and it is associated to morphological, physiological and behavioural changes in males (Lincoln et al. 1972; Oliviera 2004; Pereira et al. 2005), but furthermore it reduce the immune functions (Folstad and Karter 1992). In species with a clear seasonality of reproduction, the peak of secretion coincides with the breeding season (Hamasaki et al. 2001; Mooring et al. 2004) while in others peak during the pre-reproductive season (Pelletier et al. 2003) and it may be associated with age and social rank (Ditchkoff et al. 2001; Li et al. 2001; Pelletier et al. 2003).

Hormones are involved in the mechanism regulating rapid physiological and behavioural responses to environmental changing. To consider effects of unpredictable perturbations can be worthy of particular attention for animal living in extreme environment such as alpine habitats. Despite highly adapted, they are vulnerable to further distress that can hugely impact on an individual fitness beyond the normal reaction patterns (Wingfield et al. 2011). The combination of predictable or unpredictable changes such as alteration of diet quality, climate change, instable social status and infections can determine individual vulnerability to future perturbations (Wingfield 2008).

This study explores seasonal changes in the level of testosterone and cortisol by mean of their fecal metabolites in males of a species living in a demanding habitat like high mountains: Alpine ibex (*Capra ibex*). This ungulate is strongly sexually dimorphic, polygynous and characterized by a very high survival rate of adult males under 10 years old (Toïgo et al. 2007). We focused on the pre-reproductive season (late spring-summer), characterized by a relatively mild climate, but of outstanding importance for male success in the following wintry mating season (December-January). Males have to better exploit the short alpine growing time of the vegetation to be ready to deal with the behavioural and physiological demands of the rut period. Moreover, in the late spring and early summer they are engaged in frequents agonistic interactions to define male's social status (Bergeron et al. 2010; *Chapter 1*). In so doing, during the mating season the costs of intra-male interactions are reduced because of pre-established and consistent dominance relationships. This allows male Alpine ibex to reduce energy-expenditures and to increase their survival (Willisch and Neuhaus 2010). This splitting time between the pre-rut (frequent fights) and the rut (mainly courtship display), make male of this species a particular interesting candidate for an endocrine study. Hence, we are able to investigate the influence of seasonal environmental conditions during the period when males define hierarchy without the influence of physiological modifications typical of the mating season.

Our aims were:

- So far in literature only the measurement of fecal cortisol metabolites (FCM) has been validated (Möstl et al. 2002; Posautz 2010). Thus, as a first step of our analyses, is to validate a still missing enzyme immunoassay for the measurement of fecal testosterone metabolites (FTM) in male of Alpine ibex;
- To describe the seasonal and yearly variations in the level of FCM and FTM to define a correlation with the behavioural pattern.
- To investigate the causes at the base of the seasonal patterns of variation of FCM and FTM discerning the effects of individual traits (age, body mass, social status) from environmental characteristics (weather condition and diet quality).

Materials and Methods

Study area and population

This study was carried on from May to October in 2012 and 2013 in Levionaz, Valsavaranche, one of the main valley of Gran Paradiso National Park (GPNP; north-western Italian Alps; 45°25'N, 07°34'W). The study area lies between 1500 and 3000m above the sea level. At the lower altitude (1500-1900m), it is characterized by coniferous forest (*Picea abies, Pinus cembra, Larix decidua, Pinus sylvestris*). Above the 1900m the forests are replace by shrub (*Rhododendron ferrugineum, Vaccinum myrtillus, Juniperus nana*) and from 2200m high-altitude by alpine meadows (mainly *Festuca varia*), moraines, rock cliffs and glaciers are presents. The local climate is temperate. During the study periods, the temperature ranged from a minimum of –1.7°C to a maximum of 19.8°C in 2012 and from -8.4°C to 24°C in 2013 (fig.1). Monthly mean precipitation was 142.5 mm in 2012 and 4.4 mm in 2013. The other ungulates in the area are roe deer (*Capreolus capreolus*) at lower altitude (maximum 2300m) and Alpine chamois (*Rupicapra*

rupicapra) at the same altitude range of ibex. During the total count of July 2012 the ranger of the GPNP recorded 65 ibex males, 57 of which were marked with hear tag; in 2013 on 68 censed ibex males, 59 of them tagged. Ibex were captured by a Dan-Inject-dart gun. A mixture of xylazine HCL (Rompun, 20-40 mg) and ketamine (Inoketam, 50-100 mg) was used as anaesthetic (Bassano et al. 2004). After the animal was immobilized and blindfolded, it was measured and marked. In addiction to the application of hear tag (Allflex, USA: 5.5X6.8cm) with a unique combination of colours, in 2013, 10 individuals were fit with a GPS collar (GPS PRO Light collar, Vectionic Aerospace GmbH), set to attempt a localization once every 2 hours for the first seven day and then, once every 7 hours. The age of the captured male was determined with precision by counting the annual horn growth (von Hardenberg et al. 2004). As antagonist to xylazine, antipamezole (1,5 cc) were injected to accelerate the recovery taking on average less then 5 min (Bassano et al. 2004). The entire operation from capture to release took about 45 min and was always performed under the direct supervision of a veterinary. This population it’s extensively studied since 1999, until now we have never recorded any detrimental effect (neither behavioural nor physiological) of capture and marking operation. The research adhered to the ASAB/ABS Guidelines for the Use of Animals in Research and handling protocols were approved by the Institute for Environmental and Protection Research (ISPRA).

Data collection and data analysis

Feces collection and analysis

Fecal samples were collected immediately after the deposition, at least one per month, from all the available marked males. Identity of the male, date and time were registered. The specimen were frozen within 12 hour and stored in plastic bag at -20°C until the extraction procedure.

In order to validate group-specific immunoassays for the measurement of FTM a GNRH stimulation test was performed with two males of 4 years old at the Wildlife Park Langenberg, Switzerland. District veterinary office of the Canton of Zurich approved the treatment of the animals (N° 48/2013). Animals were captured by the veterinary of the park by mean of a Dan-Inject-dart gun, injecting to each animal 150mg of Xylazine (Rompun) and 120mg of Ketamine (Narketan) for anaesthesia. Once narcotized, they were caught, blindfold, separated from the herd and brought in two different enclosures. Here they received via intramuscular 4ml Vit E/Selenium (Tocoselenit), with a protective effect against anaesthesia complication and 0.01 µg of Buserelin (Receptal) as GNRH agonist. As antagonist they received 15mg of Antipamezole (Alzane). Animals were monitored for 24h, collecting all the fecal samples and registering time of defecation. The first sample was collected before Buserelin injection. All the specimens were stored at -20°C until the analysis.

We extracted fecal steroid metabolites by shaking for 1-2 min on a handvortex (Vortex mixer, company Velp), 0.5 g of wet faeces suspended in 5 ml of 80% methanol (Palme et al. 2013). The samples were centrifuged (Allegra X-12R, company Beckmann / GS-6KR) for 15 min at 2,500 g and subsequently, 300 µl of the supernatant were diluted (1:10) with assay buffer (ph 7.5), and stored at -20°C until analysis with EIA (enzyme immunoassay).

We use two different EIAs to measure the FTM levels in the samples. The standard for assay A (measuring 17β-OH-androgens) was testosterone while the standard for assay B (measuring 17-oxo-androgens) was epiandrosterone. Details of the two EIAs, including cross-reactions of the antibodies, are given by Palme and Möstl (1994). FCM levels were measures with an 11-oxoetiocholanolone EIA. The validation of this EIA for measuring FCM in ibex was performed by Posautz (2010). Möstl et al. (2002) provides the detailed information about the 11-oxoetiocholanolone EIA.

Behavioural observation

We registered the agonistic interactions involving marked individuals by mean of *ab libtum* sampling (Altmann 1974) using binoculars (8x42 and 10x42). For each observation identity of the contenders, date, time and outcome of the fight were registered. Following Bergeron et al. (2010), the identification of four main displays was used to discriminate between dominant (male A) and the submissive (male B): mounting (male A mount male B), displacement (male A go towards male B threatened it with the horn, the interaction end with the exit of male B), horn fight (after a fight characterized by contact display, such as horn clash or pushing, male B avoid male A while male A attempt to interact with male B), procession (at the end of an horn fight, male B move away followed closely by male A).

The linearity of the hierarchy was estimated using the de Vries's linearity index (*h'*; de Vries 1995, 1998), with 10000 randomizations, to determine statistical significance. The index range from 0 to 1 where 1 represents a perfectly linear hierarchy among individuals and zero the lack of it. The directional consistencies (DC) index (van Hooff and Wensing

1987) was also calculated to estimate the predictability in the outcome of encounters within each dyad. The DC ranges from 0 (unpredictability of the outcome of a dyad encounter) to 1 (complete directionality). These two indexes were implemented using Matman 1.1 (Noldus). Afterwards we assigned dominance ranks of marked individual by means of Elo-rating (Albers and de Vries 2001; Neumann et al. 2011). This method is based on the sequence of dominance interactions and provides sequential estimation of individual dominance strengths (Albers and de Vries 2001). Specifically, after each contest the winner gain points (and in the same way the loser's decreases) as function of the expected probability of that individual to win the interaction (i.e: the increase of rating is small if the chance of winning is high and vice versa). In respect to other ranking methods, Elo-ratings has several advantages that make it the more profitable for our purpose: it is independent of the number of individuals, deals very well with missing data (dyads that do not interact), takes into account the sequence of the interactions and allows to follow the temporal dynamics of interactions (Neumann et al. 2011). The stability of the hierarchical order was assessed estimating the stability index (S), proposed by McDonals and Shizuka (2013). This index unlike the one proposed by Neumann et al. (2011), is independent from group size and range from 0 (low level of stability) to 1 (high stability). Elo-ratings and Neumann stability index were calculated using scripts included in Neumann et al. (2011), we subsequently recalculated S following McDonals and Shizuka (2013).

Body mass estimation

Data on body mass were collected weighting male ibex (without handling) on platform scales placed in proximity to salt licks (Bassano et al. 2003). An operator checked weight, which appeared on a digital display, and individual identity with binoculars from such a distance (about 50-60 meters) as to avoid disturbance. In order to obtain comparable data, ibex body weights were adjusted to the 1st of August (von Hardenberg 2005) by means of a linear mixed effect model (Pinheiro and Bates 2000), with restricted maximum likelihood (REML) method and individual identity modelled as a random factor (Pelletier et al. 2007). We collected 403 measurements (190 in 2012 and 213 in 2013, respectively) of weight and we estimated the weight at the 1st of August for 44 marked males in 2012 and 41 in 2013 observed at least twice during data collection.

Environmental data

We used data collected by the National Aeronautics and Space Administration (Land Processes Distributed Active Archive Center http:// LPDAAC.usgs.gov, User Service Geological Survey – USGS – Earth Resousrces Observation and Science – EROS). NDVI is calculated from the red/near –infrared reflectance ratio [NDVI=(NIR-REF)/(NIT+RED)], where NIR and RED are the amounts of near-infrared and red light, respectively, reflected and captured by a satellite's sensor. We considered the Moderate resolution Imaging Spectroradiometer (MODIS-AQUA) dataset (250 m resolution available on a bimonthly basis, fig 1). To account for the seasonal space use of the species, we calculated the overall home range of the 10 collared males, as minimum polygon convex (MCP 100%), using software Arcmap version 10.0 (ESRI, Redlands, CA, U.S.A.) and used it as proxy of the study area.

An automatic station, located near the study area at 1951 m a.s.l, registered temperature, radiation and precipitation (24 records/d Property of Meteorological Service of Aosta Valley region). We assumed that 18h of time lag for excretion time (Huber et al. 2003; Corlatti et al. 2014), so in our analysis we included data determined one day before the fecal sample collection.

Statistical analyses

The first step of analysis was focused on the monthly variation of FTM and FCM. We fitted two general additive models (GAMM) with a Gaussian distribution of errors and ibex identity set as random factor to control for repeated data per individual. To better fit the normal distribution of the residuals, the level of both fecal metabolites (FMs) were log transformed. FMs are defined as the response variables and the Julian date was considered as continuous independent variable.

In the second phase, we examined in further details the biological and environmental causes behind these fluctuations. We explored the variations of the level of FTM and FCM through 112 (56 for each metabolites) GAMMs with the same assumptions of the previous models (Appendix A and B). The independent continuous variables taken into account were: Julian date, maximum temperature, mean precipitation, radiation, mean NDVI, age, body mass and rank position. The association between explanatory variables were examined by means of Pearson correlation coefficient. Age, body mass and rank position were used alternatively in the models in consideration of the collinearity revealed by the test. The selection of the best model was assessed via single-term addiction from the simplest model (single term) to the fullest model involving all dependent variables. AIC values and AIC weights of the models were compared and following Symonds and Moussalli (2011) we selected models with ΔAIC values <2 (tab. 1-tab. 2). We used graphical inspection to ensure that the residuals from the models were normally distributed and to check for the homogeneity of variance. We used R software version 3.0.2 (R Core team 2013) in Rstudio 0.98-953 (Rstudio 2013) to perform all analyses.

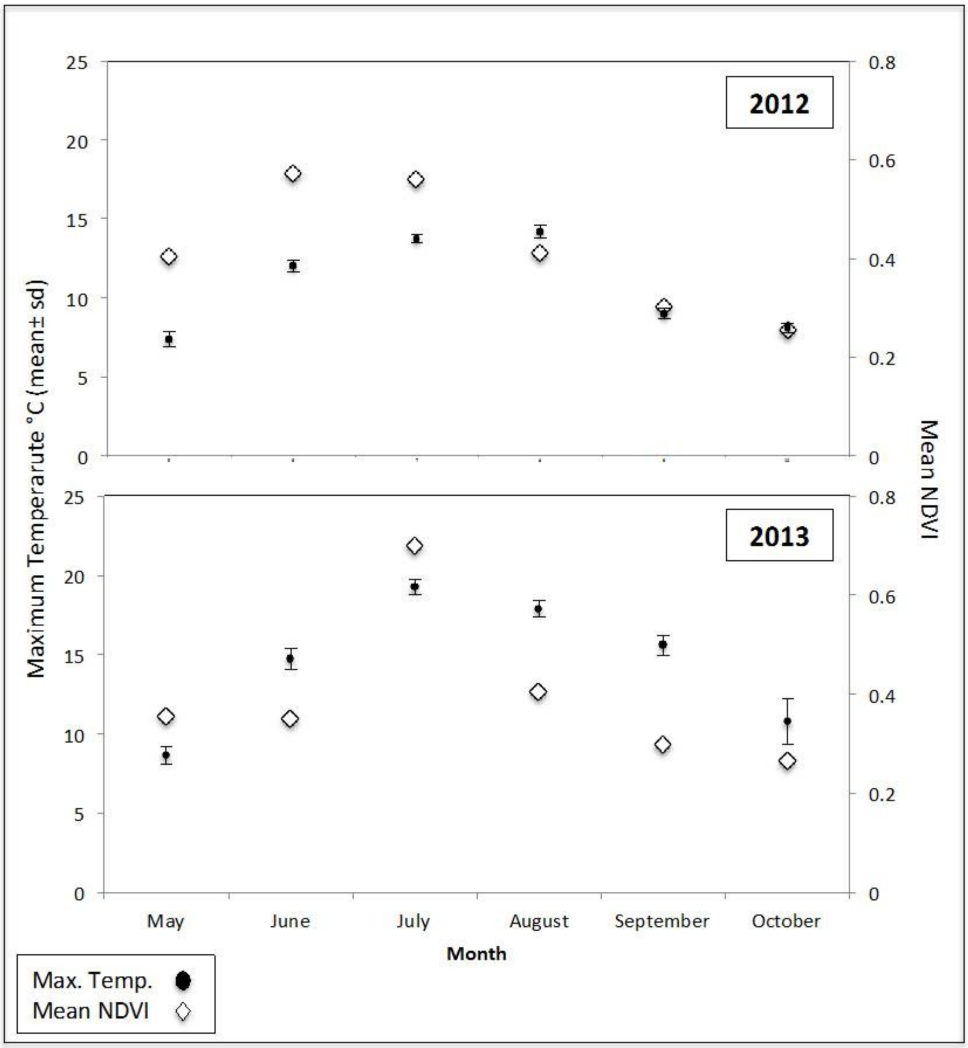

Figure 1. Monthly maximum temperature (mean±s.d.) registering by an automatic station, located at 1951 m a.s.l, (Property of Meteorological Service of Aosta Valley region) and mean NDVI derivated from moderate resolution Imaging Spectroradiometer (MODIS-AQUA) dataset (data collected by the National Aeronautics and Space Administration).

Table 1. Set of the best Generalized Additive Mixed Models that predict the variation of fecal cortisol metabolites (FCM) from May till October (2012-2013) in male Alpine ibex in the Gran Paradiso National Park. FCM were modelled by body mass (bm), maximum temperature (temp), mean NDVI (NDVI), radiation (rad), Julian date (JD).

Model term	df	AIC	ΔAIC	AIC weight
FCM{age+temp+NDVI+JD}	11	1553.511	0	0.780
FCM{age+NDVI+JD}	9	1556.054	2.543	0.219

Table 2. Set of the best Generalized Additive Mixed Models that predict the variation of fecal testosterone metabolites (FTM) from May till October (2012-2013) in male Alpine ibex in the Gran Paradiso National Park. FTM were modelled by body mass (bm), maximum temperature (temp), mean NDVI (NDVI), radiation (rad), Julian date (JD).

Model term	df	AIC	ΔAIC	AIC weight
FTM{bm+temp+rad+NDVI+JD}	13	1580.584	0	0.276
FTM{bm+temp+NDVI+JD}	11	1508.653	0.069	0.266
FTM{bm+rad+NDVI+JD}	11	1509.312	0.728	0.191
FTM{bm+NDVI+JD}	9	1510.100	1.516	0.129

Results

The epiandrosterone assay detected the highest percentages of increase in immunoreactive metabolites (17-oxo-androstanes) prior and after GnRH injection in fresh fecal samples (fig. 2).

Dominance relationship (see tab. 3 for descriptive statistic) among male Alpine ibex was significantly linear each year. The value of *h'* was comparable to the one estimated by Bergeron et al. (2010) for the same population. The high DC indicates that for a given dyad, the outcome of an interaction was consistent with the results of the previous encounter. The hierarchical order estimated by Elo-ratings was stable as indicated by S index, suggesting little changes within hierarchical order among individuals.

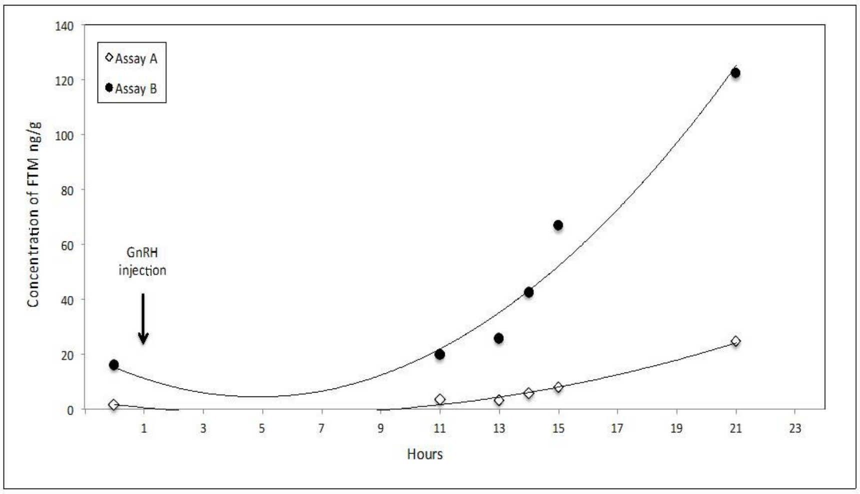

Figure 2. Concentration of FTM in fecal samples of male ibex over the whole observation period. Assay A: 17-β-OH-androgens (standard: testosterone); Assay B: 17-oxo-androgens (standard: epiandrosterone).

Table 3. Dominance matrices of Alpine ibex males in 2012 and 2013 in Gran Paradiso National Park, Italy

Year	*n*	Interaction observed	*h'*	P- value	DC	S
2012	53	574	0.125	0.001	0.950	0.992
2013	45	564	0.136	<0.001	0.930	0.996

The levels of FCM varied through the pre-reproductive seasons (tab. 4). Interestingly the models showed a divergent trend in 2012 and 2013 (fig. 3). Specifically, FCM concentrations appear to grow gradually as the season progresses in 2012; whereas in 2013 the curve estimated by the model remains most of all stable.

Table 4. Generalized Additive Mixed Model (GAMM) of the effects of the day of the year, express as julian day, (JD) on the level of fecal cortisol (FCM) and fecal testosterone metabolites (FTM) of male Alpine ibex in the Gran Paradiso National Park, Italy.

Model	*Intercept*				*Approximate significance of smooth terms (JD)*				R^2_{adj}	*Scale est.*	*n*
	Coeff.	**SE**	**T**	**P(>|t|)**	***edf***	***d.f.***	$F_{edf,\ d.f.}$	***p-value***			
FCM_{2012}	4.498	0.054	82.63	<0.001	2.91	2.91	59.17	<0.001	0.324	0.500	321
FCM_{2013}	4.629	0.066	69.64	<0.001	2.119	2.119	9.357	<0.001	0.048	0.522	357
FTM_{2012}	6.038	0.065	92.16	<0.001	4.844	4.844	25.96	<0.001	0.207	0.327	321
FTM_{2013}	5.614	0.075	74.49	<0.001	7.228	7.228	7.919	<0.001	0.107	0.554	357

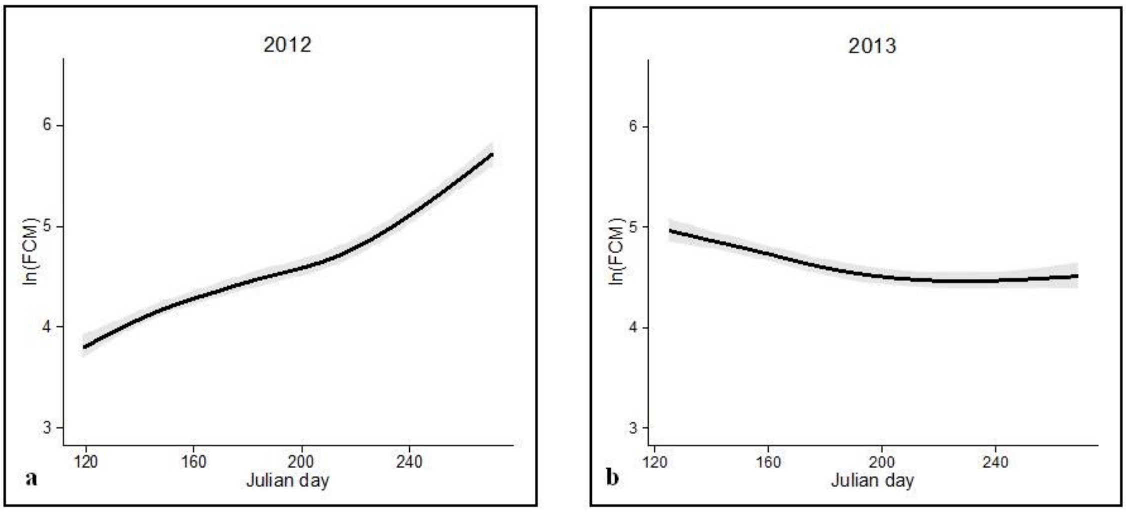

Figure 3.Daily variation of fecal cortisol metabolites (ln-transformed, ηg/g) predicted by the Generalised Additive Mixed Model for male Alpine ibex (including the individual as a random effect) from May till the beginning of October in 2012 (a) and in 2013 (b) in Gran Paradiso National Park, Italy.

The best model (tab. 1) describing the variation of FCM (AIC weight 0.780) includes four predictors: maximum temperature, age, mean NDVI and Julian date. The model showed a positive linear correlation with temperature (0.025±0.009). There is a slightly appreciable quadratic effect of age (fig. 4.) with the level of FCM that increase till the age of 10 years old. The influence of NDVI is described by a sinusoidal curve (fig. 5) that estimated the highest levels of FCM in correspondence of the lowest value of mean NDVI.

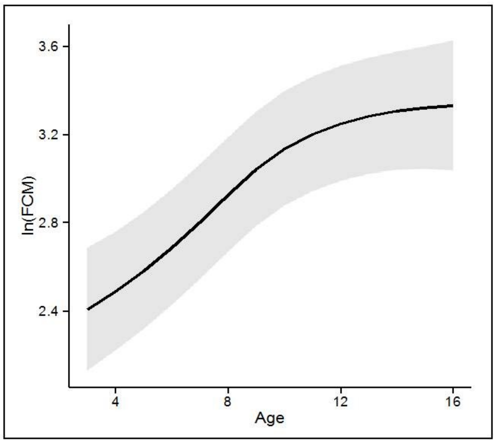

Figure 4. Variation of fecal cortisol metabolites (ln-transformed, ηg/g) predicted by the most solid General Additive Mixed Model for male Alpine ibex (including the individual as a random effect) as a function of age, in Gran Paradiso National Park, Italy.

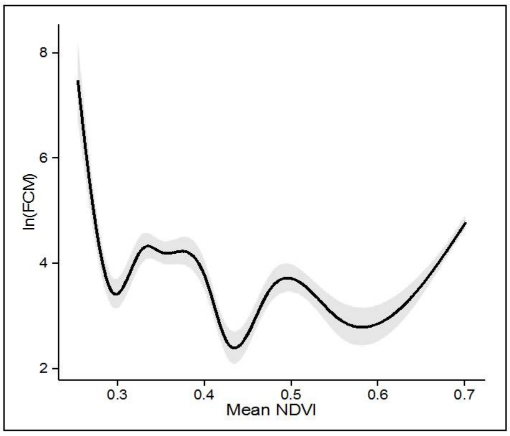

Figure 5. Variation of fecal cortisol metabolites (ln-transformed, ηg/g) predicted by the most solid General Additive Mixed Model for male Alpine ibex (including the individual as a random effect) as a function of the mean NDVI, in Gran Paradiso National Park, Italy.

FTM levels were not constant across the season (tab. 4). Specifically, as well rendered by the two sinusoidal curves (fig. 6) estimated by the model, in 2012 and in 2013, FTM had two important increases: one from May to June (1st of June= Julian date 152) and the second started at the end of the summer (1st of September= Julian date 244).

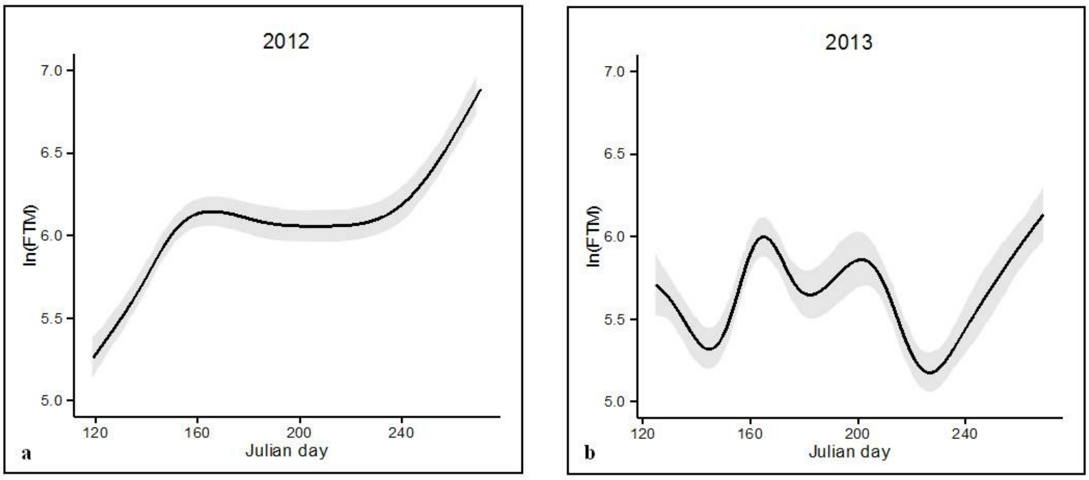

Figure 6. Monthly variation of fecal testosterone metabolites (ln-transformed, ηg/g) predicted by Generalised Additive Mixed Model for male Alpine ibex (including the individual as a random effect) from May till the beginning of October in 2012 (a) and in 2013 (b).

The four models (tab. 2), with ΔAIC<2 (Symonds and Moussalli 2011), best describing the variation of FTM (AIC weight respectively: 0.275; 0.266; 0.191; 0.129) include the variables body mass, maximum temperature, radiation, mean NDVI and Julian date. The model with the lowest AIC predicted an increase in the level of FTM with body mass (0.023±0.003) and maximum temperature (0.020±0.010) and as described by the curve (fig. 7) with radiation. On the other hand, the relation with mean NDVI is described by a the sinusoidal curve with a irregular trend (fig. 8).

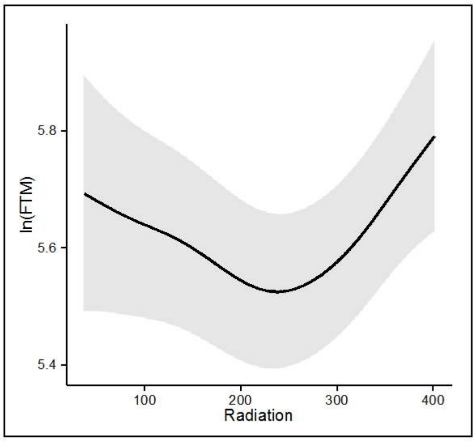

Figure 7. Variation of fecal testosterone metabolites (ln-trasformed, ηg/g) predicted by the most solid General Additive Mixed Model for male Alpine ibex (including the individual as a random effect) as a function of the radiation.

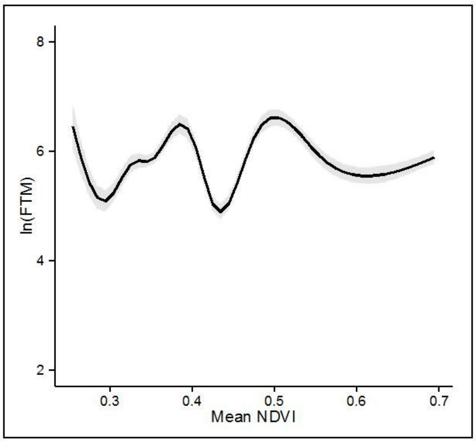

Figure 8. Variation of fecal testosterone metabolites (ln-transformed, ηg/g) predicted by the most solid General Additive Mixed Model for male Alpine ibex (including the individual as a random effect) as a function of the mean NDVI.

Discussion

Our study examines the variation of FCM and FTM in male Alpine ibex. The prospect of research allowed by the analysis of endocrinological differences at individual levels is ample, but to have reliable result a careful validation for each species and sex is needed (Touma and Palme 2005). A validation for the measurement of FCM in males of this species was already performed (Posautz 2010), but for FTM it was still missing. We filled this gap by performing a GnRH stimulation test. Epiandrosterone EIA (measuring androgen metabolites with a 17-oxo group) have been shown to reliability reflect testicular endocrine function in a variety of mammalian species (Ganswindt et al. 2002; Seeber et al. 2013). We recommend the use of this EIA for the analysis of FTM in male Alpine ibex, due to its more sensitive response the GnRH injection.

Alpine ibex males' life history is characterized by two important events: pre-reproductive season (from early spring till late autumn) and rut (December-mid January). In spring male ibex aggregate at the lowest altitude meadow, engaging frequent fights to define individual's social status. Thanks to the established hierarchy, male ibex will reduce the conflicts during the demanding reproductive season (Willisch and Neuhaus 2010). Monitoring FCM and FTM in the pre-reproductive season allowed us to follow the endocrinological changing (in response to behavioural and environmental stimuli) that prepare male to deal with the wintry reproductive season. Our study, showing the different yearly trends of each FMs, highlights these correlations. The level of FCM appears to be more stable in 2013 in respects to 2012, where it increased from May to October, (fig. 3), while FTM peaked, both years, in late spring and in late summer (fig. 6). In spring high testosterone levels can promote competiveness despite their potential costs (Folstad and Karter 1992), while as the reproductive season gets closer (from the beginning of autumn) the levels of FTM began to increase. The pattern of increase of androgens levels in the pre-rut, till a maximum reached during the mating season, has already been described in other ungulate species, like red deer (*Cervur elaphus hispanicus*, Gaspar-Lopez et al. 2010) and Pampas deer (*Ozoteceros bezoarticus bezoarticus*). In bison bulls (*Bison bison*) Mooring et al. (2004) found androgens level more than two times higher compared with the pre-rut level.

The level of FCM increased with age till 10 years old (fig. 4) while FTM grew linearly with body mass. Younger males, with lower body mass and horn length, are less competitive and generally subordinate (Bergeron et al. 2010). The growth in size is slow (Toïgo et al. 1999), so that male ibex reach the body mass asymptotically and late (according to our data at an age of about 10 years). Therefore, from about 9 years old, males are heavy and with horn long enough to compete for the top of the hierarchy (Bergeron et al. 2010; Willisch and Neuhaus 2010). In mammals, testosterone increases muscle volume, body mass (Kousteni et al. 2001; Hartgens and Kuipers 2004) ornaments and armaments (Bartoš et al. 2009). According to our models, in male Alpine ibex the production of testosterone follow a quadratic effect driven by the growth in body mass that reaches the maximum at about 10 years of age, when males are potentially at the best physical condition. After that, probably due to a different allocation of energy in consequence of senescence (about 13 years old, Toïgo et al. 2007), the increase of production stops. In alpine ibex, as in bighorn sheep (*Ovis canadensis*, Martin et al. 2013), testosterone may be correlated with aggressiveness, endurance or other factors that improve ability to fight.

The increase in glucocorticoid level with age has already been documented in other species (Sapolsky et al. 1983; Meaney et al. 1988; Dellu et al. 1996; Mooring et al. 2006) as a results of an effect of age on stress response and in particular an impairment of negative feed back system regulating the glucocorticoid response to stress (Reeder and Kramer 2005). Moreover we have to add the potential stress induced by repeated aggressive interactions, and endurance competition engaged by males during the pre-reproductive season (Muller and Wrangham 2004; Sands and Creel 2004). This result is in accordance with the stress of domination hypothesis which predicts that high ranking individuals are more stressed than subordinates because they must engage in energetically expensive display and fights and/or because of instable hierarchy order (Creel 2001). Finding this correlation already in the summer season highlights the need to careful examine this endocrinological aspect during the wintry rutting season when males are exposed to a greater amount of stress (environmental and behavioural) that can be potentially detrimental for individuals fitness.

On the environmental level the models predict correlations with maximum temperature and mean NDVI for both FMs and more with radiation for FTM. Maximum temperature exerts an influence on cortisol secretion. Ungulate living in Alpine habitat are well adapted to cold climate (Corlatti et al. 2014), but can experienced heat stress at lower temperatures than species adapted to warmer environments (Schab and Pitt 1991; Owen-Smith 1998).

These species can cope with environmental changes such as a rise in temperature, with behavioural (i.e shifting the peak of activity from midday to morning and evening; Roberts and Dunbar 1991) or physiological responses (i.e increase metabolic rate*,* Renecker and Hudson 1986). It has already been shown that Alpine ibex handle heat stress in summer, moving to higher elevation, expanding its home range (Aublet et al. 2009; Grignolio et al. 2004) and constraining foraging activities during the day (Neuhaus and Ruckstuhl 2002): our work highlights an additional response to thermal stress. We found a direct proportionality also between maximum temperature and FTM. Seasonal variation of testosterone has always been related to behavioural changes or to photoperiod (Wingfield et al. 1990; Ketterson and Nolan 1992) but not to temperature. This result could be related to other modification like weight gain, but to correctly interpret it further studies are needed.

The sinusoidal curves accounted for the effect of mean NDVI have no clear biological interpretation by itself. It's shape is probably driven by temporal (day) or spatial (seasonal movement of ibex) changing, for this reason it could be necessary to set an appropriate sampling design to explore these preliminary results into more detailed.

In conclusion, in the pre-reproductive season the peak of FTM provides an impulse to compete for a high ranking position and is correlated to individual body mass. FCM responded to environmental stress like high temperature and behavioural demands like to compete for high position of dominance. However, we have to consider that cortisol is an adaptive mediator of the stress response by helping animal redirect activities. An increase in FCM does not automatically equate to a state of distress (Romero 2004). To know the basal level of FMs is crucial for conservation biology because allowed to correct evaluate the reaction to environmental or behavioural perturbation. Furthermore our work pointed out different hormonal patterns for the two year of studying. It's important not to underestimate the relevance of this result because it implies the need of a long time series of data in order to assess the endocrinological state of a population. It's a call to carefully assess the background in which they are integrated to make reliable comparisons (Corlatti et al. 2014). Without this information, the influence of natural or human-induced effects on wildlife populations becomes difficult to assess (Millspaugh and Washburn 2004). As mediator of life history trade-offs, monitoring FCM give the possibility to identify and prevent the impact of stressful events from climate change to anthropic disturbance, while the analysis of FTM can be an important tool for the analysis of male endocrine basis of dominance and subsequently male mating tactics.

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Appendix A. Set of the Generalized Additive Mixed Models tested ordering by AIC vaule. The models predict the variation of fecal testosterone metabolites (FTM) from May till October (2012-2013) in male Alpine ibex in the Gran Paradiso National Park. FTM were modelled by age, body size (BS), rank, maximum temperature (TEMP), radiation (RAD), precipitation (prec), mean NDVI (NDVI) and Julian date (JD). Degree of freedom (df), AIC value, ΔAIC value and weights are shown.

AGE	BS	RANK	TEMP	RAD	PREC	NDVI	JD	df	AIC	ΔAIC	Weight
	x		x	x		x	x	13	1508.584	0.000	0.275
	x		x			x	x	11	1508.653	0.069	0.266
	x			x		x	x	11	1509.312	0.728	0.191
	x					x	x	9	1510.100	1.516	0.129
	x				x	x	x	11	1511.661	3.077	0.059
	x			x	x		x	11	1512.238	3.654	0.044
	x		x	x	x	x	x	15	1514.046	5.462	0.018
	x		x	x	x		x	13	1515.402	6.818	0.009
x			x			x	x	11	1517.698	9.114	0.003
x						x	x	9	1518.459	9.875	0.002
x				x		x	x	11	1519.412	10.828	0.001
x			x	x		x	x	13	1521.088	12.504	0.001
	x			x			x	9	1521.112	12.528	0.001
x					x	x	x	11	1522.458	13.874	0.000
x			x	x	x	x	x	15	1523.037	14.453	0.000
		x	x			x	x	11	1525.014	16.430	0.000
	x		x	x			x	11	1525.112	16.528	0.000
	x		x		x		x	11	1525.435	16.851	0.000
		x				x	x	9	1526.001	17.417	0.000
	x						x	7	1526.005	17.421	0.000
	x				x		x	9	1526.517	17.933	0.000
		x		x		x	x	11	1526.676	18.092	0.000
		x	x	x		x	x	13	1528.598	20.014	0.000
	x		x				x	9	1529.058	20.474	0.000
		x			x	x	x	11	1529.982	21.398	0.000
		x	x	x	x	x	x	15	1530.671	22.087	0.000
			x			x	x	9	1538.651	30.067	0.000
x				x	x		x	11	1540.293	31.709	0.000
						x	x	7	1541.167	32.583	0.000
				x		x	x	9	1541.281	32.697	0.000
			x	x		x	x	11	1542.314	33.730	0.000
x			x	x	x		x	13	1543.386	34.802	0.000
				x	x	x	x	11	1544.862	36.278	0.000
					x	x	x	9	1544.990	36.406	0.000
			x	x	x	x	x	13	1547.928	39.344	0.000
		x		x	x		x	11	1550.392	41.808	0.000
		x	x	x	x		x	13	1553.343	44.759	0.000
				x	x		x	9	1556.436	47.852	0.000

AGE	BS	RANK	TEMP	RAD	PREC	NDVI	JD	df	AIC	ΔAIC	Weight
x				x			x	9	1556.932	48.348	0.000
x			x		x		x	11	1557.908	49.324	0.000
x					x		x	9	1558.112	49.528	0.000
			x	x	x		x	11	1559.604	51.020	0.000
x			x	x			x	11	1560.085	51.501	0.000
x							x	7	1567.102	58.518	0.000
		x	x		x		x	11	1567.497	58.913	0.000
		x			x		x	9	1567.968	59.384	0.000
		x		x			x	9	1568.074	59.490	0.000
				x			x	7	1569.325	60.741	0.000
			x		x		x	9	1570.694	62.110	0.000
x			x				x	9	1570.909	62.325	0.000
		x	x	x			x	11	1571.108	62.524	0.000
					x		x	7	1571.636	63.052	0.000
			x	x			x	9	1573.089	64.505	0.000
		x					x	7	1575.647	67.063	0.000
			x				x	7	1579.383	70.799	0.000
		x	x				x	9	1579.603	71.019	0.000

Appendix B. Set of the Generalized Additive Mixed Models tested ordering by AIC vaule. The models predict the variation of fecal cortisol metabolites (FCM) from May till October (2012-2013) in male Alpine ibex in the Gran Paradiso National Park. FCM were modelled by age, body size (BS), rank, maximum temperature (TEMP), radiation (RAD), precipitation (prec), mean NDVI (NDVI) and Julian date (JD). Degree of freedom (df), AIC value, ΔAIC value and weights are shown.

AGE	BS	RANK	TEMP	RAD	PREC	NDVI	JD	df	AIC	ΔAIC	Weight
x			x			x	x	11	1553.511	0.000	0.780
x						x	x	9	1556.054	2.543	0.219
	x		x			x	x	11	1567.598	14.087	0.001
		x	x			x	x	11	1569.322	15.811	0.000
		x				x	x	9	1571.874	18.363	0.000
	x					x	x	9	1573.315	19.804	0.000
			x			x	x	9	1580.517	27.006	0.000
x			x	x	x	x	x	15	1580.643	27.132	0.000
x				x		x	x	11	1580.898	27.387	0.000
x			x	x		x	x	13	1581.746	28.235	0.000
						x	x	7	1585.461	31.950	0.000
	x		x	x		x	x	13	1593.284	39.773	0.000
		x	x	x	x	x	x	15	1594.758	41.247	0.000
	x		x	x	x	x	x	15	1594.867	41.356	0.000
	x			x		x	x	11	1594.885	41.374	0.000
		x		x		x	x	11	1595.687	42.176	0.000
		x	x	x		x	x	13	1596.032	42.521	0.000
x					x	x	x	11	1596.218	42.707	0.000
x			x	x			x	11	1602.975	49.464	0.000
x			x	x	x		x	13	1606.506	52.995	0.000
			x	x	x	x	x	13	1606.648	53.137	0.000
			x	x		x	x	11	1606.797	53.286	0.000
x				x			x	9	1607.170	53.659	0.000
x				x	x		x	11	1608.665	55.154	0.000
				x		x	x	9	1608.866	55.355	0.000
	x				x	x	x	11	1610.413	56.902	0.000
		x			x	x	x	11	1612.014	58.503	0.000
				x	x	x	x	11	1612.184	58.673	0.000
		x	x	x			x	11	1614.884	61.373	0.000
x			x		x		x	11	1615.945	62.434	0.000
		x	x	x	x		x	13	1617.800	64.289	0.000
		x		x			x	9	1618.932	65.421	0.000
		x		x	x		x	11	1620.748	67.237	0.000
	x		x	x			x	11	1621.468	67.957	0.000
					x	x	x	9	1622.349	68.838	0.000
	x		x	x	x		x	13	1624.092	70.581	0.000
	x			x			x	9	1627.355	73.844	0.000
	x			x	x		x	11	1627.675	74.164	0.000

AGE	BS	RANK	TEMP	RAD	PREC	NDVI	JD	df	AIC	ΔAIC	Weight
		x	x		x		x	11	1628.317	74.806	0.000
x			x				x	9	1629.103	75.592	0.000
x							x	7	1630.328	76.817	0.000
			x	x			x	9	1631.244	77.733	0.000
x					x		x	9	1631.876	78.365	0.000
			x	x	x		x	11	1634.016	80.505	0.000
				x	x		x	9	1637.547	84.036	0.000
				x			x	7	1638.072	84.561	0.000
	x		x		x		x	11	1638.762	85.251	0.000
		x	x				x	9	1642.924	89.413	0.000
		x					x	7	1644.147	90.636	0.000
			x		x		x	9	1644.328	90.817	0.000
		x			x		x	9	1644.728	91.217	0.000
	x		x				x	9	1646.279	92.768	0.000
	x						x	7	1650.540	97.029	0.000
			x				x	7	1653.716	100.205	0.000
	x				x		x	9	1655.664	102.153	0.000
					x		x	7	1661.698	108.187	0.000

Chapter 3

Are males more timorous or females more cautious? Sexual different responses to a change in the landscape of fear.

Nicoletta Sica [a], Stefano Grignolio [a], Francesca Brivio [a], Marco Apollonio [a]

[a] University of Sassari, Department of Science for Nature and Environmental Resources, via Muroni 25, I-07100, Sassari, Italy.

Abstract

Predators may influence their prey populations not only through direct lethal effects, but also through indirect behavioural changes. The natural expansion of the wolf (*Canis lupus*) in North-Western Alps provided the rare opportunity to monitor the behavioural responses of a prey species to the arrival of a predator which had been absent for about a century. Sex-specific density effects have rarely been considered in the study of antipredator strategies. We examined the effects of the wolf recolonization and density modifications on group size and on use of safe areas by male and female Alpine ibex (*Capra ibex*) in the Gran Paradiso National Park (Italy), a location where no terrestrial predators were formerly present from about one century. We documented that the change of the landscape of fear induced, in only a few years, significant modifications in ibex behavioural patterns that can not been explained if we considered only density changes. Male groups decreased in size and moved closer to safer areas. On the contrary, the distance of female groups from refuge sites was not affected and their propensity to live in group was only partially modified by the modifications in the environmental conditions. Our results showed that male and female ibex, which are characterized by a strong dimorphism, adopted different strategies to solve the conflicting demands of nutrient intake versus predation avoidance. Results also strongly suggest that, in polygynous species, sexual selection implies different behavioural patterns in males and females. Behavioural modifications likely caused a reduction of nutrients intake in males, because they tended to use lower-quality feeding patches.

Keys words: Alpine ibex, antipredator behaviour, behavioural modification, density modification, risk effects, wolf recolonization.

Introduction

In the past, predator-prey models typically focused on lethal (predation) events only (Holling 1959), while more recent works reconsidered the importance of indirect effects of predators' presence on the population dynamics of prey species (e.g., altered prey behaviour or physiological modifications, cf. Creel and Christianson 2008). While the benefits of antipredator behaviours are fairly obvious (decreased risk of predation), their costs (risk effects) are less apparent and have often been disregarded in the analyses of vertebrate predator–prey dynamics (Creel and Christianson 2008). Predator-prey models that neglect prey's behaviour may fail in describing population dynamics adequately. The example of the wolf-elk models explain well this topic: the theoretic predator-prey models built with data collected in Yellowstone National Park predicted a decline of 10-25% in the elk (*Cervus elaphus*) population after the recolonization of wolf (*Canis lupus*; Boyce and Gaillard 1992; Garton and Crabtree 1992). In Banff National Park (Alberta, Canada), however, the elk population declined by 50-70% (Hebblewhite et al. 2002) after the return of wolves. The difference between the estimates based on theoretical models (that did not considered induced behavioural modifications) and the actual decrease in elk population of Banff National Park was explained by the interaction between wolf predation and snow depth, which probably caused modifications in elk grouping behaviour (Hebblewhite and Pletscher 2002).

One of the main risk effects assessed in vertebrates is the alteration of prey habitat use, with prey trading security for a reduction in forage quality and/or quantity (Brown 1999), as a consequence of modifications in perceived predation risk. For example, when predators are present, small blue gill sunfish (*Lepomis macrochirus*) switch to safer but less profitable habitats. As a result, their intake rate and growth rate decrease (Werner et al. 1983). Prey's responses include changes in activity patterns (Fenn and Macdonald 1995) or in vigilance levels (Ciuti et al. 2012), as well as variations in group size (Lima 1995), or interactions among these behavioural patterns (Roberts 1996; Childress and Lung 2003). Embar et al. (2011) showed that gerbils (*Gerbillus andersoni allenbyi*) harvested fewer seeds, allocated less time to foraging in dangerous patches, and were more vigilant while foraging where and when risks were higher. Several studies have measured the effects of modification in predation risk on habitat selection or on vigilance behaviour, but in most cases group size is considered as an independent variable. More recently, researchers have related group size, considered as a dependent variable, to independent measures of risk (see, for example, Creel and Winnie 2005). As yet, though, few works have examined simultaneously how group size and use of safe areas are affected by a natural variation in predation risk.

In the analyses of antipredator behaviour and group size dynamics of social species it is important to consider that density-dependent processes have a fundamental effect on them (Rangeley and Kramer 1998; Vander Wal et al. 2013). However, in the analyses of behavioural patterns it is often difficult to disentangle the roles of predation risk and density. When these two factors act simultaneously, they may amplify or cancel out the effects of each other. Anyway, the new findings on behavioural ecology can help to understand their interplay. For example Vander Wal et al. (2013), assuming a random Brownian model of animal movements, obtained evidence for more pronounced negative density dependence in female groups' size.

The synergism between morphological and behavioural traits constraints the antipredator strategy adopted by animals (Cott 1940), so that different individuals of the same species may use different defences against the same predator (Caro 2005). One factor that plays an important role in this respect is the animal body size, which generally influences the individual sensitivity to predation risk (Cohen et al. 1993; Sinclair et al. 2003). Accordingly, in species which are characterised by sexual body size dimorphism, antipredator strategies of females should be expected to differ from those of males. Several studies on sexual dimorphic ungulates have shown that smaller females are more likely to be found in safer habitat (hiding in bushes, staying close to cliffs etc.), while males can better exploit areas of greater predation risk because their larger size makes them less vulnerable to predators than females (see for example: *Ovis canadensis nelsoni*, Bleich et al. 1997; *Ovis dalli dalli*, Corti and Shackleton 2002; *Ovis canadensis mexicana*, Mooring et al. 2003; *Dama dama*, Ciuti et al. 2004; *Syncerus caffer*, Hay et al. 2008). In mouflon (*Ovis aries*), the grouping patterns of males and females reflected different anti-predator tactics, with females forming larger groups, particularly during the lambing season (Pipia et al. 2009).

As yet, the response of prey species to a modification in the landscape of fear has been mostly evaluated on the basis of the information obtained after the reintroduction of the grey wolf in the Yellowstone National Park, Wyoming, where the wolf has been absent for about 50 years. During this period, ungulates (i.e., elk and bison, *Bison bison*) were still preyed by grizzly bears (*Ursus arctos horribilus*), coyotes (*Canis latrans*), and mountain lions (*Puma concolor*). The study case of Yellowstone National Park (Fortin et al. 2005), as well as the case of Serengeti National Park, Tanzania, where the lion (*Panthera leo*) was reintroduced (Hunter and Skinner 1998), documented how prey species changed their behaviour when the landscape of fear was modified by the presence of newly reintroduced predators, in addition to those already present. What can happen if a predator re-establishes where prey species have been living free from terrestrial predators for a long time? The natural wolf expansion in North-Western Alps (Scandura et al. 2001; Fabbri et al. 2007; Marucco and McIntire 2010; Palmegiani et al. 2013) provides an opportunity for ecologists to accept the challenge proposed by Creel and Christianson (2008) to design field studies that determine the relative magnitudes of risk effects and direct predation in wild populations that are not manipulated. In the present paper we assessed the indirect effects of the natural expansion of the grey wolf on the antipredator behaviour of Alpine ibex (*Capra ibex*) taking into account that wolf predation has neglected consequence for population dynamic (Palmegiani et al. 2013). Alpine ibex is a good model species to test the influence of sex on antipredator behaviours by virtue of its sexual dimorphism (Bassano et al. 2003), which is one of the most accentuated among ungulate species (Loison et al. 1999; Ruckstuhl and Neuhaus 2005).

We formulated two alternative sets of hypotheses for females and males to disentangle the effects due to the increase of predation risk and the changes in the population size (tab. 1). Ibex, males and females, live in open areas in social groups throughout the year. Based on the wide range of available literature on social ungulates (Caro 2005), we predicted that both sexes form larger groups with increasing the predation risk. Instead, we predicted that ibex density reduction don’t influence the size of the male groups. For male the costs of living in a group are far outweighed by the positives effects (Brivio et al. 2014). For female similar information are not available, so their group size is expected to reduce with reduction of population density – due to lower number of conspecifics – or to remain constant.

With regard to habitat use, we know that ibex of different age-sex classes showed different antipredator behaviour (i.e. use of safer areas) also in absence of predator (Grignolio et al. 2007 a,b). Hence, we foresee that wolf recolonization forced male and female groups to live closer to rocky slopes (refuge areas). But, it is important to consider that females groups, also before the return of the wolf used the meadows very close to the rocks, so they could find more difficult to get more adjacent or they could consider the areas used secure enough from the predator. Instead, in the light of the available information, we have not reasons to predict a change in the use of refuge areas due to a modification in the population size.

Table 1. Expected effect due to the reduction of population size or the increase of predation risk on Alpine ibex group size and the distance from refuge areas in Gran Paradiso National Park, Italy.

		Group size	**Distance from refuge areas**
Reduction of population size	***Male groups***	↔ No changes because for males is favourable living in group (Brivio et al. in press)	↔ No change is expected
	Female groups	↓ Decrease to reduce intrasexual competition	↔ No change is expected
Increase of predation risk	***Male groups***	↑ Increase to increase safety	↓ Reduction to increase safety
	Female groups	↑ Increase to increase safety	↓ Reduction to increase safety, but less than males considering that females are living very close to rocky slopes

Materials and Methods

Study area and population

This study was conducted in the GPNP (northwestern Italian Alps; 45° 25′ N, 07° 34′ E) in the Levionaz basin (about 1700 ha, fig. 1). The main habitats in the study area were rocks, glaciers, stone ravines and alpine meadows. Forests represented less than 10% of the area and were below 2000-2300 metres above sea level. Upper forest vegetation was mainly composed of conifers (*Picea abies* and *Larix decidua*). Above the timberline, slopes were covered by meadows (*Festuca spp* and *Carex curvula*) and shrubs (*Rhododendron ssp., Vaccinium vitis-idaea, Vaccinum myrtillus* and *Juniperus nana*). Areas used by ibex ranged from 1500 m to 3300 m above sea level. Hunting was not allowed in the GPNP since the ban of legal trophy hunting in 1960s and the park wardens never reported poaching events inside the study area during the period of data collection. The most relevant natural predators, such as lynx (*Lynx lynx*) and wolf, had been absent for about a century. The only potential predator for ibex present in the study area was the Golden eagle (*Aquila chrysäetos*), but it exerted only a very scarce impact on kids of a few months of age. During summer 2006, the first signs of the presence of a breeding wolf pair were found and the presence of a wolf pack was confirmed during data collection. The wolf pack ranged from four to six individuals (Palmegiani et al. 2013).

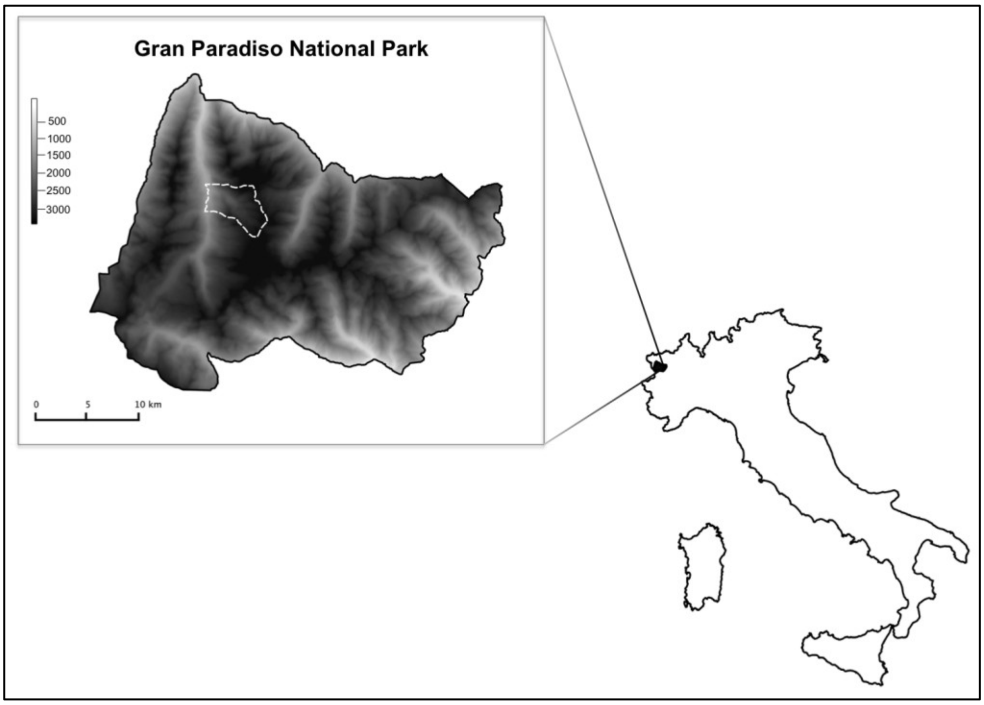

Figure 1. Map of Italy displaying the location of the Gran Paradiso National Park and the map of the park with the border (white broken line) of the Levionaz basin (study area). Shading indicates altitude in metres.

The Alpine ibex population of the GPNP grew to almost 5,000 individuals in the 1990s, and then began to decline (Jacobson et al. 2004). Ibex density in the Levionaz basin during the years of data collection ranged from 19.0 individuals/Km^2 (in 2004) to 9.0 individuals/Km^2 (in 2008; tab. 2). Every year the wardens and vets of the GPNP captured some ibex by telenarcosis to monitor the prevalence of infectious diseases and to mark the animals. Throughout the present study, ibex were captured using the same methodology described in Bassano et al. (2004) and reported in Grignolio et al. (2007a). Ibex were aged by counting the clearly separated annuli on their horns (Ratti and Habermehl 1977; von Hardenberg et al. 2004) and then marked with livestock ear tags (Allflex, USA: 5.5 x 6.8cm) and visual collars. In 15 years of ibex behavioural research, we never recorded any adverse effects of marking on animals. During the manipulation, no ibex showed any particular signs of respiratory, cardiac, or physical pain. The research adhered to the ASAB/ABS Guidelines for the Use of Animals in Research and the handling protocols were approved by the Italian National Wildlife Institute.

Table 2. Alpine ibex population size and ratio of marked animals during the years of data collection in the study area of Levionaz basin, Gran Paradiso National Park, Italy.

Year	**Males (N)**	**% of marked males**	**Females (N)**	**% of marked females**	**Sex ratio**
2000	114	0.46	113	0.11	0.99
2003	108	0.55	119	0.23	1.10
2004	104	0.56	129	0.17	1.24
2005	98	0.56	117	0.19	1.19
2008	85	0.69	81	0.12	0.95
2009	64	0.81	44	0.20	0.69
2010	62	0.68	50	0.16	0.81

Data collection

Data collection was conducted from May to October: before (2000, 2003-2005) and after wolf recolonization (2008-2010). In order to monitor habitat use and grouping patterns, we used 10 line transects of 3-6 km each. The transects were designed to provide an adequate coverage of the study area and a proper sampling of the habitat types typically used by female and male ibex. Each transect was surveyed at least 3 times per month during morning or evening hours (i.e., when animals were most active), by walking different hiking trails for an overall total of about 2,900 km. For our analyses, one or more conspecifics within a range of about 50 m were considered a group (Frid 1997). In defining a group we took into account also the habitat composition where the animals were observed, but it should be considered that the Alpine environment over the tree line is very homogeneous (Grignolio et al. 2003) and, consequently, in most cases only the distance from conspecifics was used to determine a group. We included in the analyses also the singular ibex as a group in order to better show the key role of sociality in the antipredator strategies. For each sighting, we recorded the following data:

1. Group size and composition;
2. Location of the group, determined by subdividing the study area into a grid of 125 x 125 m squares and locating each group and each fix in the centre of the corresponding square (see Grignolio et al. 2007a for more details).

Five categories of ibex groups were distinguished following Grignolio et al. (2007a): adult males (at least 75% of the group members being males, at least 50% of the males being >5 years of age); subadult males (at least 75% of the group members being males, more than 50% of the males being <5 years of age); females with young (at least 75% of the group members being females, at least 1 kid or 1 yearling present); females without young (at least 75% of the group members being females, no young present); and mixed groups (when the ratio was lower than 75% for both sexes). Given that groups of ibex move slowly and infrequently, we avoided recounting the same animal or the same group by moving rapidly along the trails and using marked animals to identify the corresponding group. During the period of data collection 62 ± 4.4 % (mean ± SE) of the males and 17 ± 1.6 % of the females present in the study area were marked (tab. 2). They were randomly distributed in the area and, consequently, in the groups observed. We measured the distance of each group location from the nearest rocky slope, that was considered refuge area in which ibex can find escape from their predators, because they move more easily there than their predators. Distances were measured by means of a geographic information system software (ArcGis 9.3), with the methodology described by Grignolio et al. (2007a).

Data analyses

In order to evaluate the response of Alpine ibex to the return of the wolf, we analysed group size (GS) and distance from rocky slopes (DRS) by means of General Linear Models (GLMs) with Gaussian distribution of errors. GS was modelled using a GLM with Poisson distribution, typically used for count data, while for DRS we used a GLM with Gaussian distribution. We compared the effect of the presence of the wolf and the population size modification on ibex GS using the period of data collection (before and after wolf recolonization for simplicity) as a categorical fixed effect in the model. We considered also group type and month of data collection as fixed effects; distance from rocky slopes was included in the model as a covariate. We excluded from our analyses the effect of year of data collection because it was collinear with the presence of wolf. The DRS of each ibex group was modelled following the same assumptions (i.e., the same fixed effects) used for GS. In this case, we included group size as a covariate in the model. All main effects and all two-way interactions were initially included in both GLMs and, subsequently, dependent variables were modelled excluding the interaction terms that were not found to be significant. An estimate of marginal means was performed to show differences among values of significant variables.

Results

We recorded 3,267 ibex group locations, among which 1,639 were collected before, and 1,628 after, wolf recolonization. Group size ranged from 1 to 56 (4.59 ± 0.11) and mean distance from rocky slopes was 135.19 ± 1.94 meters (ranging from 0 to 557.38m). Table 3a shows the results of the final model, after model simplification, assessing the effects of the fixed factors (period of data collection, group type, distance from rocky slopes, month of data collection) and 2-way interactions (period x group type, group type x month) on GS. Group size significantly differed from the first to the second period of data collection. Specifically, it decreased in the period after wolf recolonization (GS = 5.74 ± 0.94) with respect to the period with no predators (GS = 3.95 ± 0.84). Moreover, group composition was found to significantly affect group size (fig. 2a). For instance, groups of females with young were, by far, the largest group type observed (GS = 7.97 ± 0.14), followed by mixed groups (GS=5.74 ± 0.29) and groups of males (prevalence of adult males: GS = 5.55 ± 0.73; prevalence of subadult males: GS = 4.02 ± 0.13). Female groups without young (GS = 2.39 ± 0.70) were the smallest ones. The two-way interaction term period x group type was significant (fig. 2a). More precisely, GS of female with young slightly decreased in the period after wolf recolonization (GS=7.27 ± 0.19), compared to the period with no predators (GS=8.75 ± 0.18), whereas the GS of females without young did not vary significantly. The size of both kinds of male groups was smaller when the predator was present (prevalence of adult males: GS =3.98 ± 0.07; prevalence of subadult males: GS=3.13 ± 0.10) than before wolf recolonization (prevalence of adult males: GS =7.75 ±

0.13; prevalence of subadult males: GS=5.17 ± 0.28). In May, June and July, ibex formed significantly larger groups than in the other months of data collection. Distance from rocky slopes did not affect ibex group size.

Table 3. General Linear Models of the effects of period of data collection (before and after wolf re-colonization), month of data collection (May, June, July, August, September, and October), group type (adult males, subadult males, females with young, females without young, mixed groups), distance from rocky slopes (integrated in the model A as covariate), and group size (integrated in the model B as covariate) on Alpine ibex group size (A) and on distance of groups from rocky slopes (B), in the Gran Paradiso National Park, Italy.

Dependent variable	Explicative variable	Wald-value	df	P-value
(A)	intercept	7947,028	1	<0.001
Group size (GS)	period	211,632	1	<0.001
	month	93,287	5	<0.001
	group type	1405,957	4	<0.001
	distance from rocky slopes	5,753	1	0.512
	period* group type	223,629	4	<0.001
	month * group type	149,584	20	<0.001
		F-value	**df**	**P-value**
(B)	intercept	1406.220		<0.001
Distance from rocky slopes	period	0.170		0.680
(DRS)	month	7.284		<0.001
	group type	145.586		<0.001
	group size	0.476		0.490
	period* group type	3.574		0.006
	month * group type	3.712		<0.001

Table 3b reports the effects of all fixed factors (period of data collection, group type, group size, month of data collection) and 2-way interactions (period x group type, month x group type) on DRS. Group type, month and their two-way interaction term significantly affected DRS. Groups of females were the closest to rocky slopes (females with young: DRS = 82.370 ± 4.581 m; females without young: DRS = 89.604 ± 4.418 m), whereas male groups were the farthest (prevalence of adult males: DRS = 186.847 ± 2.857 m; prevalence of subadult males: DRS = 155.947 ± 6.683 m; fig. 2b). Ibex were closer to rocky slopes in

May (DRS = 106.563 ± 6.055 m) and October (DRS = 102.889 ± 7.019 m), and farther in July (DRS = 142.959 ± 6.698 m) and August (DRS = 147.510 ± 7.684 m).

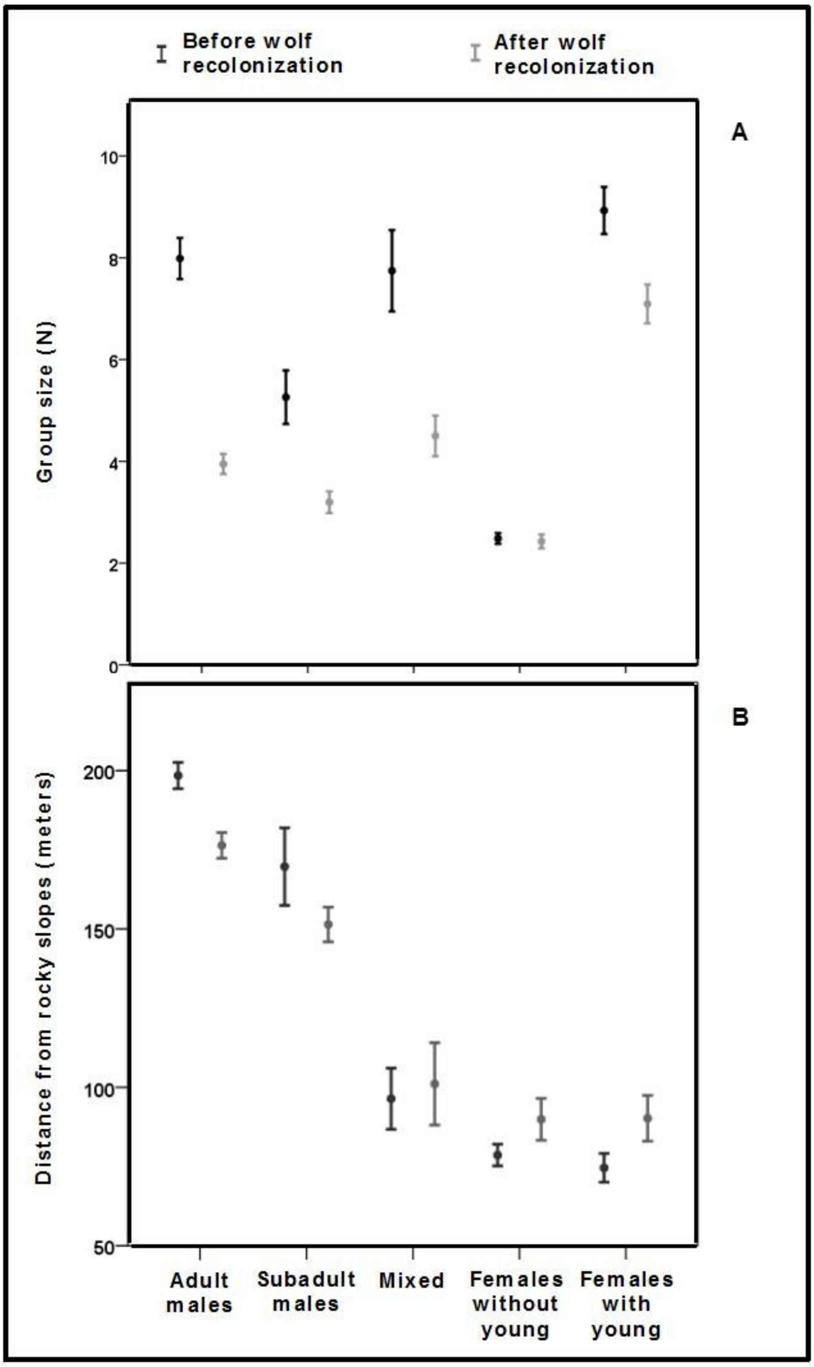

Figure 2. (A) Mean (±SE) group size and (B) mean (±SE) distance from rocky slopes (refuge areas) in relation to group composition of ibex (see the text for group classification) before and after the recolonization by wolf (Gran Paradiso National Park, Italy).

Moreover, different group types showed different behavioural patterns over the months. Adult male groups were always the farthest from rocky slopes, with scarce variation over the months. Groups of females with young reduced their DRS during the birth period and the first part of the weaning period, i.e. in June and July. DRS before and after predator recolonization did not significantly change, but the combined effect of presence of wolf and group type had a significant effect on DRS (fig. 2b). In particular, groups mainly composed of adult males were observed to be closer to rocky slopes after wolf recolonization, whereas the behaviour of other group types did not change significantly.

Discussion

Our analyses showed that within few years after a modification of the landscape of fear (i.e. the settlement of a breeding wolf pack and a reduction of ibex population size), male and female ibex modified differently their defensive strategies. Males formed smaller groups during the second period of our research. Females without young did not modify their average group size, whereas females with young slightly reduced their group size. Moreover, when wolves were present, male ibex groups only modified their habitat use in favour of sites closer to refuge areas. Generally we argue that a modification of the landscape of fear induce a greater unexpected change on ibex male' behaviour, rather than in the female' ones.

The modifications in male grouping behaviour recorded in the second period of our data collection would be partially unexpected if we took into account only the decrease in population density as a possible explanation. The size of adult male groups decreased more than expected (more than 50%) when wolves were present, whereas the overall number of males declined by about 30%. This mismatch leads to the assumption that the cause inducing male ibex to form smaller groups is not simply the decrease of population size. In the light of a recent research, we expected that males did not modify their social behaviour. Indeed, Brivio and colleagues (2014) showed that males foraging in groups with conspecifics did not incur in negative effects (i.e. no reduction of food intake or diet selection), instead they benefit of a reduction in the time spent in vigilance. In other words, they showed that forage in group is particularly rewarding for males. For this reason, we predicted that a change in population density did not affect male grouping behaviour. Instead, our results point out that when male live in groups they incur in negative factors, other than the competition and aggressive interactions, possibly related to the risk of predation.

Ibex males provided mixed responses to of the landscape of fear, modifying both distance from safe areas and group size. Before wolf recolonization, adult males lived mainly in flat areas, that were more dangerous, but also richer in forage (Grignolio et al. 2007a). After the settlement of a wolf pack, male groups reduced their size and distance from rocky slope; they nevertheless still used areas where wolves could move easily. We supposed that the choice of richer but riskier feeding sites was compensated by a reduction in the group size, in order to reduce predator encounter and/or attack, as found also in elk in North America (Hebblewhite and Pletscher 2002). Some authors have reported that large groups are more often detected and attacked and that the predators' success increases when they attack such groups (Hirsch and Morrell 2011). In particular, Creel and Winnie (2005) documented that elk herd size decreased when wolves were present, even when groups were far from timber (safe areas), thus suggesting that elk may disaggregate to reduce the likelihood of being detected by the predator. Hebblewhite and Pletscher (2002) also found that wolves encountered larger groups more often than expected by chance. On the basis of such findings, we concluded that in male ibex the trade-offs between encounters with predators, dilution effect and forage needs provide a coherent explanation for male mixed responses that clearly favoured food intake.

Females' propensity to live in group for social or antipredator needs was poorly affected by population density and, differently from our expectations, after wolf recolonization female ibex did not adapt their habitat selection, despite the presence of young. It is clear that for females it is convenient to live in groups and that their size is relatively stable. It remains to understand because it occurs. This behavioural pattern could be due to an antipredator strategy rather than it is forced by the distribution of resources or by other reasons. We state that females could not move nearer to rocky areas because they already used sites and meadows very close to such safe sites. The quality of forage in these meadows being lower compared to that of flat areas (Grignolio et al. 2007a), females were probably unable to increase the energetic costs of their antipredator behaviour. It is possible that females considered the areas used before wolf recolonization secure enough to avoid the risk of wolf predation; they had therefore no need to further decrease the distance from refuge areas.

Previous research documented behavioural modifications in ungulate populations after a modification of the landscape of fear due to the arrival of a predator (e.g. Hunter and Skinner 1998; Hebblewhite and Pletscher 2002), but in those cases other predator species were already present. The present case study, instead, provided new information about ungulate antipredator responses when a modification in the landscape of fear occurs in an area formerly free from predators. In other words, we provided evidence of the prey's ability to modify their behaviour in a limited amount of time (in 3-4 years) when confronted with the arrival of wolves after a century-long absence of predators. The phenomenon of "ghost of predator past" (Byers 1997) is an important key-factor in engendering quick prey responses. Toward the end of the 19th century, the GPNP was free of predators for adult ibex. Notwithstanding, ibex showed typical antipredator behaviour depending on sex, age, month and reproductive status (Grignolio et al. 2007a; Grignolio et al. 2007b). Behavioural patterns have coevolved in this prey species and its predators for thousands of years. The absence of predators for about one hundred years was not sufficient to remove antipredator behaviours in the GPNP population. This phenomenon seems more pronounced in females that were more cautious and have less need for modify their defensive strategies when occurs a perturbation in the landscape of fear.

We argue that the different behavioural patterns of females and males of this species are due to their high sexual dimorphism. Adult males weigh twice as much as females and their massive horns are more effective weapons (Bassano et al. 2003). These differences between the sexes may contribute to understand the different behavioural responses to a change in the landscape of fear. Males and females should be expected to seek a different trade-off between maximizing foraging benefits and minimizing predation risk: females preferred safer but poorer habitat and had a greater propensity to aggregate with conspecifics than males, as found in other sexual dimorphic ungulates (Corti and Shackleton 2002; Ciuti et al. 2004; Hay et al. 2008; Pipia et al. 2009). Therefore, our findings highlighted the role of perceived predation risk in the ecological sexual segregation of dimorphic mammals, in accordance with the postulates of the reproductive strategy-predation risk hypothesis (reviewed in Main et al. 1996; Ruckstuhl and Neuhaus 2000; Mooring et al. 2003).

It is worth noting that the GPNP ibex population is the only one that has not been reintroduced when the species was endangered and is therefore well adapted to its environmental conditions. So, when a modification occurs in a natural animal ecosystem, modifications in the behavioural patterns are often the first response. Consequently, individual behavioural characteristics are the first to affect the life history and population dynamics and to bear, in their turn, the effects of natural and/or artificial selections. These behavioural modifications entail costs (i.e. risk effects) on prey dynamics that can be as large as, or even larger than, direct effects (Schmitz et al. 1997; Nelson et al. 2004; Preisser et al. 2005; Pangle et al. 2007). Even though ibex were not so much affected by direct predation (i.e., lethal effects), being often less than 10% of the total biomass of ungulates eaten by wolves (Palmegiani et al. 2013), males suffered from important indirect effects. They were forced to forage in meadows which were safer (i.e., closer to rocks and slopes), but characterised by a worse forage quality and quantity. Such trade-offs likely caused a reduction of nutrients intake, thus compromising individual body conditions and growth. Moreover, we cannot exclude that other negative effects may be related to wolf recolonization. As shown in other ungulate species, modifications of the landscape of fear may affect movements (Fortin et al. 2005; Frair et al. 2005; Winnie et al. 2006), activity rhythms (Jeppesen 1987; Yarmoloy et al. 1988), and/or individual physiology (Bateson and Bradshaw 1997; Creel and Christianson 2008). Indeed, Creel et al. (2007) provided evidence that wolf predation risk alters reproductive physiology and demography of elk. The influences of risk effects on individual life history may play a relevant role in Alpine ibex, a species currently characterised by a negative trend in population dynamics (Jacobson et al. 2004).

Finally it may be interesting to assess whether the ibex behavioural modifications pointed out in this study could affect trophic cascades. Trophic cascades generally arise from direct population-level effects, when predators prey on herbivores, thereby decreasing the impact of the latter on plant trophic levels (Schmitz et al. 1997). But trophic cascades may also arise from prey behavioural shifts that can result in reduced feeding intake, thus lowering the impact of herbivores on plants. For instance, Ripple et al. (2001) showed that, in the Yellowstone ecosystem, aspen benefited from the reintroduction of wolves because elk were forced to modify their distribution and foraging strategy, thus reducing the browsing pressure on that tree species. Accordingly, it is possible that ibex responses to the predator's presence also modify their pressure on Alpine meadows. Future studies may be implemented to evaluate the influence of this ungulate on the Alpine ecosystem.

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Chapter 4

Effects of capture by chemical immobilization on Alpine ibex.

Francesca Brivio [a], Stefano Grignolio [a], Nicoletta Sica [a], Stefano Cerise [b],
Bruno Bassano [b]

[a] University of Sassari, Department of Science for Nature and Environmental Resources, via Muroni 25, I-07100, Sassari, Italy

[b] Alpine Wildlife Research Centre, Gran Paradiso National Park, Via della Rocca 47, I-10123 Torino, Italy

Abstract

The importance of capturing and marking wild animals for research or conservation projects is widely shared. As this activity continue to become more common, increases the need to assess its risks and negative effects in order to ensure ethical standards and the validity of research results. Increasing evidences have revealed that indirect (physiological and behavioural) effects of capture are as important as direct risks (death or injury). The various capture methodologies can induce heterogeneous effects on animal biology. We investigated the influence of chemical immobilization on Alpine ibex in the Gran Paradiso National Park, where is adopted one of the less invasive methodology used to capture ungulate species. For ten days after the capture we collected data on spatial behaviour, activity levels of 10 males and 9 females and male hormonal levels. Moreover, for each marked female captured since 2000 (61 individuals), during each breading season, we recorded its reproductive status. We constructed several a priori models to investigate the effects of the capture taking into account the changes in environmental conditions. Our results showed that this capture methodology did not affect spatial behaviour (both males and females), testosterone and cortisol levels of ibex males. Instead, up to two days after the capture, both sexes showed reduced activity levels. The capture did not significantly affect the probability for a female to give birth in the following summer. The other environmental variables analysed influenced behavioural patterns accordingly with previous knowledge. Our findings highlighted a high reduced impact of the chemical immobilization on ibex biology. Nonetheless, in areas characterised by high predator density, we suggest to carefully check freed animals for some hours after the capture. Researchers should avoid considering data of the first days after the manipulation in order to avoid biased information.

Key words: activity rhythms, *Capra ibex*, chemical immobilization, handling effects, spatial behaviour, hormone levels, reproductive success.

Introduction

A wide array of research, conservation and management programmes on wildlife require the capture and handling of animals. Certainly, the developments of no or- less invasive procedures (e.g. collecting faecal or hair samples for DNA analysis or hormonal levels) allow to obtain information without the need of handling animals. Nonetheless, some information can be collected only by capturing animals (e.g. morphometric measurements, age determination, or serum biochemistry for population sanitary controls; Powell and Proulx 2003; Garshelis 2006). Captures are also important for marking individuals: ecological and behavioural studies require data from individually recognizable animals. New technological advances such as global positioning system (GPS) collars (Cagnacci et al. 2010), heat sensitive vaginal implant transmitters indicating the birth of neonates (Bishop et al. 2007), and advanced physiological monitoring equipment (Laske et al. 2011) allow detailed and novel researches on wildlife, but necessitate the capture and handling of the animals. Live captures are also required in conservation biology for animal translocations (i.e. reintroductions or population restocking). Moreover, it may be an alternative to lethal control in management situations, where human safety or property may be at risk (Linnell et al. 1997).

While there are clear reasons to perform capture of wild animals, only recently researchers paid attention to assess the risks and negative effects of this activity, particularly about the delayed and latent effects. The captures of wild animals have potential to involve the risk of mortality and the reduction in survival probability (Jacques et al. 2009) or the injury of focal individuals (Cattet et al. 2008). Mortality rate is absolutely important and rather easy to measure. Anyway, it cannot stand-alone as the measure of capture success and immobilization safety. The full impact of capture and handling procedures cannot be determined without evaluating physical, physiological behavioural effects on animals at the time of capture and in the days that follow. Indeed, haematological and biochemical blood constituents show that capture and immobilisation of wild ungulates is likely to be one of the most stressful events in their lives (Wesson et al. 1979a,b; Spraker 1993). The duration and the magnitude of the stress affect significantly animal physiology and are reflected in an increase in glucocorticoid levels (Romero 2004). As glucocorticoids (cortisol in large mammals) regulated animals’ behavioural responses (DelGiudice et al. 1990; Diverio et al. 1996; Ingram et al. 1999; Montané et al. 2003), changes in behavioural pattern related to physiological alterations could also be expected after capture events. Several studies have assessed effects of captures on behavioural metrics in free ranging wildlife. The potential impacts include displacement from areas around capture sites (Chi et al. 1998; Moa et al. 2001), altered space and habitat use (Morellet et al. 2009), depressed movements (Cattet et al. 2008; Quinn et al. 2012; Nurthrup et al. 2014) and reduction in activity patterns (Morellet et al. 2009).

The effects of immobilization can differ considerably according to the method of capture used (e.g. DeNicola and Swihart 1997; Haulton et al. 2001; Langkilde and Shine 2006; Cattet et al. 2008). A method that is becoming prevalent in capturing large mammals is remote delivery of immobilizing agents via free-range darting (Ferris 1990; Casady and Allen 2013). Benefits of this approach are lower mortality and injury rate with respect to other methods (Haulton et al. 2001; Cattet et al. 2008), low capture stress as animals are manipulated when already sedated (Peinado et al 1993; DeNicola and Swihart 1997) and ability to select specific individuals. Conversely, drawbacks include drug-induced side effects depending on the specific drug used (e.g. Yadav et al. 2008; Ghurashi et al. 2009; Mohammed et al. 2011). By improving capture protocols and adopting improvements in drugs and doses, researchers were able to reduce the mortality rate of this methodology at minimum levels (e.g. Havernick et al. 1998; Kilpatrick and Spohr 1999; Bassano et al. 2004; Dematteis et al. 2009; Casady and Allen 2013). Anyway, anaesthetic drugs are never completely devoid of toxicity and induction of anaesthesia invariably carries risk of severe side effects, e.g. respiratory depression, shock, hyperthermia, asphyxia due to tympany or vomiting. Of special concern is the capture and immobilization of pregnant female, as drugs could reduce reproductive output and survival of offspring. Findings in this regards are not homogenous: chemical immobilization before the rut decreased female productivity the following year in mountain goats (*Oreamnos americanus*, Côte et al. 1998), and postnatal calf mortality rates increased after winter immobilization of pregnant moose cows (*Alces alces*) in Canada (Larsen and Gauthier 1989). Conversely, in other species no negative effect of chemical immobilization has been reported on either reproduction or infant survival (e.g., wild horse, *Equus caballus*, Berger et al. 1983; caribou, *Rangifer tarandus*, Valkenburg et al. 1983; white-tailed deer, *Odocoileus virginianus*, DelGiudice et al. 1986).

Chemical immobilisation can be obtained approaching animals and shooting a dart by helicopter, snowmobile, off-road vehicle or walking. The latter is likely the methodology more difficult to adopt because it requires high detectability and confidence of the animals to man. On the other hand, it reduces the approach stress and it is used in several study areas, particularly in protected areas. Nevertheless, to date no study has evaluated the effects of a capture by chemical immobilisation with walking approach, i.e. with the lowest stress conditions, on ungulate behaviour. In our study we investigated the influence of captures in the Alpine ibex (*Capra ibex*) population of the Gran Paradiso National Park (GPNP). The personnel of this protected area experimented the first capture of this species by chemical immobilisation more than 50 years ago. Since then, a hard work has been done in order to increase the success and to minimize the impact of the captures by improving drug composition and doses, by reducing handling time and by putting a lot of attention in causing the least stress possible to animals (e.g. approach only on foot, no manipulation till the sedation, no animal displacement). We expected reduced alteration on ibex physiology and behaviour; anyway, based on our literature search, we predict capture and handling to cause subtle or short-term changes in ibex movements and total activity and male hormone levels. Finally, in order to evaluate possible long-term effects of capture and handling procedures, we compared the female productivity of marked females in the years in which they have undergone the capture and in the subsequent years.

Materials and Methods

Study area

The study took place in the Valsavarenche Valley, within the Gran Paradiso National Park (GPNP; 45°35’N, 7°12’E; north-western Italian Alps). The study area is a mountainous region with steep glacial valleys ranging from 1500 to 3300 m a.s.l.. Rock cliffs, moraines and alpine meadows are the dominant habitats. The vegetation of this area includes conifer woods (*Picea abies*, *Larix decidua* and *Pinus cembra*), scrubs (*Rhododendron* and *Vaccinium* ssp.) and grassland, where the most common grass genera are *Festuca, Carex*, *Poa*, *Achillea*, and *Trifolium*. The local climate is temperate, with snowfall mostly occurring from November to April, the warmest period generally occurs from June to September. An automatic station recorded temperature, radiation, precipitation and wind speed data (24 records⁄d, Property of Meteorological Service of Aosta Valley Region).

Data collection

Adult ibex were captured between April 2000 and September 2014 as part of on going research on the ethology, ecology, and sanitary conditions of the only autochthonous population of this species. The captures were performed by a team with a long experience in the use of chemical immobilizations on mountain ungulates. The team is composed by the rangers (at least 3 people) and the veterinarian of the Park. To reduce considerable distress to the animals during the first part of capture only the shooter with a CO_2 injection rifle (Daninject) approached walking the ibex. The operator measured the distance from the animal by a laser rangefinder to properly regulate the pressure of the shot of the dart. Animals were darted at a distance of 26.6 ± 6.3 m (mean ± SD) far from cliffs to prevent potentially dangerous situations for the animals and operators. In order to immobilize the ibex we used a mixture of xylazine HCl (Rompun, 20-40 mg) and ketamine (Inoketam, 50-100 mg). Xylazine is an alpha-2-adrenergic agonist acting as a nonnarcotic sedative analgesic, and ketamine is a dissociative anesthetic. The combination of the two drugs enables their dosages to be reduced, enhances muscle relaxation and duration of effect, and has been associated with faster and smoother induction (Lin 1996).

The shot ibex was observed using binoculars and about 10 minutes after the injection the ibex lay down. After further 5 minutes three operators approached it and if the animal did not show any signs of alert, they simultaneously immobilised the ibex hobbling and blindfolding it. Upon the capture the animal was placed in right lateral recumbency and the tongue was adjusted to ensure open air-ways. We collected biometric data, took biological samples and weighted the animals with a digital scale. Ibex was aged by counting the clearly separated annuli on its horn (von Hardenberg et al. 2004) and at the end, marked. During the manipulation the ibex were constantly monitored to assess signs of stress related to capture. Heart rate, respiratory rate, and rectal temperature were recorded. Finally, in order to accelerate recovering of the animal and reduce the risks of hypothermia we injected a specific alpha-2-adrenergic antagonist, the atipamezole (1.5 cc), that has the property to reverse the effects of xylazine; currently there are no effective antagonists for ketamine (Kreeger et al. 2002). Ibex were marked with ear tags and with visual or VHF collars. We freed the ibex after about 45 min in the same place where it was captured. At least one operator checked the ibex for about half an hour or until the animal reached a safe location and did not showed sign of distress. The capture and handling protocol was approved by the Italian Ministry of Environment (protoc. n. 25114/04).

From May 2013 to September 2014 we fit 10 males and 9 female ibex with a GPS radio collar (GPS PRO Light collar, Vectronic Aerospace GmbH) set to attempt a relocation once every 2 hours. Moreover, these collars were equipped by an activity sensor that measure activity in two axes based on the true acceleration experienced by the collar.

Activity is measured four times per second simultaneously on each axis as the difference in acceleration between two consecutive measurements, and is given within a relative range between 0 and 255, characterizing the mean activity/acceleration (Krop-Benesch et al. 2011). Measurements are averaged over a sampling interval of 4 minutes and stored with the associated date and time. Localisation and activity data were downloaded by a handheld terminal (VHF connection).

During the capture section of the year 2013 we collected faecal samples from 9 marked male just before or during the capture. After the capture, we collected samples twice per days for five consecutive days. We recorded the date and time of collection and stored in plastic bag at -20°C until the immune assay analysis.

For each marked female captured since spring 2000 (N=61), each summer, we recorded its reproductive status. The reproductive status was scored with 1 if the female was followed by a kid during June - July and 0 if no kid was present near the female. Variations in female reproductive performances were monitored from the year of capture to the year of their last appearance in our population survey.

Data analysis

We performed statistical analyses under 3 broad themes: effect of capture on behavioural patterns (movement and activity), on male hormonal levels and on female productivity. To account for the nested nature of the data, we used hierarchical models (General additive mixed models – GAMMs) in all analyses performed, using ibex identity as a random factor in all models performed. The goodness of fit of each model (homoscedasticity, normality of errors and independence) was checked by visual inspection of residuals. When necessary, the dependent variables were transformed to improve normality of residuals and reduce skew. Statistical analyses were implemented using R 2.14.1 (R Core Team 2013).

Behavioural patterns

Analysis focused on data (localisations and activity values) collected by the GPS collars during the 10 days following the capture. We used localisation data to examine the effect of capture on male and female movement behaviour. We removed any localisation recorded by the collar with less than 4 satellites and with a dilution of precision (DOP) greater than 10. With the resulting data, we calculated movement rates (MR, m/h) for ibex as straight-line distance (m) between consecutive localisations divided by time interval (h). We transformed MR with a natural logarithm and we used it as depend variable in

GAMMs. With data of the activity sensor we calculated the mean activity values in the same time interval used for MR (2 hours). From these values we calculated activity rate (AR), as a value on a scale from 0 to 1, dividing it by the maximum value recorded by the collar sensor (255). AR was arcsin square root transformed and used as dependent variable in GAMMs.

To explore the potential for effects of capture and handling on ibex behaviour we constructed two separate sets of models using the function "dredge" (MuMIn package) in RStudios. We developed an *a priori* set of models predicting the variation of our two response variables (namely: MR and AR) using a set of factors (predictor variables) that previous researches found to be important to explain variation in ibex movement (Grignolio et al. 2004; Aublet et al. 2009) and activity patterns (Signer et al. 2011). The effect of the capture event was evaluated by including in the models the number of hours post captures as a continuous variable. To account for the daily and seasonal changes of the behavioural patterns we included the sampling time (hour) and day (Julian day). As behaviour is proven to be influenced by individual and environmental factors, we also used as predictor variables: ibex age and sex, temperature (C°), radiation (W/m^2), precipitation (mm), wind speed (m/sec) and, only for MR, altitudinal differences between the arrival and the starting location divided by time interval (m/h). Precipitations were calculated as the sum in each time interval. All other meteorological data were calculated as mean, minimum and maximum values during the respective time interval of each displacement and activity value. We built a correlation matrix (Pearson correlation coefficient) within each variable to avoid collinearity (Sokal and Rohlf 1995) and in order to select the variables that were better correlated with the response variables (i.e., MR and AR). For each response variable, we ranked and weighed the alternative models using the minimum AIC criterion (Symonds and Moussalli 2011): models with $\Delta AIC < 2$ were considered to be essentially as good as the best model (Symonds and Moussalli 2011). To avoid retention of overly complex models (i.e., models having additional parameters that result in a minimal increase of fit), we excluded models that were only more complex versions of those with a lower AIC value (Richards et al. 2011).

Hormonal levels

We extract steroid metabolites from 0.5g of wet faeces samples well-homogenized suspended in 5 ml of 80% methanol (Palme and Möstl 1997). Fecal metabolites were measured with validated EIAs, specifically: Epiandrosterone EIA measuring androgens metabolites 17-oxo group for fecal testosterone metabolites (Palme and Möstl 1994; *chapter 1*) and 11-oxoetiocholanolone EIA measuring 5 β–androstano-11,17-dione structure for fecal cortisol metabolites (Posautz 2010; Möstl et al. 2002).

We performed statistical analyses on the level of both faecal hormone metabolites (cortisol and testosterone) in two different sets of GAMMs. The number of hours after capture was included in the model to account for the effect of capture. Moreover we included the Julian date and male age as a previous study has shown that these variables could affect the level of hormone metabolites (*chapter 2*) to account respectively for the effect of capture and seasonal changes on ibex hormonal levels.

Female productivity

Variations in female annual kidding success were analysed using GAMMs with Binomial distribution. The age of the female and the year of data collection were inserted as continuous variables in the model. To account for the effect of the capture event we also inserted in the model a dummy variable scored 1 if the female was captured in the current year, 0 if the female was captured in the previous years.

Results

We captured 10 male ibex, aging from 8 to 13 years, between the 7th of May and the 28th of June 2013. 9 females (4-15 years old) were captured from the 22nd of April and the 16th September 2014. For each marked ibex, during the 10 days following their capture we obtained 90.58 ± 8.60 valid relocations, from which we calculated the MR. The total values of AR of ibex obtained were 112.53 ± 1.47. We collected a total of 83 faecal samples from 9 males (9.11 ± 3.14 for each male), from which we measured both testosterone and cortisol faecal metabolites levels. From 2000 to 2014 in the study area were captured 61 females, aging from 2 to 15 years. During the period of data collection we recorded a total of 229 observation/year/female in order to analyse the annual kidding success.

Movement behaviour

The best model (tab. 1) explaining ibex MR included five predictor variables: sex, hours post capture, interaction between hour of the day and sex, Julian day and altitudinal difference rate. According to the prediction of the GAMM, ibex MR did not significantly differ between sexes. Moreover, no influence of hours post capture was detected for both sexes. During the period of data collection MR increased from the 22nd of April up to the end of June and then decrease till September (fig. 1a). The pattern of variation predicted by the model for MR as a function of the hour of the day was different for the two sexes. For males the pattern was described by a sinusoidal curve with two positive picks (about at 9:30 and at 20:00) and a negative pick (about at 4:00). For females the model identified two picks, a positive one at about 9:30 and a negative one at about 4:00 (fig. 1b). Finally, results of the model showed that MR were minimum when ibex reached areas with low differences in altitudes and increased with increasing altitudinal differences till ±50 m/h, beyond which was found a different pattern for positive and negative values (fig. 1c).

Activity patterns

The best model explaining activity patterns (tab. 1) included six predictor variables: sex, hours post capture, interaction term between sex and hour of the day, Julian day, interaction between maximum temperature and sex, and the precipitations. Female AR was higher than that of males ($\beta= 0.08 \pm 0.02$). According to the prediction of the model, AR was lower immediately after the capture and showed an increase over time up to about 48 hours, after which the activity appears to stabilize showing little variations (fig. 2c). Ibex AR fluctuated during the days of data collection, with a general increase of AR up to the end of June and a subsequent decrease till September (fig. 2a). Daily pattern of activity showed by the model's predictions was slightly different for the two sexes. Male AR was described by a sinusoidal curve with two positive picks at about 9:30 and 20:00 and a negative pick at about 4:00. Females showed lower levels of AR during the night (21:00-5:00) and three positive picks during the day: at about 9:30, 15:00 and 20:00. Both sexes had higher activity levels in the evening (fig. 2b). AR linearly decreased with increasing precipitation levels ($\beta= -0.012 \pm 0.003$, $P<0.001$). Both the interactions between age and sex and between temperatures and sex were included in the best models, but without a significant influence.

Hormonal levels

Results on hormone levels showed that in ibex males both cortisol and testosterone faecal metabolites did not significantly change with increasing the number of hours after the capture event. Moreover, no pattern was found investigating the influence of the Julian day

Table 1. Top 10 Generalised Additive Mixed Models predicting movement rate and activity rate in Alpine ibex during the 10 days following their capture, in the Gran Paradiso National Park, Italy.
x Terms included in each model, logLik logliklyhood of each AIC akaike information criterion; DAIC difference in the AIC value between a given model and the most parsimonious one. See the text for description of predictor variables.

!!	model #	sex	age	age * sex	hour after capture	hour after capture * sex	hour of the day	hour of the day * sex	Julian day	temperature	temperature * sex	radiation	radiation * sex	precipitations	precipitations * sex	altitudinal difference rate	altitudinal difference rate * sex	**logLik**	**AIC**	**DAIC**
MOVEMENT!RATE!	1	x			x			x	x					x		x		-2343.7	4715.4	0
	2	**x**			**x**			**x**	**x**							**x**		**-2345.8**	**4715.7**	**0.24**
	3	x			x			x		x						x		-2346.8	4717.6	2.19
	4	x			x			x		x				x		x		-2344.9	4717.7	2.31
	5	x			x			x				x				x		-2347.0	4718.0	2.59
	6	x			x			x	x		x					x		-2343.3	4718.7	3.24
	7	x			x			x	x		x			x		x		-2341.5	4719.1	3.63
	8	x			x			x	x			x				x		-2345.7	4719.4	3.94
	9	x			x			x	x						x	x		-2343.8	4719.6	4.15
	10	x			x			x								x		-2349.9	4719.8	4.35
ACTIVITY!RATE!	**1**	**x**			**x**		**x**		**x**		**x**			**x**				**1482.7**	**-2933.4**	**0.00**
	2	x		x	x		x		x		x			x				1486.6	-2933.2	0.24
	3	x		x	x		x		x	x				x				1484.2	-2932.5	0.95
	4	x		x	x		x		x		x	x		x				1487.7	-2931.4	2.05
	5	x			x		x		x		x	x		x				1483.6	-2931.2	2.18
	6	x			x		x		x	x				x				1479.3	-2930.7	2.75
	7	x			x		x		x		x		x	x				1485.3	-2930.6	2.82
	8	x		x	x		x		x		x		x	x				1489.0	-2930.1	3.35
	9	x			x		x		x	x		x		x				1480.8	-2929.7	3.73
	10	x			x		x		x		x				x			1482.7	-2929.4	3.99

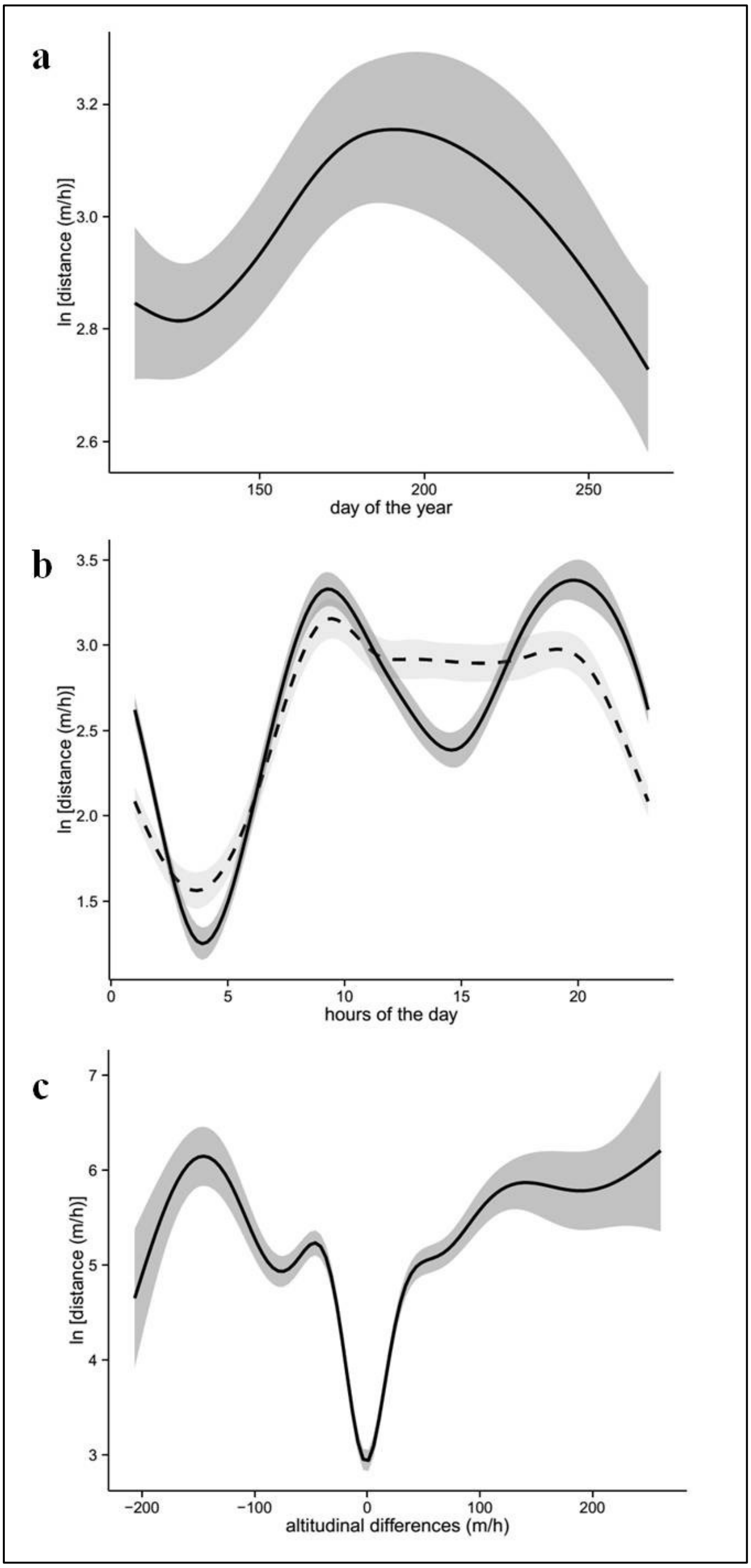

Figure 1. Predicted value by the best General Additive Mixed Model (see the text for more details) of movement rate of Alpine ibex in the Gran Paradiso National Park (Italy) during the ten days after the capture. The figure showed the effect of the day of the year (a), the time of day (b) and the altitude difference rate (c). In the graph (b), males are represented by continuous line, female by broken line.

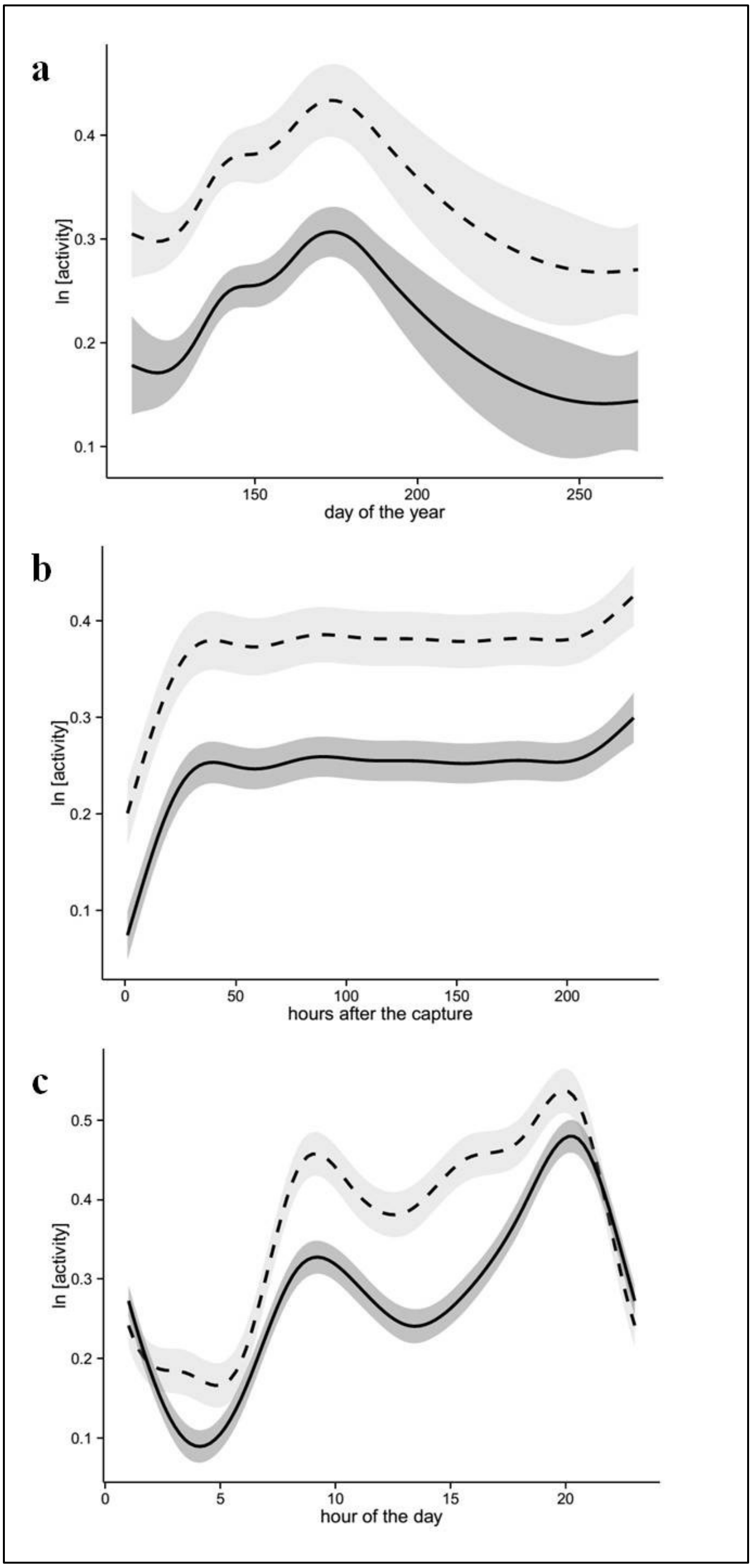

Figure 2. Predicted value by the best General Additive Mixed Model (see the text for more details) of activity rate of Alpine ibex in the Gran Paradiso National Park (Italy) during the ten days after the capture. The figure showed the effect of the day of the year (a), the time of day (b) and the hours after capture (c): males are represented by continuous line, female by broken line.

and of male age on both metabolites. Also looking at individual trends did not emerge a significant influence of the capture on male hormonal excretion (fig. 3).

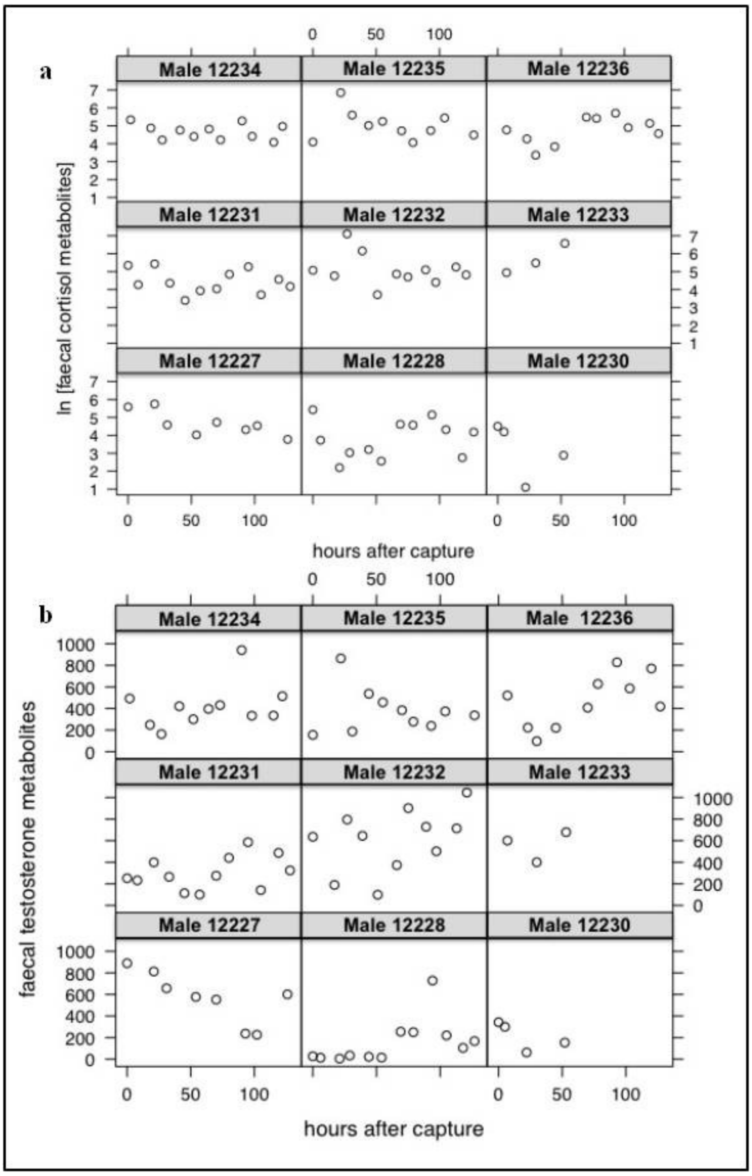

Figure 3. Variation of values of faecal cortisol metabolites (a) and faecal testosterone metabolites (b) for each sampled male Alpine ibex in the Gran Paradiso National Park (Italy) as a function of number of hours after the capture event.

Female productivity

Results of the GAMM performed to explain female productivity showed that capture events did not significantly influence the annual kidding success (fig. 4). The model detected a linear and significant increased of female kidding success from 2000 to 2014 (β= 0.21 ± 0.04). Moreover the female productivity was significantly affected by female age, with a pattern that increase up to 6-8 years of age and a subsequent decline in breeding success for older females (fig. 4).

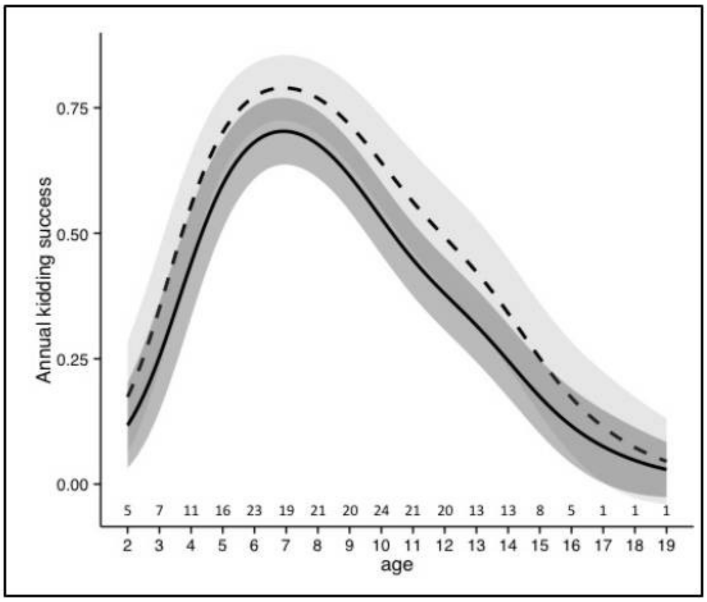

Figure 4. Predicted value by the best General Additive Mixed Model (see the text for more details) of female productivity rate of Alpine ibex in the Gran Paradiso National Park (Italy). The figure showed the effect of age on female productivity in the capture year (broken line) and in the years following capture event (continuous line).

Discussion

We showed that our capture methodology (approach by walking, reduce drug doses and no displacement of the animals) had slight and short-term impacts on Alpine ibex, with a similar reaction by the two sexes. Indeed, we found alterations only in short-term analysis of overall activity levels during the first two days after capture. We did not find differences in the other short-term effects (spatial behaviour, male hormonal levels) or in long-term effects (female productivity). The latter one can directly affect life history, animal fitness, and, consequently, population dynamics. Our analyses on 14 years of female capture activities revealed that in our studied population the probability for a female to give birth, taking into account age effect, was not affected by the capture event. Also Valkenburg et al. (1983) and DelGiudice et al. (1986), on caribou (*Rangifer tarandus*) and on white-tailed deer (*Odocoileus virginianus*) respectively, did not found any negative effects of immobilization by xylazine on female reproductive output and offspring survival.

Conversely, several studies reported that immobilization and handling could increase fetal loss or early offspring mortality in different large herbivores (e.g., mountain goats, Côté et al. 1998; black rhino, *Diceros bicornis*, Alibhai et al. 2001; moose, Solberg et al. 2003). Such effects could clearly have important consequences on population dynamics. Sakamoto et al. 1996 found that administration of xylazine to pregnant goats results in an alteration of the mother and fetus physiological parameters (decrease in uterine blood flow arising from the induction of uterine contractions, decrease in circulating blood volume, hypoxemia and acidosis in the mother) that could lead to abortion.

In our models we took into account some variables that are know to affect behaviour of ibex, this allowed us to tease apart the effects of capture on ibex behaviour from natural seasonal patterns. We found that movements and activity were highest at the begging of summer, as could be expected for herbivores living in alpine environment where quality of forage picks just in this period (Pettorelli et al. 2007). Results highlighted picks of movements and activity during morning and evening hours, as typically found in ungulate species living in temperate regions (e.g., Georgii 1981; Singer et al. 2009; Pagon et al. 2013). Our analyses did not bring out any influence of meteorological data, probably due to the brief period of data collection.

Analyses on movements showed that immediately after the capture both sexes had MR comparable to the following days. This finding is partially surprising because we could supposed that the stress of the capture induced ibex to adopt a flight behaviour, increasing movements in order to move away from the capture area. This kind of behavioural pattern was found in other ungulates species, like mule deer (*Odocoileus hemionus*, Nurthrup et al. 2014) or moose (Neumann et al. 2011), probably because in these cases animals were displaced far from the capture site. Nurthrup et al. (2014) suggested that after capture animals typically made long movements to return to their home range, causing elevated movement rates and daily displacements in the first days after capture. Conversely, we could also expect a reduction of MR, as found after capture methodologies where immobilised individuals were not traslocated. Morellet et al. (2009), working with roe deer (*Capreolus capreolus*), and Quinn et al. (2012) on white-tailed deer, found decreased movements following capture, which they interpreted as recovery from immobilization or drugs effects. In this respect, capture and handling appeared to have the least impact on ibex accordingly with our prediction of low stress levels of our capture methodology.

The model on activity patterns provides results according to our expectations: the capture affected ibex activity levels. The best model assessing the AR showed that both sexes were significantly less active after capture. This type of effect has also been observed on roe deer (Morellet et al. 2009), in which the stress of the capture induced a reduction in the overall level of activity in the following 10 days. Ibex needed two days to regain normal activity rhythms after capture. This result is not in contrast with our findings on spatial behaviour. The new marked ibex moved together with the groups of conspecifics. But, whereas other ibex fed, the just captured individuals lay down and slept, in spite of the administration of an antagonist of xylazine. Marked animals preferred make an effort to move with other ibex, rather than look for a refuge area and wait to feel better as observed in roe deer (Morellet et al 2009). Our conjecture is supported also by several occasional observations made by rangers and people of capture team. In the hours after the capture, the personnel of the Gran Paradiso National Park is used to carry out short and repeated sessions of control by binoculars on captured animals. During these observations, it is easy to identify the marked ibex inside a foraging group, because often it is lying on the ground. Our findings highlight the key role of sociality as antipredator behavioural strategy in response to a stress event such as the captures, handlings and fitting of a collar. This strategy to move in-group with other conspecifics, but reducing total activity, could be dangerous in area with high-density predator population. A lying ibex inside a foraging group undergoes higher predation risk in case of an attack, for example from a wolf pack. Consequently, the managers should take into account this potential behavioural effect of the chemical immobilization. In area characterised by high density of predator could be dangerous to free an animal without providing adequate precautions. For example, could be necessary to foresee a continuous direct control of the released individuals during the first hours after the capture in order to deter a potential attack by a predator.

The behavioural changes induced by the capture could affect animals’ energy balance. As matter of fact, the reduction of total activity after captures likely results in a reduction in food intake. Blanc and Brelurut (1997) found a decrease of 40% in grazing activity of red deer hinds over a period of eight days after fitting of a GPS-collar. A similar result was observed directly on red grouse (*Lagopus l. scoticus*, Boag 1972) and indirectly on bears (*Ursus arctos*, *U. americanus*), in which a decrease in body conditions was detected (Cattet et al. 2008). Our analyses reported a reduction of total activity, but it is important to consider that ibex recover gradually a normal activity up to about two days (fig 2). Thereby, it is clear that even if ibex reduced the foraging time for 48 hours, this cost rarely may be risky for the life history or the survival of the individual. Moreover, it is useful to note that only in the first hours post-capture the overall activity is very low and after tends to increase quickly. Hence, the period with a significant reduction of feed intake is short and with scarce effect on animal fitness.

The analysis about males' hormone levels did not show significant systemic modifications. Certainly the capture and the manipulation induced an acute stress pointed out by the increase in faecal cortisol levels (detected in two marked males, fig. 3), but this alteration had a short duration. In fact, we are not able to measure a significant increase or decrease in males' hormonal levels using faecal metabolites (cortisol and testosterone). Consequently, we can affirm that chemical immobilization did not cause serious alteration of hormonal levels that could potentially be dangerous for individual wellness. These findings are in accordance with results found for African wild dogs (*Lycaon pictus*, Creel et al. 1997) and for red colobus monkey (*Procolobus rufomitratus*, Wasserman et al. 2013) in which measures of faecal cortisol levels indicated no chronic stress in captured and collared individuals.

Our results point out that our method of chemical immobilization generated short-term and slightly hazardous behavioural modifications. The impact of any manipulation in a wild species is mediated by the way it is performed (Arzamendia and Vila 2012). We demonstrated minor impacts of captures on ibex, above all because we worked under a strict animal welfare framework, where several adjustments to reduce stress are implemented: we approach animals by a walking shooter, we use reduced doses of drugs and we don't move the animals away from the capture site. Finally, we recommend researchers to exclude from the their analyses data collected in the first days following capture (2-3 days in our case) to avoid biased results.

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Conclusion

The two main components of male ungulates life cycle are the pre-reproductive season and the rut. In Alpine ibex the first one (from late spring to late autumn) matches the green up of the vegetation, it is characterized by high degree of sociality (males' interactions) and mild climate condition. Ungulates living in Alpine habitat are well adapted to cold climate (Corlatti et al. 2014), but can experienced heat stress at lower temperatures than species adapted to warmer environments (Schab and Pitt 1991; Owen-Smith 1998;). It has already been shown that Alpine ibex has to handle heat stress in summer (Neuhaus and Ruckstuhl 2002; Grignolio et al. 2004; Aublet et al. 2009; Signer et al. 2011): my results highlighted an additional response of ibex to thermal stress, finding a direct correlation between fecal cortisol metabolites and maximum temperature during summer (**chapter 2**). The rut period (from December to half January) is characterized by poor food resources, sever climatic conditions and high energy expenditures to support the courting patterns. I showed that these selective pressures have favoured the evolution of an earlier definition of hierarchical order, that remains stable till the rut and affects male reproductive success (**chapter 1**). This strategy is not common among male polygynous ungulates, in which the social status is usually defined during the reproductive season with an almost full overlap between the interaction and courtship periods (Clutton-Brock et al. 1979; Apollonio et al. 1990; McElligott et al. 1998). It is clearly advantageous because allows ibex to decrease time and energy spend in male-interaction to regulate the access to oestrus females and reallocated it in display directly connected with fitness (reproduce and survive).

Dominance is usually correlated with phenotypic quality that enhances fighting ability in polygynous ungulates (McElligott et al. 2001; Pelletier et al. 2003). My findings confirmed the results of Bergeron et al. (2010): the major determinant of social status is the combined effect of individual size and age. On the other hand, differences in fecal hormones metabolites did not affect dominance (**chapter 1**). Direct associations between testosterone, rates of aggression, and dominance rank are often been related to situation of social instability or to the establishment of territorial boundaries (Wingfield et al. 1990; Corlatti et al. 2014). The absence of a relationship between testosterone and hierarchy in ibex supports the prediction of the presence of a stable social dominance among males. The level of testosterone is influenced by males size and by cortisol level (**chapter 1**); its basal level has a peak in correspondence of the per-reproductive season (**chapter 2**). My results are then consistent with a permissive role of testosterone: it strengthens pre-existing pattern of aggression and provided an impulse to compete for a high ranking position.

The positive correlation between cortisol and testosterone supports an absence of chronic stress in the population (**chapter 2**). In absence of chronic stressor (e.g. dominant animal in a stable hierarchy population) an acute stress is expected to increase the concentration of testosterone in the initial stage of stress reaction, contributing to reinforce the initial impulse to compete, as explained by Chichinadze and Chichinadze (2008).

The specie showed quickly responds to environmental changes, even in response to an increase in predation risk triggered by the return of the main predator. My results shown that a natural modification of the landscape of fear induces responses in group behaviour. Adult males changed their behavioural patterns moving closer to safe areas (i.e. rock and steep slopes) at the expenses of a suboptimal food intake (**chapter 3**). Instead, the impact of a most punctual disturbance (capture activities) caused short time changes which allow a quick recovery to daily routine. I showed that the capture could provoke an acute stress in the individual (pointed out in the increase in fecal cortisol metabolites detected in two males), but magnitude and duration of the disturbance are not sufficient to generate a significant modification of the hormonal levels detectable in faecal hormones metabolites. The consequently behavioural changes are short-lasting. Indeed, I detected only a decrease in the level of activity that disappeared in about two days (**chapter 4**). The integration of behavioural and physiological data gave the possibility to demonstrate that the capture methodology employed (the chemical immobilization) had a reduce impact on ibex biology and for this reason it is suitable for conservation projects.

This results suggest that Alpine ibex is able to respond efficiently as to predictable as to unpredictable disturbance, but stress the importance of the pre-reproductive season to allow ibex to cope with the seasonal shortage of winter. The findings of this thesis highlights the preeminent role of the environment in shaping Alpine ibex physiology and behaviour in order to increase fitness and consequently its chance of survive and reproduce.

My results emphasize the importance and the need for further investigations into the effects of the social environment on individual stress and energy budgets in mountain-living animals.

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Annex

Do relatives like to stay closer? Spatial organization and genetic relatedness in a mountain roe deer population

Daniela Biosa[a], Stefano Grignolio[a], Nicoletta Sica[a], Nives Pagon[a], Massimo Scandura[a], Marco Apollonio[a]

[a] University of Sassari, Department of Science for Nature and Environmental Resources, via Muroni 25, I-07100, Sassari, Italy

Abstract

The fine-scale genetic structure of mammal populations arises from the social and spatial behaviour of individuals. In wild ungulates gene flow is usually mediated by males, being the dispersing sex. The roe deer (*Capreolus capreolus*) represents an exception: males and females are mostly sedentary and disperse in similar proportions, while mechanisms for inbreeding avoidance are more complex and not fully known. We investigated the seasonal variation in the relationship between genetic relatedness and spatial behaviour in a sample of 69 roe deer, monitored from 2002 to 2010 in a high-density population in Italy. Genetic and spatial analyses, based on individual positions, did not reveal any population structure, neither in the whole sample, nor in the two sexes separately. Our results confirm the absence of a sex bias in roe deer dispersal and suggest the existence of a high gene flow across a continuous forest habitat, thus preventing the onset of population structuring at a local scale. We found that genetic relatedness tended to increase with home range proximity, but mostly in winter. Nevertheless, when the extent of overlap between seasonal home ranges with respect to genetic relatedness was considered, males and females seemed to preferably share their home range with relatives of the same sex, reducing the overlap with relatives of the opposite sex during the rutting period. We conclude that home range rearrangements during the breeding season may afford a certain level of spatial segregation between closely related potential mates, thus reducing inbreeding risk.

Key words: *Capreolus capreolus*, social organisation, microsatellites, spatial behaviour, genetic structure.

Introduction

Reproduction represents a crucial step in animal life. Approaching the mating season many species modify their spatial and social habits in order to maximize their fitness, by increasing mating opportunities or exerting their mate choice. In some cases sexually active individuals increase their overall mobility (Labisky and Fritzen 1998) or enlarge their home range, by roaming over a wide area (Fisher and Lara 1999; Dahle and Swenson 2003) or by undertaking temporally and spatially limited excursions outside the usual home range (Liberg et al. 1998; Kolodzinski *et al.* 2010; Stopher et al. 2011). So doing, they increase the chance of finding a mate (e.g. in low density populations) or of breeding with an unrelated or high quality mate. As a consequence of these behaviours, socio-spatial organization during the mating season may deviate from that observed during the rest of the year. Inbreeding avoidance is one of the main selective forces which promotes these behaviours (Blouin and Blouin 1988; Pusey and Wolf 1996). Philopatry and social viscosity (delayed dispersal) indeed may induce individuals to live in kin clusters (Fowler 2005), where social bonds are stronger than in groups of unrelated individuals (Wolf and Trillmich 2008). Under these conditions, in most cases initiatives are taken to reduce the risk of mating with close relatives, though some species show tolerance or even preference for inbreeding (see Szulkin et al. 2013)

Several authors tried to disentangle the relationships between social and mating system, dispersal patterns, and gene flow in mammals (Greenwood 1980; Clutton-Brock and Lukas 2012), showing that having a sex-biased dispersal is the usual way to cope with inbreeding, where males are the dispersing sex while females are philopatric (Dobson 2012). Under this perspective, among wild ungulates, the roe deer (*Capreolus capreolus*) represents an exception. Unlike the majority of cervids, roe deer shows a very slight sexual dimorphism and very limited differences in spatial behaviour between sexes (Gaillard et al. 2008, Cagnacci et al. 2011). Its mating system (Liberg *et al.* 1998; Vanpé et al. 2009) does not conform to the expected pattern of a conventional resource-defence polygyny described by Greenwood (1980) and is characterized by low levels of polygyny (Vanpé et al. 2008). This suggests that maximizing mating opportunities is not the primary selective force driving the evolution of dispersal patterns in this species (Coulon et al. 2006). Rate and extent of dispersal are region-dependent (Coulon et al. 2004), but common patterns are observed. Natal dispersal (sensu Howard 1960) occurs in individuals between 10 and 36 months (mostly around 12 months, Debeffe et al. 2012), often covers a few kilometres only (Linnel et al. 1998) but can exceptionally cover several tens of kilometres (Wahlström and Liberg 1995). No difference in dispersal propensity and covered distances is observed between sexes (Gaillard et al. 2008; Bonnot et al. 2010; Debeffe et al. 2012). A high but variable percentage of individuals is philopatric, showing a high annual and lifetime fidelity to the natal range, whilst the rest of the population either disperse early in their life or undergoes seasonal migrations (Cagnacci et al. 2011; Wahlström and Liberg 1995). Dispersal is condition-dependent, being more frequent in heavier animals of both sexes (Wahlström and Liberg 1995; Debeffe et al. 2012). In addition, some females undertake short-range movements, classified as ‘breeding excursions’, during the mating season (Liberg et al. 1998; Lovari et al. 2008; Richard et al. 2008).

Finally, roe deer have adapted to a variety of habitats, developing a high variability in social structure and spatial behaviour among populations (Hewison et al. 2001; Hewison, Vincent and Reby 1998; Cargnelutti et al. 2002; Lamberti et al. 2006). Accordingly, the fine-scale genetic structure is expected to vary in relation to landscape structure (Coulon et al. 2004), climatic conditions (Cagnacci et al. 2011), habitat (Lamberti et al. 2006), population structure, and density (Wahlström and Liberg 1995). In this broad frame, given the quite different levels of sociality and space occupancy shown by the roe deer during the territorial and the non-territorial period (Danilkin and Hewison 1996), we specifically examined the socio-spatial structure of a population in summer and in winter. Spatial behaviour during summer is indeed relevant to the social system of roe deer (Kjellander et al. 2004), as males are solitary and territorial on account of the approaching rutting season (i.e., July-August), while females’ spatial behaviour is influenced by their social bond with fawns, as well as by the active search for a partner during the oestrus (Lovari et al. 2008). Given the philopatric nature of the majority of individuals in a roe deer population, we would expect a general correlation between spatial proximity and genetic relatedness. However, considering the risk of inbreeding that would arise from mating with neighbours, short-range movements (including seasonal migration) could be aimed at reducing the average relatedness with potential mates during the breeding season. In this case, we would predict that roe deer show a lower relatedness to surrounding individuals of the opposite sex in summer than in winter.

Here we present data on the fine scale spatial and genetic structure in a high-density, forest-dwelling roe deer population by comparing pairwise genetic relatedness between individuals with two spatial variables, i.e., the distance between individual home range centroids and the overlap between individual home ranges.

Materials and Methods

Study area

The study was carried out between 2002 and 2010 in a mountainous area of ca. 120 km^2 in Central Italy. One fourth of it is inside of a protected area, the Oasi Alpe di Catenaia (OAC). Elevation ranges from 330 to 1,514 m a.s.l.. The study area is covered mainly by mixed forests (83%), consisting of beech *Fagus sylvatica* and silver fir *Abies alba* at > 900 m, and of Turkey oak *Quercus cerris*, chestnut *Castanea sativa*, black pine *Pinus nigra*, and Douglas-fir *Pseudotsuga menziesii* at lower altitudes. Shrubs and pastures cover around 16% of the area. Roe deer and wild boar *Sus scrofa* are the only ubiquitous ungulates in the study area. Natural predators of roe deer are grey wolf *Canis lupus* and red fox *Vulpes vulpes* (Bassi et al. 2012). In the OAC, hunting activities are permanently banned, whereas in the rest of the study area wild boar hunting with hounds and roe deer stalking from fixed high seats are permitted (Grignolio et al. 2011). Roe deer density is routinely assessed in spring by drive census (Davis et al. 2012). During the study, post-birth roe deer density in the study area averaged 39.1 ± 5.0 heads/km^2 (mean ± SD), ranging annually between 33 and 48 heads/km^2.

Spatial behaviour data collection

Between March 2002 and March 2010, 69 roe deer (38 females and 31 males) were captured by means of vertical drop nets, sampled for genetic analysis and fitted with VHF radio-collars (Televilt, Sweden). All animals but one were captured as yearlings (18) or adults (50) in late winter/early spring (February – March; only five of them were captured out of this period). Radio-collared deer were monitored by discontinuous radio-tracking obtaining individual locations by the triangulation method, on the basis of three bearings. We uniformly distributed the locations over the 24 hours on a monthly basis and separated consecutive locations by at least 12 hours, in order to minimise temporal and spatial autocorrelation. Further details on field procedures are described in Grignolio et al. (2011).

Genetic analysis

DNA was extracted from plucked hairs (≥ 10 bulbs) using Instagene Matrix (Bio-Rad, Hercules, California) and then stored at -20°C. DNA samples were genotyped with a panel of eleven polymorphic microsatellites: Roe1; Roe6; Roe8; Roe9 (Fickel and Reinsch 2000); NVRTH16; NVRTH21; NVRTH24 (Røed and Midthjell 1998); ILSTS011 (Kemp et al. 1995); OarFCB304 (Talbot, Haigh and Plante 1996); BMC1009 (Kappes et al. 1997) and RT1 (Wilson et al. 1997). They were amplified by three multiplexed (multiplex A: Roe1, Roe8, Roe9; multiplex B: RT1, NVRTH21, BMC1009; multiplex C: Roe6, NVRTH16, ILSTS011) and two single PCRs (NVRTH24 and OarFCB304). PCR conditions are available upon request. Fluorescently-labelled PCR products were sized by capillary electrophoresis on an ABI PRISM 3730 automatic sequencer (Applied Biosystems).

MicroChecker 2.2.3 (Van Oosterhout et al. 2004) was used to check for possible genotyping errors, allelic dropout, or null alleles at the 11 loci, while deviations from Hardy-Weinberg (HWE) and linkage equilibria were assessed by Genepop 4 (Raymond and Rousset 1995). The significance of tests was corrected for multiple comparisons with the Bonferroni sequential procedure (Rice 1989). Genetix 4.05 (Belkhir et al. 2001) was used to assess the level of genetic variability in the sampled population, through the estimation of observed (H_O) and expected heterozygosity (H_E), the mean number of alleles per locus (k) and the allele frequencies in the sample. F_{IS} was calculated for the overall population and its significance was tested by permutations.

The matrix of pairwise relatedness among the sampled individuals was calculated with GenAlEx 6.4 software (Peakall and Smouse 2006). In order to improve the calculation of relatedness coefficients, we enlarged the dataset by adding 64 roe deer genotypes from surrounding areas, so as to correct for possible distortions in allele frequencies in the monitored sample. As coefficient of relatedness, we estimated the r_{xy} statistic (Queller and Goodnight 1989) ranging between -1 and +1, which quantifies the probability of sharing two alleles by descent. Highly positive relatedness values are expected for pairs that are more closely related than random pairs in the population.

Analyses on spatial behaviour and genetic structure

Summer (1st June – 31th August, mating period) and winter (1st December – 28th February, grouping period) home ranges were estimated using Kernel method 90% by means of

Ranges VI software (Kenward et al. 2003). Each home range used in the analyses had been obtained by at least 18 fixes per season (on average 24 fixes) evenly distributed among the three months. Kernel centroids were obtained for every individual home range, and Euclidean distances between centroids were estimated.

Correlations between genetic relatedness and geographical distance were performed by Mantel test in GenAlEx 6.4. The test compares the correlation between the observed spatial and genetic matrices, to correlations between 9,999 random matrices obtained by permuting columns and rows in each matrix (Smouse et al. 1986). This computation was performed both jointly and separately for the two sexes. All considered seasons had ≥ 3 monitored roe deer.

For each dyad of individuals (male-female, male-male and female-female), we calculated the extent of the seasonal home range overlap using the Intersect tool in ArcGis 9.3 (ESRI, Environmental Systems Research Institute Inc. Redlands, California) and Minta’s index (Minta 1992) as follows:

$$Minta'sindex(\mathrm{MI}) = \sqrt{\frac{\mathrm{HR(AB)}}{\mathrm{HR(A)}} \times \frac{\mathrm{HR(AB)}}{\mathrm{HR(B)}}} \times 100$$

where HR(AB) is the area shared by individuals A and B, while HR(A) and HR(B) are the home ranges of individuals A and B, respectively (Minta 1992). Thereby, MI represents the mean potential interaction probability and may range between 0 and 100, with 100 indicating full overlap. On the basis of this parameter, we then classified seasonal roe deer dyads into three categories representing different extents of overlap: no overlap (O_0: MI = 0), low overlap (O_1: $0< \mathrm{MI} \leq 40$), and high overlap (O_2: MI >40). The adopted threshold was selected in order to have similar numbers of dyads in the two classes with overlapping home ranges. We assessed whether genetic relatedness increased with the extent of home range overlap by means of non-parametric randomisation tests in R 2.15.1 (R Development Core Team 2011). Specifically, we calculated the significance of the difference between means of two compared classes by computing the number of times a higher difference was obtained in 1,000 permuted datasets. This calculation was performed by sex and season, in order to check for different patterns of spatial and social organisation. Furthermore, we tested whether males and females tended to overlap their summer home range with unrelated potential partners, by comparing summer and winter relatedness in male-female dyads belonging to the O_2 class. Significance was estimated by randomisation test in R.

Results

A total of 11,061 individual positions were recorded in eight years. The mean home range size (± SE) in summer was 94.70 ± 15.90 ha and did not show significant differences between sexes (males: 87.25 ± 16.90 ha, females: 98.15 ± 21.20 ha; two-sample t-test: t =1.979, d.f. = 120, p = 0.412). In winter, home range size tended to be lower (69.18 ± 58.13 ha), again without a significant difference between males (67.38 ± 39.53 ha) and females (70.25 ± 67.13 ha; two-sample t-test: t =1.991; d.f. = 76; p = 0.813).

In total, 133 multilocus genotypes were obtained (69 monitored + 64 non-monitored roe deer). The proportion of missing data was 2.93%. No evidence of allelic dropout was detected by MicroChecker, while the presence of null alleles was suspected at three loci (NV21, NV24 and BMC1009, but in none of them the estimated frequency of nulls was > 0.1). A total of 71 different alleles (3-10 alleles per locus, k = 6.45) were found in the population. Average observed and expected heterozygosity amounted to H_O = 0.581 and H_E = 0.616, respectively. The overall F_{IS} in the population amounted to 0.057 and a deficiency of heterozygotes was observed at several loci (i.e., deviation from HWE; p<0.001). All pairs of loci but two (RT1-NVRTH16 and RT1-OarFCB304, $\alpha_{5\%}$=0.00093) were in linkage equilibrium. The average relatedness coefficient in the sample of monitored roe deer was 0.098 ± 0.215 (mean ± SD, N=2,347 pairs). The overall distribution of r_{xy} values fitted a normal distribution (Kolmogorov-Smirnov normality test: p = 0.774; fig. 1a) and did not differ between males and females (two-sample t-test: t = 2.101; d.f. = 18; p = 0.965; mean r_{xy}= 0.097 ± 0.214 in females and 0.087 ± 0.212 in males; fig. 1b).

In summer, correlation coefficients (R) between relatedness and spatial distance were mostly negative and not significant (Mantel test, p > 0.05 for the overall population, and for the two sexes separately, tab. 1). In winter, instead, a significant negative correlation was found for the overall population in 2003 (p = 0.009), and females showed only negative correlations which were weakly significant, or marginally significant, in three years (2002: p = 0.050, 2003: p = 0.033, and 2004: p = 0.030). No significant correlation was found in males.

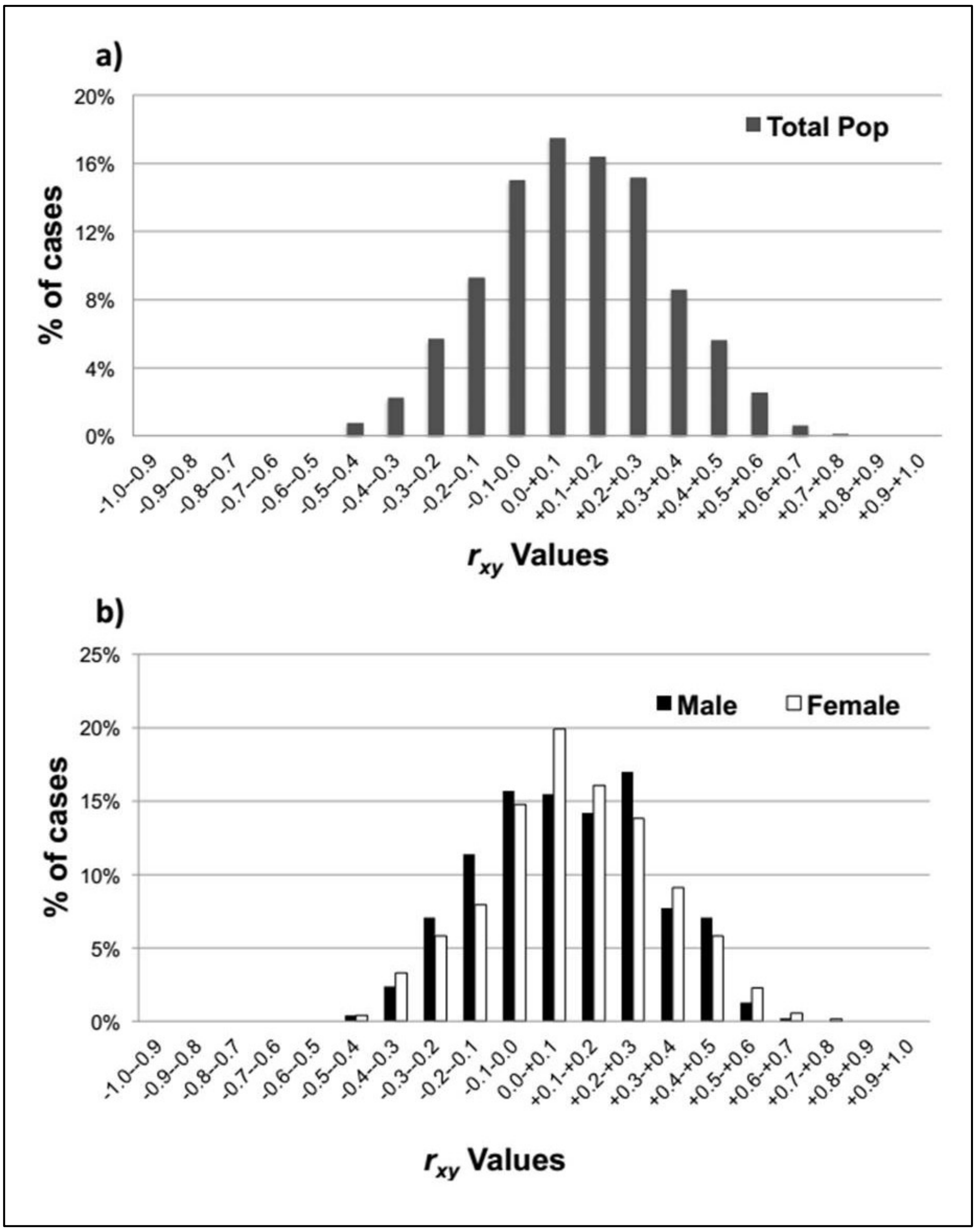
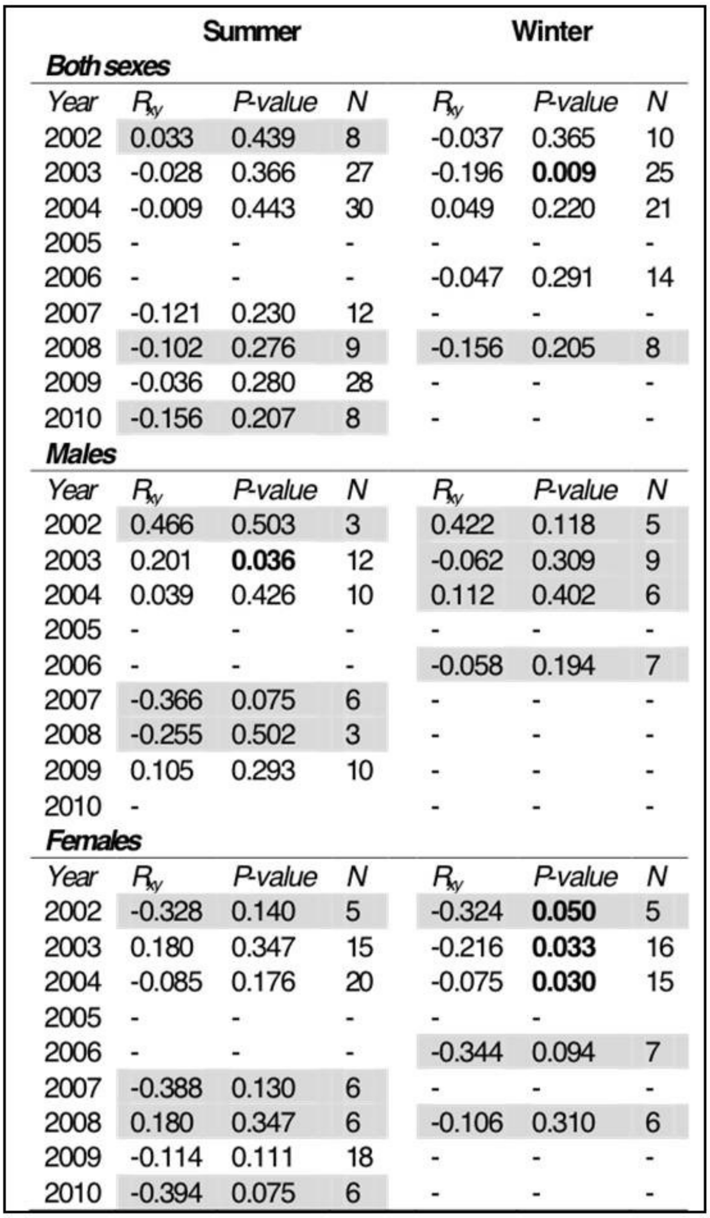


Figure 1 Distribution of pairwise relatedness (r_{xy},Queller& Goodnight, 1989) in 69 roe deer captured from 2002 to 2010 in Alpe di Catenaia, Central Italy. a) Overall distribution, b) separated distributions of males and females.

In summer, the extent of overlap in roe deer dyads sharing a portion of their home range was on average 37.4 ± 12.5 % and no difference was detected between sexes across years (two-sample t-test: t = 2.44; d.f. = 6; p = 0.90). As for intra-sex comparisons, dyads of females (n=92 pairs) shared, on average, 30.5 ± 15.8% of their summer home range, whereas the overlap for dyads of males (n=38) averaged 30.1 ± 26.4%. As expected, the overall overlap increased in winter (66.7 ± 10.9 %, for the overall sample, n = 238 pairs), averaging 55.1 ± 9.5 % and 41.1 ± 29.9 % for dyads of females and males, respectively.

Table 1. Correlations (R_{xy}) between genetic relatedness and Euclidean distance among monitored roe deer in Alpe di Catenaia, Central Italy (2002 – 2010). Spatial distances were calculated between summer and winter Kernel home range centroids. N = number of monitored individuals, P-value = significance of correlation (in bold significant values at α = 0.05). Shadowed cells refer to seasons in which the sample size was small (i.e. < 10 individuals).



	Summer			Winter		
Both sexes						
Year	*R_{xy}*	*P-value*	*N*	*R_{xy}*	*P-value*	*N*
2002	0.033	0.439	8	-0.037	0.365	10
2003	-0.028	0.366	27	-0.196	**0.009**	25
2004	-0.009	0.443	30	0.049	0.220	21
2005	-	-	-	-	-	-
2006	-	-	-	-0.047	0.291	14
2007	-0.121	0.230	12	-	-	-
2008	-0.102	0.276	9	-0.156	0.205	8
2009	-0.036	0.280	28	-	-	-
2010	-0.156	0.207	8	-	-	-
Males						
Year	*R_{xy}*	*P-value*	*N*	*R_{xy}*	*P-value*	*N*
2002	0.466	0.503	3	0.422	0.118	5
2003	0.201	**0.036**	12	-0.062	0.309	9
2004	0.039	0.426	10	0.112	0.402	6
2005	-	-	-	-	-	-
2006	-	-	-	-0.058	0.194	7
2007	-0.366	0.075	6	-	-	-
2008	-0.255	0.502	3	-	-	-
2009	0.105	0.293	10	-	-	-
2010	-			-	-	-
Females						
Year	*R_{xy}*	*P-value*	*N*	*R_{xy}*	*P-value*	*N*
2002	-0.328	0.140	5	-0.324	**0.050**	5
2003	0.180	0.347	15	-0.216	**0.033**	16
2004	-0.085	0.176	20	-0.075	**0.030**	15
2005	-	-	-	-	-	
2006	-	-	-	-0.344	0.094	7
2007	-0.388	0.130	6	-	-	-
2008	0.180	0.347	6	-0.106	0.310	6
2009	-0.114	0.111	18	-	-	-
2010	-0.394	0.075	6	-	-	-

Also in winter the two sexes did not show any significant difference (two-sample t-test: t = 2.77; d.f. = 4; p = 0.64).

In both sexes, relatedness was higher between individuals with overlapping home ranges (classes O_1 and O_2) than between O_0 dyads (tab. 2). This pattern was more evident and statistically significant in winter (randomisation test, p = 0.020). In inter-sex dyads showing high overlap (O_2 class only), relatedness was low in summer (r_{xy}=0.072 ± 0.03) and significantly higher in winter (r_{xy} = 0.154 ± 0.03, randomisation test, p = 0.046).

Table 2. Distribution of pairwise relatedness (r_{xy}) in dyads belonging to three different home range overlap classes (O_0, O_1 and O_2) in Alpe di Catenaia, Central Italy. Spatial data refer to summer and winter, separately. FF, female-female dyads; MM, male-male dyads; FM, female-male dyads.

		SUMMER			**WINTER**		
		FF	*MM*	*FM*	*FF*	*MM*	*FM*
	N°pairs	410	144	505	176	56	202
O_0	*Mean*	0.075	0.054	0.095	0.053	0.03	0.08
	SE	0.011	0.018	0.010	0.016	0.024	0.015
	N°pairs	66	23	82	53	12	54
O_1	*Mean*	0.123	-0.057	0.149	0.15	0.069	0.18
	SE	0.030	0.040	0.024	0.029	0.057	0.030
	N°pairs	25	15	49	42	15	62
O_2	*Mean*	0.107	0.125	0.072	0.12	0.141	0.154
	SE	0.048	0.040	0.031	0.035	0.047	0.028

Discussion

Patterns of genetic relatedness did not differ between sexes in the forest-dwelling roe deer population under study. In the two sexes, we detected no significant negative correlation between genetic and spatial distance in summer (mating period), while a weak correlation was found in females but not in males in some winters (grouping period). The general lack of difference in spatial behaviour between the two sexes in roe deer agrees with previous studies on this species (Bonnot et al. 2010; Coulon et al. 2006; Debeffe et al. 2012; Gaillard et al. 2008) that showed the absence of sex-biased dispersal in mosaic landscapes.

The model on dispersal proposed by Wahlström and Liberg (1995) suggests dispersal in roe deer being related to population density and fawns' body conditions, and predicts no difference in dispersal rate between sexes at high densities, if fawns reach a critical weight in winter. Despite the high population density in our study area, fawns' body weight in winter was relatively high (on average 16.1 ± 2.0 Kg in a sample of 53 females and 16.9 ± 2.2 Kg in 43 males, i.e., beyond the thresholds reported for a fragmented habitat in France - Debeffe et al. 2012). We can thus hypothesize that the environmental quality of our study area allows juveniles of both sexes to grow quickly becoming potential dispersers. The combination of habitat conditions and high population density therefore offers to roe deer in the area a spectrum of different possible strategies to limit inbreeding and to shuffle genes in the population. Unfortunately, in our study we could not estimate the dispersal rate in the population, as most animals were monitored when they were 21 months or older, and in our sample we recorded only two cases of natal dispersal (two yearlings: one male and one female).

It is important to notice that, unlike other studies on this issue (e.g. Bonnot et al. 2010), we calculated pairwise distances between individuals' home range centroids, and not between the geographical coordinates of capture or kill sites. Accordingly, we could have expected more pronounced differences between sexes on account of their dissimilar spatial behaviour during the mating season. Instead, when we compared genetic relatedness at different degrees of home range overlap, we observed that it tended to increase with overlap, in both sexes. Such tendency was more pronounced in winter probably because of the scarcity of food resources and the consequent formation of small groups, usually consisting of relatives, in the most suitable areas (Danilkin and Hewison 1996).

During the breeding period, intra-sex dyads of both males and females showed a general preference in sharing their home range with relatives. This suggests that a large proportion of the population is philopatric (see also Debeffe et al. 2012), without great distinction between sexes. Summer represents the period when males are territorial and undertake aggressive interactions with other males, especially at high densities due to increased male-male competition for mating opportunities (Kjellander et al. 2004). However also in this period, males seem to share their home range preferentially with their relatives. This behavioural pattern (i.e., kin tolerance) could be a strategy to reduce costs of territory defence by limiting the number of aggressive interactions with neighbouring males (Brown and Brown 1993). A similar pattern was observed in swift foxes (*Vulpes velox*) where territory holders tolerated a high home range overlap with related neighbours (Kitchen et al. 2005). Our findings comply with the mating system generally adopted by roe deer: approaching the breeding season males become territorial, but their territoriality would be relaxed if the neighbours are relatives. Through the reduced costs of territory defence males can save energy for mating, and the possible costs of an increased mate competition can be balanced by the gain in terms of inclusive fitness deriving from the mating of kin.

Finally, during the rutting period, males and females seemed to occupy the space in a way to minimize the chances of inbreeding. Inter-sex dyads with highly overlapping home ranges turned out to be much less related than intra-sex pairs during summer, but not during winter. Such genetic pattern may be due to the presence of range adjustments and breeding excursions having the ultimate aim to prevent inbreeding. In fact, different spatial strategies can be used by females to avoid the mating with relatives. Previous studies in the area (Lamberti et al. 2004), for instance, identified different spatial behaviours in females during the rutting period. They distinguished ‘stationary’ and ‘migratory’ females, with the latter showing much larger summer home ranges. Short-range breeding excursions by females are also very frequent in roe deer populations (Lovari et al. 2008, Richard et al. 2008, Debeffe et al. in press). Although the causative factors of this phenomenon are not fully understood, these movements were interpreted as a specific strategy to cope with inbreeding risk (Debeffe et al. in press). The fact that spatial proximity and genetic relatedness in our study seem more strongly negatively related in females than in males might be linked to the fact that female excursions lead to a higher gene flow of the male genome in the population, thus breaking down the spatial-genetic relationship among the male segment of the population. Specific behavioural strategies adopted by females to increase the encounter probability with unrelated males during the rut (see also Liberg et al. 1998), as long with the low level of polygyny (Vanpé et al. 2008), can play a role in the maintenance of high levels of genetic variability in roe deer populations.

An apparent contradiction was the absence of correlation between genetic and spatial distances, but the association between genetic relatedness and home range overlap emphasized the need to use a multiple approach to evaluate spatial behaviour in relation to genetic relatedness in animal populations. In this regard, just a limited number of studies used home range overlap between individuals (e.g. Kitchen et al. 2005; Jesmer et al. 2011). This approach considers also the probabilities of interaction between individuals sharing the space. Further analyses on larger spatial scales in forested landscapes are needed to fully understand the spatial pattern and the genetic structure we observed, and consider the possible role of landscape features.

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Nicoletta Sica
Dipartimento di Scienze della Natura e del Territorio, via F. Muroni, 25, I-07100, Sassari, Itaty.
e-mail: nicoletta.sica@gmail.com