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Original article

Testing Cort-Fitness and Cort-Adaptation hypotheses in a habitat suitability gradient for roe deer

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ABSTRACT

According to the Cort-Fitness Hypothesis, higher stress levels (glucocorticoids) in vertebrates are correlated to lower fitness. However, recent studies have failed to validate this hypothesis. A proposed wider framework suggests that reproduction can be perceived as an overload adds up to other environmental challenges that individuals must adjust to. In this case, elevated glucocorticoids could help individuals to allocate resources to reproduction without comprising other functions, leading to the expectation of a positive cort-fitness relationship. This has been proposed as the Cort-Adaptation Hypothesis. Stress levels result from a complex interaction between the environment and the neuroendocrine system of animals. Accounting for physiological functions involved in how animals cope with their environment would help to clarify the relationship between glucocorticoids and animal performance. We used roe deer (*Capreolus capreolus*) inhabiting diverse habitats in the Iberian Peninsula to: i) test the Cort-Fitness and Cort-Adaptation hypotheses by indexing fitness using a comprehensive physiological approach which takes into account fundamental physiological functions and their trade-offs; and ii) evaluate the link between primary productivity and individuals' condition in a seasonal environment. We evaluated spatial and temporal variation in stress levels, reproductive hormone levels, nutritional status and immune function from fecal samples collected in 2010. Lower stress levels were related to better condition in non-reproductive seasons but not to higher primary productivity. In contrast, stress levels were always positively related to reproductive condition, which was better in most productive habitats. Summer and winter were the less productive seasons and the more challenging for the species in the habitat gradient studied. In winter, reproductive condition traded off against immune function being biased toward immune function in less productive habitats. In summer reduced primary productivity limited roe deer nutritional and immunological condition but not reproductive condition. Overall our results match both the Cort-Fitness and Cort-Adaptation Hypotheses.

1. Introduction

Biodiversity loss is presently occurring at a faster rate than throughout the entire history of the planet (Millenium Ecosystem Assessment, 2005) due to an increase in anthropogenic threats such as habitat loss, degradation and fragmentation, over-exploitation, pollution and climate change (Parmesan and Yohe, 2003; Vitousek, 1994). Understanding and predicting how these threats affect biodiversity and ecosystem functioning is a major focus of the scientific community (Loreau et al., 2001). However, if conservation and management efforts are to be successful, not only do we need to understand how species presence and distribution are impacted by global environmental change (Araujo and Rahbek, 2006), but we also need to identify the mechanisms which allow organisms to cope with their changing environments (Romero and Wikelski, 2001; Wingfield, 2008).

Most studies that examine how animal populations manage their variable environments have focused on the stress levels of populations to changing conditions. These studies are generally

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based on indexing stress levels using glucocorticoids (GC) or their metabolites in urine or faeces (GCm) as a quality indicator of condition (Boonstra, 2004; Busch et al., 2011; Creel et al., 2002; Gobush et al., 2008; Lanctot et al., 2003; Romero and Wikelski, 2001) since stress response is the link between an individual's perception of the environment and physiology (McEwen and Wingfield, 2003). When an animal perceives a stressor (e.g. predator, competitor), its nervous system sends a signal to the endocrine system triggering an increase in GC secretion in the blood, which allows the animal to allocate energy to respond to the stressor. However, when a stressful stimulus is present for a long period of time, high GC concentrations remain in the blood, which can have detrimental effects on certain systems, such as the immune and reproductive systems (Romero, 2004). Hence, individuals with high GC or GCm levels are expected to be in poor condition and have low relative fitness (Husak and Moore, 2008; Wingfield and Sapolsky, 2003). This hypothesis is generally referred to as the Cort-Fitness Hypothesis (Bonier et al., 2009).

In recent years, empirical evidence has cast doubts on the credibility of this hypothesis. For instance, Taillon and Côté (2008) failed to find a correlation between higher GC or GCm levels and fitness or condition in white-tailed deer (*Odocoileus virginianus*). A recent study on the relationship between GC or GCm and fitness found an array of positive, negative and non-significant results (Bonier et al., 2009). This lack of consistency could be due to the involvement of GC in several complex functions such as temperature maintenance, immunity, social interactions and reproduction (Mormede et al., 2007; Sapolsky et al., 2000). Bonier et al. (2009) have proposed a wider approach when interpreting the Cort-Fitness Hypothesis which includes reproduction as pivotal function in which GC play an important role. Reproduction is one of the most demanding processes of animal life-history (Williams, 1966), and during this period elevated GC could help to allocate the necessary resources for animals to successfully overcome reproduction without compromising other functions such as immunity or temperature maintenance (e.g. by mobilizing reserves tissues to make them available). This hypothesis is referred to as the Cort-Adaptation hypothesis (Bonier et al., 2009) according to which a positive relationship between fitness and GC could be expected during the reproductive period. Thus, the interpretation of GC levels might require complementary information on the state of reproduction and other relevant physiological functions such as immunity and nutritional conditions (Romero, 2004). In this sense, some authors have highlighted the importance of considering the trade-offs between stress response and fitness correlates of condition to accurately interpret GC or GCm results for conservation issues (Busch and Hayward, 2009; Hayward et al., 2011).

In the last few decades, roe deer have been expanding their range to the central Iberian Peninsula, forcing the species to cope with a gradient in habitat suitability from the preferred humid broadleaved forest to xeric agricultural environments with severe summer droughts (Acevedo et al., 2005; Tellería and Virgós, 1997; Virgós and Tellería, 1998). This natural expansion provides an excellent opportunity to study the physiological changes that allow animals to survive in such a wide habitat gradient. In this paper we assess roe deer (*Capreolus capreolus*) sensitivity to different habitat conditions using a holistic physiological approach. We used several indicators of fundamental physiological functions such as stress levels (indexed by fecal GCm), reproductive condition (fecal metabolites of progesterone, estradiol and testosterone), nutritional status (fecal nitrogen) and immune function (fecal IgA) to perform an empirical test of the cort-adaptation and cort-fitness hypotheses for the reproductive cycle of the species and determine the potential use of these physiological variables as indicators of habitat quality perceived by roe deer. We expected the measured physiological indicators to relate differently at the individual-level depending on the reproductive moment. During the rut season (summer), we expected to find a positive relationship between reproductive hormones (for both sexes), GCm and the measured indicators of immune function and nutritional condition, verifying the Cort-Adaptation hypothesis. Winter and spring are the periods of pregnancy and parturition/lactation, respectively, in which the reproductive hormones progesterone (P_4) and estradiol (E_2) play a major role in placenta maintenance and milk gland development (Ryg, 1986). In these periods, we expected to find a positive relationship between P_4, E_2, GCm, nutritional and immune condition indicators. During the non-reproductive season (autumn), we expected to find a negative correlation between GCm and condition-related variables (immune function and nutrition) and non-significant relationship with reproductive hormones, verifying the Cort-Fitness hypothesis.

If our expectations in relation to the Cort-Fitness and Cort-Adaptation hypotheses were validated at the individual level, we would expect the magnitude of the variables to be controlled by primary productivity at the population-level. For instance, on the reproductive seasons if the Cort-adaptation hypothesis better fits the obtained data, we would expect the levels of reproductive hormones, GCm and condition related variables to be higher in more productive habitats (Bonier et al., 2009,Pettorelli et al., 2005, 2006).

2. Material and methods

2.1. Study area

The study area occupies around 2000 km^2 in the Sierra de Guadarrama and Sierra de Altomira in central Spain (Fig. 1). We studied an environmental gradient from 800 to 1700 m. Average annual precipitation and temperature ranges are 527–974.5 mm and 7.75–13 °C, respectively (http://opengis.uab.es/wms/iberia/) (Table S1). The vegetation above 1200 m is composed of alpine grasslands, natural pinewoods and plantations of Scots pines (*Pinus sylvestris*). Between 1000 and 1200 m, the landscape is dominated by mosaics of humid pastures and broadleaved vegetation, mainly Pyrenean oak (*Quercus pyrenaica*). Below 1000 m, the most abundant formations are mosaics of pasture sclerophyllous vegetation where holm oak (*Quercus ilex*) is the predominant species. The lower parts of the mountains are occupied by extensive crops of wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), where holm forests have a patchy distribution (Tellería and Virgós, 1997). According to environmental characteristics (precipitation, temperature and vegetation type), we identified five dominant habitats (Pine, Oak, Holm, Xeric and Crops). Three to four populations were studied in each habitat (Table S1). A roe deer population was defined as an individual or group of individuals partly inhabiting a 50 ha home range centered on the sampling location (Guillet et al., 1996). To maximize the number of individuals sampled, we established three to four 50 ha plots per habitat type separated by at least 2 km (Fig. 1). Based on local densities (FIDA, 2008), we estimated that there was an average of eight roe deer in each of the study plots in the Pine, Oak, Holm and Crops habitats (range 4–12) and four in each plot of the Xeric habitat (range 3–9). More details are facilitated on the Section 2.3. In each plot we performed two linear transects of 400 m to characterize non-forest habitat composition (Table S2).

2.2. Primary productivity

Primary productivity for each habitat was assessed using the Normalized Vegetation Difference index (NDVI), which has been

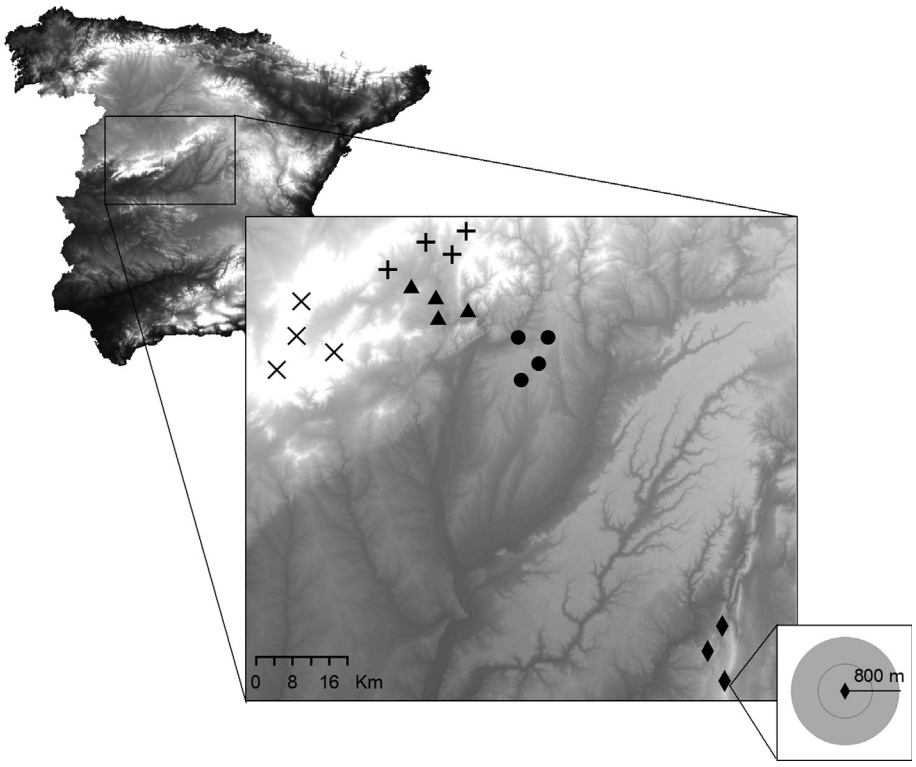

Fig. 1. The rectangle in central Iberian Peninsula represents the general location of the study area (Sierra de Guadarrama on the North-East and Sierra de Altomira on South-West). Altitudes are represented on a grayscale with black for the lowest areas and white for the highest areas. The central area of the figure represents the location of the roe deer studied populations. Different symbols are used to represent the different habitat types: X: pine; Crosses: oak; Triangles: holm; Circles: crop; Diamonds: xeric. A sample of a xeric population is shown in the lower right-hand corner. The 50 ha plot where samples were collected (small circle) and the 800 m radius buffer area used for NDVI data extraction. Different rectangles do not represent equivalent areas.

widely used as a proxy of food resources for several ungulate species (Hamel et al., 2009; Pettorelli et al., 2006, 2005). NDVI data were obtained from the Moderate Resolution Imaging Spectroradiometer (MODIS) on board the Earth Observing System-Terra platform. The collection used was MOD13Q1 with a 250 m spatial resolution and 16-day temporal resolution (Huete et al., 2002). We only considered NDVI information for the dates on which surveys were performed (Winter: 23Feb–01Apr; Spring: 06 May–10 May; Summer: 20Jul–03Sep; Autumn: 26Oct–05Nov). An index of primary productivity for each population and season considered was obtained by extracting the NDVI pixels from an 800 m radius circle from the centroid of the 50 ha plot. This was carried out to ensure that any potential home range of a roe deer sampled in the 50 ha plot was included in the analysis (Fig. 1). Raw NDVI data were then smoothed (Garonna et al., 2009), and average values were obtained for each population and season.

2.3. Fecal sample collection

Each population was sampled seasonally following the reproductive cycle of the species: gestation (winter), births (spring), rut (summer) and diapause (autumn) (Mateos-Quesada, 2011). Sampling procedures were designed according to species home range and behavior to maximize the number of individuals sampled and to minimize the effects of anonymous sampling (over- or under representation of same individuals to the detriment of others, Huber et al., 2003). Thus, our sampling plots (50 ha) are close to the average home ranges of both male and female roe deer, reviewed in (Guillet et al., 1996). As females have overlapping home ranges (Blottner et al., 1996), several females and their offspring and yearly fawns can share a same territory. In contrast, males maintain exclusive territories except during the rut season in which one male territory can overlap the territory of one or several females. Based on these evidences, our plots can maintain a minimum of 3–4 individuals around the year, a male plus a female (s) and yearly fawns and a maximum of around 10 individuals, according to local densities (FIDA, 2008). Huber et al. (2003) compared the physiological levels of a population with known and anonymous sampling and found no significant differences. These authors recommend maximizing the sampling area using linear transects. We followed this methodology replicating the 50 ha plots (3–4) in each habitat type randomly across the study area (Fig. 1, Table S1), and each plot was intensively sampled following pathways typically used by the species. Roe deer, also present a high fidelity in the use of core areas inside territories (Holand et al., 1998; Linnell and Andersen, 1998), and the different individuals (*e.g.* male and the female family group) use different areas for central activity. Therefore, sampling different linear transects within a territory maximizes the probability of sampling all individuals in the population and minimizes the over or under estimation of some individuals in detriment to others. Thus, we consider that the applied methodology and the above-mentioned typical spatial organization and space use of the roe deer allowed as to capture a reliable and representative sample of the roe deer populations inhabiting our study plots.

All faeces in the paths were labeled the day before samples were collected, so that only new fresh fecal samples with a moist layer of green mucus and no signs of dehydration were collected. If we could not collect adequate sample sizes of fresh fecal samples on one sampling day, we returned the day after. This sampling scheme ensured that collected samples which were at maximum 24 h old which does not have an effect on hormone levels (Abáigar et al., 2010; Escribano-Ávila et al. unpublished results). Fresh samples

were collected in plastic tubes and immediately stored in a portable freezer at −20 °C. They were then transferred to a conventional freezer in the laboratory and maintained at the same temperature until physiological analyses were conducted (Sheriff et al., 2011). A total number of 537samples were collected: 142 were collected in winter, 104 in spring, 135 in summer and 156 in autumn.

2.4. Fecal physiological indicators

2.4.1. ACTH challenge

We assessed several non-invasive indicators of representative physiological functions related to condition (Hayward et al., 2011; Mormede et al., 2007). One of them was fecal cortisol metabolites (CORT) which were used to index stress levels. An ACTH challenge was performed to validate the cortisol assay to be used for monitoring stress levels. The ACTH challenge was performed on two captive female roe deer which were maintained at *Centro de Fauna Cañada Real* (http://www.opennature.com/) in El Escorial, Spain. The animals lived in an out-door enclosure (20 × 40 m) with natural photoperiod and vegetation. Water and food was provided *ad libitum*. Females were individualized as H1 (5 years old, 25 kg weight) and H2 (2 years old 23, kg weight). Both animals were observed during the study period and collected faecal samples were assigned to each animal. Pre-ACTH injection samples were collected for two days (−48 h) on each animal individually immediately after defecation. A dose of 0.25 ng/ml of ACTH (Synacthen, Novartis) was blow gun injected in each female without anesthesia (0 h) and samples were collected during the subsequent three days (72 h). Fecal cortisol metabolites (CORT) were extracted and quantified according to the procedure described in the section *Fecal hormones immunoassays* using a commercial enzyme immunoassay (EIA, DRG Instruments GMBH, Marburg, Germany). To test for significant increases in CORT values after ACTH injection, data were analyzed by two-way ANOVA with CORT concentration as the response variable, and time in relation to ACTH injection (0: pre-injection time; 24, 48, 72) and animal (H1, H2) as fixed factors. Residuals were normally distributed and homoscedastic. CORT significantly increased after the ACTH treatment ($F_{4,16} = 15.8$; P *value* < 0.001; adjusted $R^2 = 0.8$), and maximum values were achieved after 24 h of ACTH injection (Fig. 2). Therefore the measurement of cortisol metabolites in faeces of roe deer by means of the commercial EIA used proved to be a useful tool for the non-invasive monitoring of adrenocortical function in roe deer. All necessary permits were obtained from the corresponding authorities (Consejería de Medio Ambiente y Ordenación del Territorio de la Comunidad de Madrid) and all animal work was conducted according to relevant Spanish and international guidelines.

2.4.2. Reproductive hormones evaluated

Reproductive status was indexed using fecal metabolites of the sex steroids progesterone (P_4), testosterone (T) and estradiol (E_2). As P_4 is essential in maintaining pregnancy, its plasma and fecal concentrations are high in pregnant females. P_4 has proven to be valuable in determining ovarian cyclicity, gestation and seasonality, allowing the identification of actively reproducing females and pregnancy in a wide number of cervids (Blanvillain et al., 1997; Garrott et al., 1998; Kapke et al., 1999; Knox et al., 1992; Monfort et al., 1991; Ramsay et al., 1994; Stoops et al., 1999) and specifically in roe deer (Hoffmann et al., 1978). E_2 has been found to increase at the end of pregnancy-parturition in several cervids and is presumably related to the development of the milk gland (Ryg, 1986 and references therein). Testosterone (T) is related to male territoriality, rut and sexual expression of secondary traits in several cervids (Bubenik et al., 1991, 1996; Gómez et al., 2006), and T plays an important role in roe deer in the rut period, antler growth (Roelants et al., 2002; Sempere and Lacroix, 1982; Sempere et al., 1992) and juvenile dispersal (Pinheiro et al., 2012).

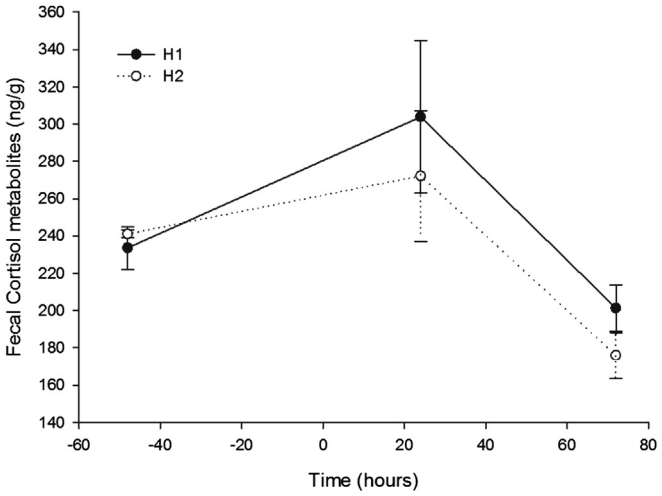

Fig. 2. On the *Y-axis* fecal cortisol metabolites (average ± SE) are shown in relation to time relative to ACTH injection on the *X-axis* for each experimental animal (H1, H2).

2.4.3. Fecal hormones immunoassays

Hormone metabolites (CORT, P, T, E_2) were extracted from fecal samples following the procedure described by Dumonceaux et al. (2006). Fecal samples were gently homogenized by applying a small amount of liquid nitrogen in a mortar and dried in an oven at 39 °C until stable weight was obtained (1–2 h). A subsample (0.7 g) was placed in assay tubes with 2.5 ml of PBS and 2.5 ml of methanol and shaken 16 h, as longer periods did not increase the amount of hormones obtained. The supernatant was centrifuged at 4000 *g* for 30 min, the pellet was discarded and samples were stored at −20 °C until assayed. Each hormone was assayed with a specific commercial enzyme immunoassay (EIA, DRG Instruments GMBH, Marburg, Germany). The cross-reactivity of the antibodies with other substances according to the manufacturer is reported when the percentage is over 1%:Cortisol: cortisone 45%, progesterone 9%, deoxycortisol <2%, dexamethazone <2%; Progesterone: 11-Desoxycorticosterone 1.10%; Testosterone: 11β-Hydroxyestosterone 3.3%, 19-Nortestosterone 3.3%. The cross-reactivity of estradiol with any other substance was insignificant (less than 1%). A parallelism test of serial dilutions of extracts was performed with dilution ratios of 1:32, 1:16, 1:8, 1:4, 1:2, 1:1, and curves parallel to those of the standard ($p > 0.05$) were obtained for each hormone. Intra- and inter-assay coefficients of variation were calculated for each hormone with extracts ($n = 8$). The obtained values for each hormone were: CORT: 7.08–10.89%; P: 7–14.5%; T: 7.2–16.5%; E_2: 3.0–9.2%. The assay sensitivity for each hormone was: CORT: 2.5 ng mL^{-1}; P_4: 0.045 ng mL^{-1}; T: 0.083 ng mL^{-1}; E_2: 9.7 pg mL^{-1}. Concentrations are expressed as nanograms per gram of dry faeces (ng g^{-1}).

2.4.4. Nutritional condition

Nutritional status was indexed using fecal nitrogen (N) as an indicator of diet quality, which is correlated with several life history traits such as body mass (Blanchard et al., 2003; Côté and Festa-Bianchet, 2001; Gendreau et al., 2005; Hewison et al., 2009). This indicator has been extensively used in ungulates (Hamel et al., 2009 and references therein) and more precisely in roe deer (Kamler and Homolka, 2005; Kamler et al., 2004; Navarro-González et al., 2011). N was quantified by elemental analyses using the dynamic flash combustion method at 1200 °C in a LECO CHNS-932analyzer (Leco,

Michigan USA). Approximately 0.1 g of the homogenized, dried sample was analyzed to determine the percentage of N. Final results were calculated by multiplying exact weight by the percentage obtained in the combustion analysis. Analyses were conducted by the certified laboratory CAI Microanálisis Elemental in Madrid (UNE-EN ISO/IEC 17025:2005).

2.4.5. Immunological condition

Immune function has been widely used as a proxy of phenotypic quality and fitness in birds and mammals (Demas et al., 2003; Dunn et al., 2009; Hasselquist et al., 2001). However, different parameters of the immune system have been used depending on laboratory and field constraints (reviewed by Demas et al., 2011). Our field constrain was that we could not capture animals so that we could only collect faeces. Accordingly, we assessed immune function by measuring immunoglobulin A (IgA) which allow as to have some information on the adaptative immune function. However for a comprehensive evaluation of immune function more information is needed. Mammals healthy gut harbors a huge collection of beneficial bacteria (*i.e.* symbionts) that – in the human body – constitute some ten times the number of cells, perhaps amounting to 10^{13}–10^{14} microbial cells and a total weight of 1–2 kg (Neish, 2009). The immune system monitors the microbiota and contributes to homeostasis by the provision of mucosal IgA secretion (Brishbin et al., 2008; Cerutti and Rescigno, 2008) Once released by mucosal gut cells, IgA promotes immune exclusion by entrapping dietary antigens and microorganisms in the mucus, downmodulates the expression of proinflammatory bacterial epitopes on commensal bacteria, and, in general, promotes the maintenance of appropriate bacterial communities within specific intestinal segments (Peterson et al., 2007; Phalipon et al., 2002). IgA has also been revealed effective combating parasitic infestations by decreasing worm fecundity (Davies et al., 2005; Smith et al., 1985) In sort, IgA is the most abundant antibody isotype produced in both mammals and birds measurable non invasively and therefore aid to monitor adaptative immune function in wild fauna however do not provide information of innate immunity (Snoeck et al., 2006). IgA was extracted from fecal samples following Peters et al. (2004). Dried faeces were incubated for 1 h in PBS 0.05% Tween 20, pH 7.4, as longer times did not enhance IgA extraction. Homogenates were centrifuged to remove solid materials (1600 *g* for 15 min at 5 °C). An aliquot of the supernatant was transferred to an Eppendorf tube containing an aliquot of a protease inhibition cocktail (Sigma, St Louis, CA, USA). A second centrifugation was carried out (10,000 *g* for 10 min at 5 °C) for optimal removal of solid material. The supernatant was collected and stored at −20 °C until assayed. IgA quantification was performed with a commercial EIA (Bovine IgA ELISA Quantitation set, Bethyl Laboratories Inc, Montgomery, USA). Samples were assayed in duplicate according to the manufacturer's recommendations. A parallelism test was performed, obtaining a curve parallel to the one performed with the standards ($p > 0.05$). Intra- and inter-assay coefficients of variation were 2.94 and 9.89% respectively. IgA concentrations are expressed as nanograms per gram of dry faeces (ng g^{-1}).

2.5. Statistical analyses

In order to establish the relationship between habitats and NDVI values, a Linear Mixed Model (LMM) was performed with average NDVI values per sampling time as the response variable, habitat and season as fixed factors and population as random factor. Residuals fulfilled the assumptions on normality and homoscedasticity.

Linear Mixed Models were performed for each physiological indicator as response variable and seasons as fixed factor to evaluate seasonal patterns. Additionally for each season we performed LMM for each physiological indicator as response variable and habitat as fixed factor to evaluate the differences among habitat types. In all cases the population was included as a random factor and residuals fulfilled the assumptions of normality and homoscedasticity.

Principal Component Analyses (PCA) were carried out to determine how the different physiological indicators were related to each other in each season at the individual level in order to validate the Cort and Adaptation Fitness hypotheses. We selected the PCA components to be retained for further analyses according to the Kaiser–Guttman criterion where only components with eigenvalues greater or equal to 1 were selected (Kaiser, 1960). For interpretation, we used the loadings definition proposed by Comrey and Lee (1992). According to these authors, loadings represent the correlation among original variables and components. Therefore, a greater loading indicates that a component is a more accurate measure of the variable. Comrey and Lee (1992) consider loadings over 0.71 (50% overlapping variance) to be excellent, 0.63 (40% overlapping variance) very good, 0.55 (30% overlapping variance) good, 0.45 (20% overlapping variance) fair and 0.3 (9% or less) poor. According to these authors, loadings under 0.3 should not be considered when defining a factor.

Once Cort-Fitness-Adaptation hypotheses were validated the retained components resulted from the PCA analyses were interpreted based on our initial expectations for each season. These were used in linear mixed models as response variables with NDVI values as predictor variable in order to verify a better population performance in habitats with higher primary productivity (NDVI). When NDVI was found to have a significant effect we performed another linear mixed model with habitat as a fixed factor and the population of each sample as a random factor. Residuals fulfilled the normality and homoscedasticity assumptions. All statistical analyses were performed in the open source software package R (R Core Team, 2011) with appropriate additional packages *ade4* (Dray and Dufour, 2007) for PCA analyses and *nlme* (Pinheiro et al., 2012) for linear mixed models.

3. Results

3.1. Environmental gradient analyses

Habitat type and season significantly differed in their primary productivity values (Habitat type: $F_{4,14} = 34.5$, *P-value*< 0.001; Season $F_{3,54} = 4.1$, *P-value* $= 0.01$). In general, habitats dominated by evergreen vegetation (pine, holm and xeric) had higher NDVI values, except in spring and summer when oak and pine habitats had the highest values. Crop habitats had the lowest NDVI values in all seasons (Fig S1, Table S3).

3.2. Fecal IgA, N and hormone metabolites

All physiological variables measured significantly varied in each season. The parameters obtained in the Linear Mixed Model were in general significant with some exceptions: IgA in summer, Estradiol was marginally significant in spring and summer and Testosterone was not significant on autumn (See Table S4, Fig. 3).

Sexual hormones results shifted according to the reproductive cycle previously described for the species. The female reproductive hormones metabolites E_2 and P_4 increased greatly in spring coinciding with the last period of gestation and parturition. These two hormones reached their highest values in this period and were between three to four times higher in spring than in the rest of year (See Table S4, Fig. 3). The male reproductive hormone, T reached its maximum in spring before the rut season (Table S4, Fig. 3). CORT, N

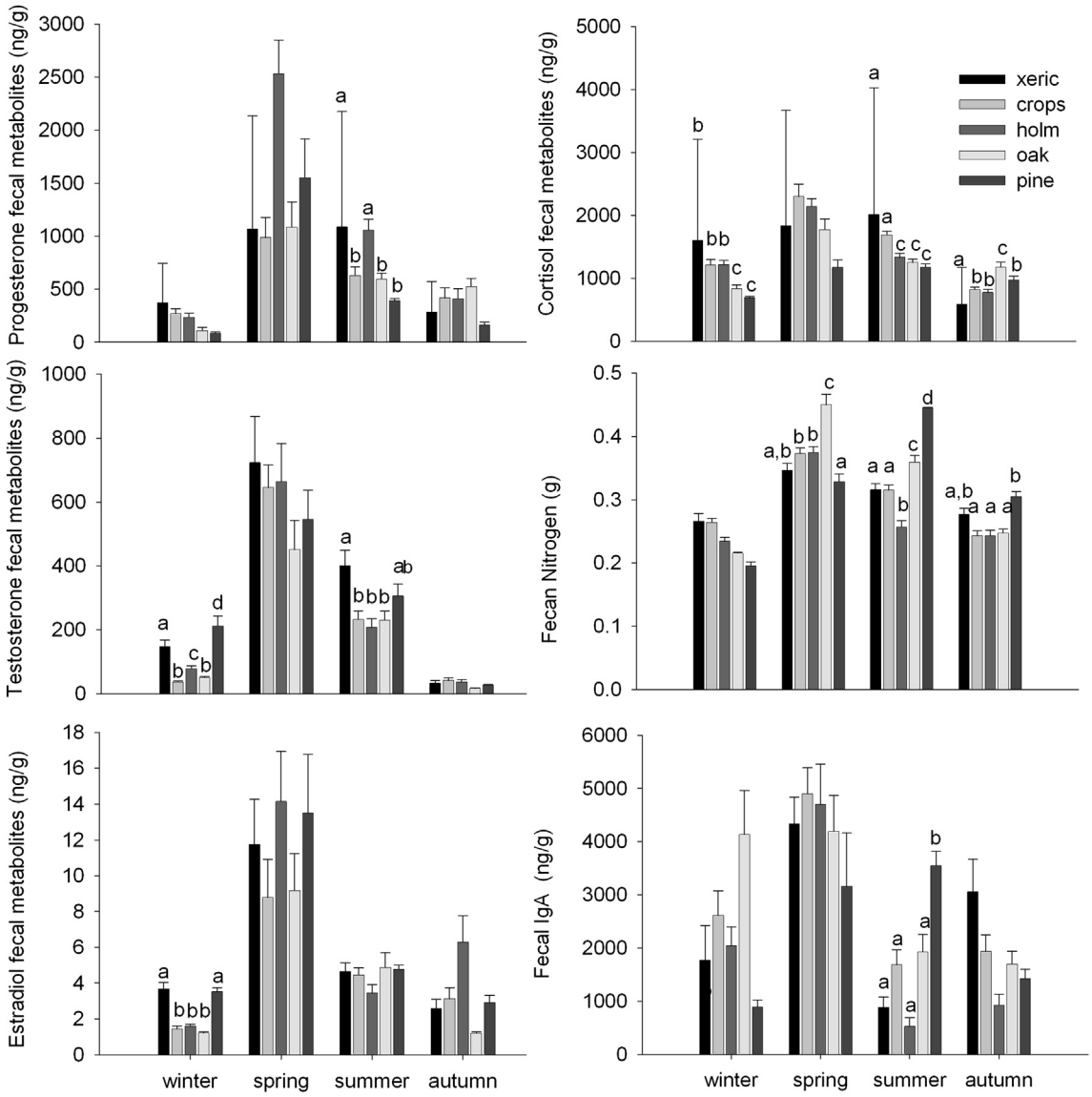

Fig. 3. Seasonal values for hormones metabolites, fecal nitrogen and IgA (average ± SE) for the five studied habitats in each season. Different letters denote significant differences among habitat types (*P-value*<0.05). If no letter is shown differences among habitats were not significant.

and IgA reached their maximum values in spring, while T, CORT, and IgA reached minimum values in autumn P_4, E_2, and N did in winter (Table S4, Fig. 3).

There were also some differences among habitats within seasons. P_4 levels were only influenced by habitat type in summer (See Table S5, Fig. 3). When habitat type had a significant effect on the physiological variables the significant differences between habitats are denoted by different letters in Fig. 3). P_4 maximum values were obtained for xeric and holm habitats while minimum values were obtained for pine (Fig. 3). E_2 levels were influenced by habitat type in winter and autumn (Fig. 3, Table S5). In winter E_2 levels in xeric and pine habitats were significantly higher than crops, holm and oak habitats while in autumn holm was the only habitat significantly different to all other habitat types (Fig. 3). T levels were influenced by habitat type in winter and summer (Fig. 3, Table S5). In winter maximum levels were reached in the most forestall habitats (in ranking position: pine, xeric, holm and oak, crops) while in summer the highest levels occurred in xeric and pine habitats which did not differ significantly and holm, oak and crops (Fig. 3). CORT levels and N were influenced by habitat type in all seasons except for summer and winter respectively. IgA levels were only influenced by habitat type in summer (Fig. 3, Table S5). CORT levels differed for each habitat and season combination. In general xeric habitat had the highest values, except for autumn in which oak habitat had the highest level of CORT. N also varied in each season and habitat combination, in summer and autumn the highest value of N was reached on pine habitats while in spring it was in oak habitats. IgA levels where maximum for pine habitats and non-significantly different for the rest of studied habitats (Fig. 3, Table S5).

3.3. Seasonal Principal Component Analyses

PCA of the physiological indicators for winter and spring produced three components which described 82 and 72% of the variance, respectively. The analyses for summer and autumn produced two components which described 66% and 60% of the variance, respectively (Table 1).

Table 1
Loading of the physiological indicators into components by PCA for each season.

Season	Indicator	C1	C2	C3
Winter	Eigenvalue	2.06	1.80	1.07
	CORT	−**0.57**	0.30	0.16
	P_4	−**0.56**	0.28	0.23
	E_2	−**0.47**	−0.36	−0.36
	T	−0.25	−**0.52**	−**0.47**
	N	−0.14	**0.49**	−**0.52**
	IgA	0.24	**0.43**	−**0.54**
	Variance (%)	34	64	82
Spring	Eigenvalue	1.79	1.38	1.18
	CORT	−**0.44**	0.23	**0.59**
	P_4	−**0.64**	−0.07	−0.12
	E_2	−**0.60**	−0.31	−0.25
	T	0.12	**0.77**	0.18
	N	0.06	−0.37	**0.60**
	IgA	0.04	−0.33	**0.42**
	Variance (%)	30	53	72
Summer	Eigenvalue	2.18	1.77	
	CORT	−**0.43**	−0.01	
	P_4	−**0.49**	0.30	
	E_2	−**0.50**	−0.32	
	T	−**0.51**	−0.29	
	N	0.06	−**0.62**	
	IgA	0.23	−**0.58**	
	Variance (%)	36	66	
Autumn	Eigenvalue	2.40	1.22	
	CORT	−0.28	−**0.42**	
	P_4	−**0.49**	−0.20	
	E_2	−**0.46**	**0.41**	
	T	−**0.48**	**0.43**	
	N	0.20	**0.64**	
	IgA	**0.43**	0.13	
	Variance (%)	40	60	

Physiological variables are abbreviated as follows: cortisol metabolites (CORT), progesterone metabolites (P_4), estradiol metabolites (E_2), testosterone metabolites (T), fecal Nitrogen (N), fecal immunoglobulin A (IgA). Component 1 (C1), component 2 (C2), component 3 (C3) The negative sign indicates a negative correlation between the variable and the component. Variables with loadings over 0.4 were used for component interpretation and are shown in bold.

Winter component 1 (WC1) accounted for 34% of the variance. Loadings of the variables in each component was interpreted according to Comrey and Lee (1992) as previously explained (See Statistical Analyses section). It was characterized by a good overlap between CORT and P_4, and a fair overlap with E_2. A strong positive correlation was found among all three variables, but they were negatively correlated to WC1 (Table 1). Hence, this component was negatively correlated to reproductive condition which coincides with pregnancy in winter. Winter component 2 (WC2) accounted for 30% of the variance. The variables which described this component were T, IgA and N. T had a good overlap and was negatively correlated to WC2, while N and IgA had a fair overlap and were positively correlated to WC2. Therefore, WC2 was negatively correlated to sexual expression but positively correlated to condition (nutritional and immunological), which suggests a trade-off between sexual expression and immune function. Winter component 3 (WC3) accounted for 18% of the variance. IgA, N and T had between a fair and good overlap and were negatively correlated to WC3. Hence, WC3 was negatively correlated to good condition (Table 1).

Spring component 1 (SC1) accounted for 30% of the variance and was characterized by P, E_2 and CORT which had an excellent, very good and fair overlap, respectively. All three variables were positively correlated to one another and negatively correlated to SC1 (Table 1). Thus, SC1 was negatively correlated to reproductive condition which coincides with births in spring. Spring component 2 (SC2) accounted for 23% of the variance and was characterized by T, which had an excellent overlap and positive correlation with SC2. Therefore, SC2 was positively correlated to male sexual expression. Spring component 3 (SC3) accounted for 19% of the variance and was characterized by N, CORT and IgA. N and CORT had a very good overlap, while IgA had a good overlap. All three variables were positively correlated to SC3. This component was positively correlated to nutritional condition, stress levels and immune function, which is considered a good condition for a reproductive period. Hence, SC3 was positively correlated to good condition (Table 1).

Summer component 1 (SUC1) accounted for 36% of the variance characterized by T, E_2, P_4 and CORT which had a fair overlap and negative correlation with SUC1 (Table 1). Hence, SUC1 was negatively correlated to reproductive condition, which coincides with the mating period in summer. Summer component 2 (SUC2) accounted for 30% of the variance characterized by N and IgA which had a good overlap and a negative correlation with C2. Hence, this component was negatively correlated to good condition.

Autumn component 1 (AC1) accounted for 40% of the variance and was characterized by P_4, E_2, T and IgA. The first three variables had a fair overlap and were negatively correlated to AC1, while IgA had a fair overlap and was positively correlated to AC1 (Table 1). As autumn is diapause time for the species, female reproductive hormones (P_4 and E_2) do not have a clear function. However, T is important for male roe deer in this season to develop the antlers. This component was related to the trade-off between sexual expression and immune function. Autumn component 2 (AC2) accounted for 20% of the variance and was characterized by N, T, E_2 and CORT. N, T and E_2 were positively correlated to one another and to AC2. N had a very good overlap, whereas T and E_2 had a fair overlap. CORT had negative correlation and a fair overlap with AC2. Hence, this component was related to good nutritional condition, good sexual expression, low stress levels and subsequently to overall good condition (Table 1).

In short, according to the physiological variables which best explained each component, these can be classified into three different groups. The first group includes reproductive variables and stress levels. The second group usually describes a trade-off between immune function and reproductive condition and the third group is related to immune function and nutritional condition.

3.4. Linear mixed model for PCA components, primary productivity (NDV) and habitat type

According to the linear mixed models, only sexual expression/immune function trade-off (WC2) in winter and good condition (SUC2) in summer were related to NDVI (Table 2). Physiological components were not related to NDVI in spring or autumn (Table 2). The trade-off between sexual expression and immune function was biased towards sexual expression in habitats with higher NDVI, while the immune function predominated in habitats with lower NDVI (*P-value* = 0.02, Fig. 4). Good condition in summer was positively related to NDVI (*P-value* = 0.01, Fig. 4). WC2 and SC2 were significantly affected by habitat type (WC2, $F_{4,11} = 3.88$, *P-value* = 0.033; SC2, $F_{4,13} = 8.1$, *P-value* = 0.002). Only the parameters for pine were significant in both habitat types while the parameter for the xeric and holm habitats were marginally significant in winter and summer respectively (See Table S.6).

4. Discussion

Measuring fitness in the field for medium–high mammals, of arround10 kg or more, is challenging, expensive, time-consuming and non-recommended for endangered species, when it implies trapping or animal handling (due to the stress suffered by the animals). Therefore it is essential to obtain reliable fitness indicators

Table 2
Linear Mixed Model on physiological components and NDVI.

Component		Value	Fixed Eff.		*t*-value	*P*-value	Random Eff.	
			SE	DF			Int.	Resd.
WC1	Intercept	1.46	1.41	126	1.03	0.330	0.83	1.24
	Estimate	−3.16	2.72	14	−1.16	0.260		
WC2		**3.74**	**1.47**	**126**	**2.54**	**0.012**	**0.91**	**0.90**
		−7.26	**2.82**	**14**	**−2.57**	**0.022**		
WC3		1.57	1.13	126	1.40	0.160	0.69	0.79
		−3.33	2.16	14	−1.53	0.150		
SC1	Intercept	0.08	1.24	88	0.07	0.950	0.65	1.22
	Estimate	−0.06	2.10	14	−0.03	0.970		
SC2		1.00	1.28	88	0.80	0.430	0.74	0.95
		−1.57	2.14	14	−0.73	0.470		
SC3		0.89	1.27	88	0.70	0.480	0.77	0.76
		−1.60	2.13	14	−0.75	0.460		
SUC1	Intercept	−1.3	0.89	138	−1.40	0.150	0.91	1.27
	Estimate	2.03	1.62	16	1.25	0.230		
SUC2		**2.03**	**0.81**	**138**	**2.5**	**0.013**	**0.88**	**0.7**
		−4.16	**1.5**	**16**	**−2.83**	**0.010**		
AC1	Intercept	−1.74	1.14	117	−1.52	0.130	0.89	1.27
	Estimate	2.89	1.91	16	1.50	0.150		
AC2		−0.59	0.94	117	−0.62	0.540	0.78	0.85
		0.91	1.60	16	0.57	0.570		

Winter component (WC), spring component (SC), summer component (SUC), autumn component (AC); Maximum values for a component are positive (+) or negative (−) as indicated by the corresponding sign between brackets. WC1: Pregnancy (−); WC2: Sexual expression (−)/Immune function (+) trade-off; WC3: Good condition (−); SC1: Births (−); SC2 Male sexual traits expression (−); SC3: Good condition (+); SUC1: Reproductive condition during mating (−); SUC2: Good condition (−) AC1: Sexual expression(−)/Immune function (+) trade-off; AC2: Good condition (+). Significant relationships between a component and NDVI ($p < 0.05$) are shown in bold.

that can be measured safely in broad spatial and temporal scales to predict how animal populations cope with their environment. The holistic physiological approach used here allowed us to determine reproductive hormone levels, nutritional condition and immune function obtained by means of non-invasive methods which were useful to validate the Cort-Fitness-Adaptation hypotheses. During the main reproductive periods (rut, pregnancy, parturition/lactation) CORT was a good proxy of fitness in agreement with the Cort-Adaptation Hypothesis while out off the reproductive periods CORT was negatively correlated to fitness in agreement with the Cort-Fitness hypothesis, even though some reproductive-related tasks were important. At the population-level winter and summer were the most demanding seasons in the studied environmental gradient. The former imposed a trade-off between sexual expression and immune function, while the latter limited roe deer condition in habitats with reduced primary productivity.

4.1. Validation of Cort-Fitness and Cort-Adaptation hypothesis at the individual level

Overall, reproductive condition was positively related to stress levels during the reproductive seasons, which agrees with our hypothesis that stress plays a positive role in the reproductive period. This is in agreement with the Cort-Adaptation hypothesis (Bonier et al., 2009, 2011). In the rut season (summer) all reproductive hormones were positively correlated to CORT, confirming the Cort-Adaptation hypothesis while CORT proved to be an indicator of roe deer fitness at the individual level during the rut. SC2 was correlated to nutritional and immunological condition; therefore this

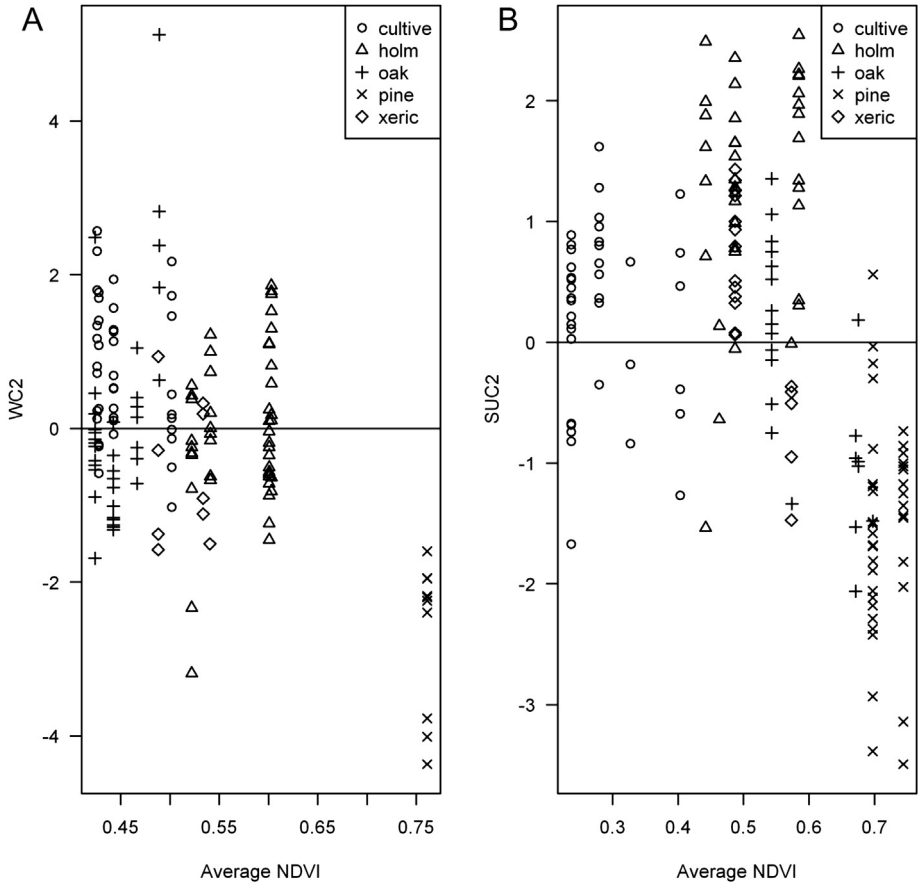

Fig. 4. Factorial coordinates of each population for winter and summer seasons as a function of NDVI. (A) Winter. High values on axis WC2 are associated with high levels of fecal nitrogen and IgA and low values of testosterone (trade-off between sexual expression and nutritional/immunological condition). (B) Summer. High values on axis SUC2 are associated with low levels of fecal nitrogen and IgA.

component was an indicator of good condition not related to reproductive condition. This implies that immune and reproductive function did not trade-off during rut season (McNamara and Buchanan, 2005; Sheldon and Verhulst, 1996) which occurred in winter (WC2) and will be discuss below.

In winter and spring, only female reproductive hormones (P_4 and E_2) were positively correlated to CORT which agrees with our predictions of the positive role of CORT in the accomplishment of reproductive tasks without compromising other relevant functions such as immunity. This was supported by SC3 in which higher levels of CORT were positively correlated to nutritional status and immune function. This pattern seems to be plausible only for females, as P_4 and E2 are mostly female hormones. In fact the main hormone for males, T, was only relevant in SC2 which it was not related to any other variable. We speculate it could be possible that higher values of SC2 could be separating males from females, however due to the anonymous sampling scheme followed it is not possible to know which samples belonged to each sex, consequently this should be considered with caution. In this sense, the application of molecular tools to determine the sex of samples would be very useful to better understand the relationship between CORT and sexual hormones in both sexes. However economical and logistical limitations could challenge the accomplishment of this task. For instance, different methods of sampling storage and homogenization are required to perform hormone and genetic analyses (hormones: freezing and complete homogenization (Sheriff et al. 2011); genetic: ethanol 96% or silica desiccation scat peeling (Flagstad et al., 1999; Wehausen et al., 2004).

Regarding the relationship between CORT and P_4 it should be noted that the immunoassays used to perform the hormone analyses of CORT cross react in 9% with P_4 (see Materials and Methods section) which could have influenced the correlations found between these two variables. Nevertheless, CORT and P_4 were correlated during female reproductive seasons (rut, gestation and parturition/lactation), as previously discussed, and were not significantly correlated during the diapause season in which roe deer females do not have to accomplish any reproductive task (autumn). Therefore, we consider our results to be reliable beyond the cross reactivity of the CORT immunoassay with P_4.

The pattern emerging during the no-reproductive season (autumn) seems to agree with our predictions and consequently with the Cort-Fitness hypothesis, as the nutritional condition was negatively correlated to CORT (AC2). We did not expect reproductive hormones to be significantly correlated to the rest of physiological variables in this season, as it is the embryonic diapause moment for females and males do not have to accomplish reproductive tasks (Mateos-Quesada, 2011). However, E_2 and T were positively and negatively correlated, respectively, with N and CORT. According to Mateos-Quesada (2011), roe deer in Mediterranean environments defend their territories during the whole year, not only when the reproductive season is approaching. This could explain the relevant role of T in our study areas in autumn. Similarly, female reproductive hormone levels were similar in winter and autumn. With autumn being the period of diapause for the species, no reproductive. Although E_2 seems to be involved in finalizing diapause and reactivating the implantation of the embryo (Lambert et al., 2001), the mechanism(s) in such a regulation which would be the whole process of delayed implantation in roe deer is still not fully understood. We speculate that reproductive hormones could be involved in such a regulation and that could be the explanation of the similar levels of female reproductive hormones found during winter and autumn. Certainly, additional research is needed to fully understand the relationship of reproductive hormones and the mechanisms regulating delayed implantation in roe deer.

4.2. Sexual expression traded off against immune function in conditions of reduced primary productivity

In conditions of limited resource availability (e.g. primary productivity), essential functions should be prioritized to maximize fitness (McNamara and Buchanan, 2005). In this study we found a trade-off between sexual expression and immune function mediated by food resources in winter. Roe deer populations inhabiting more productive habitats allocated resources to reproductive condition, while roe deer inhabiting less productive habitats allocated resources to immune function. One possible explanation for these results is that roe deer living in more productive habitats need to allocate resources to reproductive condition to maintain territories and develop bigger antlers (Gómez et al., 2006). This is most likely due to higher competition amongst males to maintain the territory in more productive habitats. In contrast, it seems that immune function is more essential for roe deer survival in less productive habitats. Nevertheless, roe deer living in more productive habitats are expected to live at higher densities, which may imply greater exposure to parasites leading to a high investment in immunity. However, densities in Mediterranean populations are usually lower than in more northern environments (28 inds/100 ha respect to 6 inds/ha; Mateos-Quesada, 2011) and may not be so dense as to have a high risk of parasite infections. An alternative explanation could be that animals inhabiting poorer habitats are not able to invest in reproductive condition, and therefore, more resources are available for the immune function independent of the risk of parasite infection. In any case, the relationship between energetic costs, testosterone, immune function and net fitness seems to be complex (Ezenwa et al., 2012; Ruiz et al., 2010). More studies in which risk of parasite infection together with immune function, reproductive performance and net fitness in different conditions of food availability are needed to clarify the optimal mechanisms which reduce energetic costs and maximize fitness in the long term (Speakman, 2008).

4.3. Primary productivity in summer but not in spring is related to roe deer condition in Mediterranean environments

In a Mediterranean environment in France, Pettorelli et al. (2006) found that INDVI in spring (the sum of NDVI throughout a period) had a significant positive effect on winter body mass of roe deer fawns. The non-significant differences between physiological components in relation to primary productivity found in our study suggest that primary productivity across all habitats was high enough to allow roe deer populations to perform their reproductive tasks- births and lactation, adult male defense of territories (Mateos-Quesada, 2011) and juvenile male start of dispersal (Bubenik et al., 1996). Reproduction is a key process in maintaining populations and permitting their expansion. As roe deer have been expanding in the Iberian Peninsula for the last two decades (Acevedo et al., 2005; Tellería and Virgós, 1997; Virgós and Tellería, 1998), reproduction does not seem to be limited in these environments.

A positive correlation was found between primary productivity and condition in summer. Oak and pine habitats (more productive, humid and cooler) had more populations in good condition, while most populations in xeric, holm and crop habitats (less productive, more xeric and higher temperatures) were in worse condition. In the aforementioned study (Pettorelli et al., 2006), the authors found a positive correlation between winter fawn body mass and bimonthly composite NDVI in August. Another study conducted in the same area found that the body mass and size of fawns was correlated to higher precipitation and lower temperatures in summer (Wahlstrom and Kjellander, 1995). These findings agree

with the maladaptation suggested for this species to xeric Mediterranean environments and its typical summer drought which result especially harsh for the species at the local scale as shown by our results and seems to be an important feature at the regional scale, as the south boundary range of the species is located in the Iberian Peninsula (Tellería and Virgós, 1997).

5. Conclusions

Our results confirm both the Cort-Fitness and Cort-Adaptation hypotheses, consequently, we consider the use of GC together with reproductive hormone levels to be a reliable indicator of fitness as long as the reproductive and non-reproductive seasons are evaluated. We encourage researchers to supplement stress and reproductive measures with other fundamental physiological functions such as immune function and nutritional status, as this information has proven to be very useful in understanding the holistic functioning of the physiological system in roe deer. The holistic physiological approach and the consideration of food resources (primary productivity) in different habitats allowed us to detect that winter and summer are the most limiting seasons in Mediterranean environments for roe deer. Therefore, we suggest that managers should try to avoid carrying out stressful tasks during these periods (e.g. hunting). We also demonstrated that the relationship and trade-offs among physiological indicators change according to food resources and seasons. Hence, researchers and managers should avoid reaching general conclusions and carrying out management tasks based only on physiological studies conducted in a specific period or habitat for seasonal species which occupy wide environmental gradients.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.actao.2013.08.003.

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