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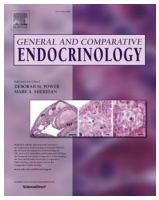

Age and spatio-temporal variations in food resources modulate stress-immunity relationships in three populations of wild roe deer

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ABSTRACT

Living in variable and unpredictable environments, organisms face recurrent stressful situations. The endocrine stress response, which includes the secretion of glucocorticoids, helps organisms to cope with these perturbations. Although short-term elevations of glucocorticoid levels are often associated with immediate beneficial consequences for individuals, long-term glucocorticoid elevation can compromise key physiological functions such as immunity. While laboratory works highlighted the immunosuppressive effect of long-term elevated glucocorticoids, it remains largely unknown, especially in wild animals, whether this relationship is modulated by individual and environmental characteristics. In this study, we explored the co-variation between integrated cortisol levels, assessed non-invasively using faecal cortisol metabolites (FCMs), and 12 constitutive indices of innate, inflammatory, and adaptive immune functions, in wild roe deer living in three populations with previously known contrasting environmental conditions. Using longitudinal data on 564 individuals, we further investigated whether age and spatio-temporal variations in the quantity and quality of food resources modulate the relationship between FCMs and immunity. Negative covariation with glucocorticoids was evident only for innate and inflammatory markers of immunity, while adaptive immunity appeared to be positively or not linked to glucocorticoids. In addition, the negative covariations were generally stronger in individuals facing harsh environmental constraints and in old individuals. Therefore, our results highlight the importance of measuring multiple immune markers of immunity in individuals from contrasted environments to unravel the complex relationships between glucocorticoids and immunity in wild animals. Our results also help explain conflicting results found in the literature and could improve our understanding of the link between elevated glucocorticoid levels and disease spread, and its consequences on population dynamics.

1. Introduction

The neuroendocrine stress response helps animals to cope with recurrent stressful situations in natural environments (Sapolsky et al., 2000; Wingfield and Romero, 2001). Exposure to stressors stimulates the hypothalamic–pituitaryadrenal (HPA) axis and leads, among others, to an increase in the secretion of glucocorticoids by the adrenocortex (Sapolsky et al., 2000; Sheriff et al., 2011). Glucocorticoids, which

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contribute to the control of an individual's energy balance through acquisition, storage and mobilization, also coordinate the body's overall response to stressors through metabolic changes that depend on their concentration and duration of secretion (Hau et al., 2016). This hormonal cascade is then subjected to negative feedback from glucocorticoids on their own secretion (Romero, 2004; Sheriff et al., 2011), facilitating a return to a baseline level that ensures maintenance of daily activities according to the current life-history stage (Möstl and Palme, 2002; Wingfield and Sapolsky, 2003). While short-term elevation of glucocorticoid levels promotes survival (Sapolsky et al., 2000; Breuner et al., 2008), chronic elevation of glucocorticoids may alter physiological functions and ultimately compromise both survival and reproductive success in the long run (Boonstra, 2005). Among these functions, the potential immunosuppressive action of glucocorticoids is one of the most discussed effects of chronic stress (Martin, 2009).

Immunity, which helps to reduce disease susceptibility, is a key physiological function of vertebrates: it includes innate and adaptive components (Stanley, 2002), each comprising cellular and humoral effectors (Stanley, 2002). While innate immunity sets up rapidly (within hours) and is mostly non-specific, adaptive (memory-based) immunity deals with repeated infections and selectively eliminates pathogens (Lee, 2006). Like any other physiological function, the maintenance and functioning of the immune system requires energy (Lee, 2006; Martin, 2009). Hence, immunity has been hypothesised to trade-off against other energy demanding physiological functions (Martin, 2009). Glucocorticoids may mediate these trade-offs, with elevation of these hormones redirecting the energy away from immunity towards functions promoting immediate survival but with deleterious effects in the long term (Lee, 2006; Martin, 2009). However, energy costs vary depending on the stage (development, maintenance, or activation) and component of immunity (Klasing, 2004, Lee, 2006). The adaptive immune response is thought to have a higher development cost, but lower maintenance and activation cost than the innate immune response, the inflammatory response entailed by innate response being particularly energy demanding (McDade et al., 2016). Moreover, energy saving is probably not the only mechanism explaining trade-offs among immune traits and with other traits (Eraud et al., 2005; Lee, 2006). Therefore, differential effects of trade-off mediated by glucocorticoids can be expected between the components of immunity.

The commonly accepted finding is that chronically elevated glucocorticoid levels are immunosuppressive (Sapolsky et al., 2000; Dhabhar, 2009), while transient response levels can enhance immune functions (Dhabhar and McEwen, 1997; Shini et al., 2010; Koutsos and Klasing, 2014; Dhabhar, 2014), in both innate and adaptive arms (Dhabhar, 2002). In response to an acute stressor, glucocorticoids elevation has been shown to induces the trafficking of immune cells (e.g., neutrophils and lymphocytes) out of compartments such as the spleen, bone marrow, and lymph nodes into the blood, to ultimately reach target organs where an individual is most likely to be injured (e.g. skin, gastrointestinal tract, or lungs) (Dhabhar et al., 2012). So far, most empirical studies documenting a detrimental effect of chronic stress on immune functions have been conducted on laboratory or domestic animals (e.g. Dhabhar et al., 1994; Dhabhar et al., 1995; Wada et al., 2010; Moazzam et al., 2012) and much less studies have been conducted in wild populations (e.g. Bourgeon and Raclot, 2006; Brooks and Mateo, 2013; Josserand et al., 2020).

In wild populations, exposure to fluctuating environmental conditions is more intense than in captivity in terms of resources, variation of temperature, predator risk or disease threats for instance, which could lead to different outcomes regarding the relationship between glucocorticoids and immunity. In addition, a large body of literature shows that diverse internal and external factors could influence both immunity (Lee, 2006; Martin, 2009) and glucocorticoid secretion (Hau et al., 2016). For instance, poor environments (in terms of resource quantity or quality) can lead to both increased glucocorticoid levels (Fokidis et al., 2012; Carbillet et al., 2020) and decreased concentrations of immune parameters (Dhabhar et al., 1994; Dhabhar et al., 1995), without any causal relationships. It has also been shown that baseline glucocorticoid levels are related to age (Sapolsky et al., 1983) as are several immune parameters (e.g. immunosenesence, see Nussey et al., 2012; Cheynel et al., 2017). To date, the contribution of these individual and environmental factors has not been investigated and their role in modulating the relationship between immunity and glucocorticoids remains poorly understood.

The aim of this study was to analyse whether the correlatation between integrated glucocorticoid levels and immunity is modulated by both individual and environmental factors in three wild populations of roe deer (*Capreolus capreolus*) living in habitats with contrasting environmental conditions. Integrated glucocorticoid levels were assessed non-invasively by measuring faecal cortisol metabolites (FCMs), which reflect overall circulating glucocorticoid levels that an individual has experienced over a particular time-period which is species-specific (Palme et al., 2005; Sheriff et al., 2011; Palme, 2019). The immune function was assessed to the same years as FCMs by measuring twelve immune parameters encompassing the innate (neutrophils, eosinophils, basophils, monocytes, hemagglutination, hemolysis), inflammatory (alpha-1-globulin, alpha-2-globulin, beta-globulin, haptoglobin) and adaptive (lymphocytes, gamma-globulin) immunity (Cheynel et al., 2017).

Based on current knowledge, we expected that individuals with higher integrated glucocorticoid levels would exhibit weaker overall immunity than those with lower integrated glucocorticoid levels. More precisely, we expected (prediction 1) that high levels of FCMs would be associated with low levels of parameters measuring innate (cellular and humoral) immunity (neutrophils, eosinophils, basophils, monocytes, hemagglutination, hemolysis), as well as inflammation (alpha-1-globulin, alpha-2-globulin, beta-globulin and haptoglobin) due to their high activation cost (Dhabhar et al., 2012; Brooks and Mateo, 2013; McDade et al., 2016). In contrast, we expected a weaker relationship between FCMs and cellular and humoral adaptive immune parameters (lymphocyte and gamma-globulin concentrations) due to their lower activation cost (Klasing, 2004, Lee, 2006). In addition, we expected (prediction 2) that the negative relationship between FCMs and immune parameters would appear, or be stronger, in individuals facing poor environmental conditions such as low availability in food resources, compared to individuals living in favourable environments (Hau et al., 2016). Finally, we expected (prediction 3) that the negative relationship between FCM levels and immune parameters would appear, or be stronger, in older individuals compared with younger ones, due to impairment of both the adrenocortical stress response (Sapolsky et al., 1983) and the immune response in old individuals (Cheynel et al., 2017).

2. Material and methods

2.1. Study populations

This study was conducted on three wild populations of roe deer living in contrasting habitats.

First, the Trois-Fontaines population is located in an enclosed forest (1,360 ha) in the north-east of France (48°43′ N, 4°55′ E). The climate is continental, and the soil is particularly rich, making it a very productive forest offering a homogeneous and high-quality resources habitat for roe deer (Pettorelli et al., 2006). Second, the Chizé population is located in an enclosed forest (2,614 ha) in western France (46°50′ N, 0°25′ W). The climate is temperate oceanic, with Mediterranean influences. Due to poor soil quality and frequent summer droughts, the forest productivity is low compared to Trois-Fontaines (Pettorelli et al., 2006), making it a relatively poor-quality habitat for roe deer in terms of resources (Gaillard et al., 1993, Gaudry et al., 2018). At a finer scale, three sectors are distinguished in this population according to the quantity and quality of resources (Pettorelli et al., 2001). Sector 1 is composed of oaks (*Quercus*

spp.) and hornbeams (*Carpinus betulus*) and is considered to be of better quality than the other two sectors, sector 2 is composed of oaks and Montpellier maples (*Acer monspessulanum*) and is considered to be of intermediate quality, and sector 3, composed of beeches (*Fagus sylvatica*) is the sector of worse quality. Pettorelli and colleagues (2003) reported that these differences in habitat composition result in differences in roe deer body mass, with juveniles in sector 1 being on average 2 kg heavier than those in sector 3. Finally, the Aurignac population is located in an agricultural landscape (10,000 ha), in south-western France (43°13′ N, 0°52′ E) and is part of a Long-Term Socio-Ecological Research platform called ZA PYGAR. This site is exposed to an oceanic climate, with summer droughts (Hewison et al., 2007). It provides a highly heterogeneous environment with a fragmented landscape composed of forests, grassland and cultivated fields (see Martin et al., 2018 for details). This study site provides overall high-quality habitat for roe deer (as Trois-Fontaines) but can also be divided into three sectors according to resource quality and habitat openness (see Morellet et al., 2009 for details). The most open habitats (sector 1) offer more important and high-quality food resources for roe deer during most of the year (Abbas et al., 2011), but can also be a source of higher exposure to stressors (Bonnot et al., 2013), such as road and human dwellings leading to higher FCM levels (Carbillet et al., 2020), compared to the partially wooded area (sector 2) and woodland (sector 3). Hewison and colleagues (2009) have shown a beneficial effect of landscape openness on roe deer reproduction, demographic performance, and juveniles body mass, with juveniles in sector 3 weighing on average 2.0 kg less than in sector 2, and 3.1 kg less than in sector 1 (Hewison et al., 2009).

2.2. Data collection

As part of long-term capture-mark-recapture programs initiated in 1975, 1977 and 1996, in Trois-Fontaines, Chizé and Aurignac respectively, 6 to 12 days of drive net captures are organised between December and March each year. At each capture session, between 30 and 100 beaters push roe deer towards nets deployed over 4 km. Once captured, each animal is marked, weighed (to the nearest 100 g), sexed, and age is determined by tooth eruption patterns in Aurignac (Hewison et al., 1999), with 2 age classes: juveniles (<1 year) and adults (>1 year). For the Trois-Fontaines and Chizé populations, the exact age (in years) is known since the individuals were all captured and marked during their first year of life when the age can be estimated without error. Blood samples are taken from the jugular vein (up to 1 mL/kg), a drop is used to realise a blood smear, either immediately after sampling (Aurignac) or on arrival at the lab (Trois-Fontaines and Chizé), and part of the collected blood is transferred to a dry tube, centrifuged, and the serum conserved at −20 °C for biochemical analyses. The remaining blood is transferred to a tube containing ethylenediaminetetraacetic acid (EDTA) for further determination of immune parameters (see below). For each individual, faeces are collected rectally and stored at −20 °C until extraction. For the Trois-Fontaines and Chizé populations, blood was maintained in a cooler box with freeze-dry ice packs upon arrival to the lab and realisation of blood smears (Corrons et al., 2004).

Due to conservation issues of faeces, the available data encompassed different year ranges according to the population. Data were available for the years 2010 to 2019 in Trois-Fontaines, while only the years 2013, 2014 and 2016 to 2019 were available in Chizé. For the Aurignac population, neutrophils, esosinophils, basophils, monocytes and lymphocytes concentrations were available for the years 2012 to 2017. For hemagglutination and hemolysis data were available from 2013 to 2017, while for gamma-, alpha-1-, alpha-2- and beta-globulin, data were available for the years 2014 to 2017. Consequently, the number of samples differed according to the population and immune marker considered.

2.3. Measurement of FCMs

Faecal cortisol metabolites were extracted at the University of Veterinary Medicine in Vienna, following the protocol developed by Palme and colleagues (2013). Briefly, for each individual, 0.5 g of faeces were suspended in 5 mL of 80% methanol, vortexed for 30 min and centrifuged for 15 min at 2500*g*. The supernatants were diluted 1:10 with assay buffer before FCM concentrations were determined with a group-specific 11-oxoaetiocholanolone enzyme immunoassay as previously described (Möstl et al., 2002) and validated for roe deer (Zbyryt et al., 2017). Measurements were carried out in duplicate. Intra-assay coefficients of variation were below 10%, and inter-assay coefficients for a low and high concentration pool sample were 11.7% and 13.9%, respectively. Results are expressed as nanograms of FCMs per gram of wet faeces (ng/g).

2.4. Measurement of immune parameters

2.4.1. Innate cellular immunity

White blood cells (WBC) counts were carried out by impedance technology (ABC Vet automaton, Horiba Medical) and the proportions of each WBC type (neutrophils, monocytes, lymphocytes, eosinophils and basophils) were quantified under microscope (x1000) by counting the first 100 WBC on blood smears, previously stained with a May-Grünwald and Giemsa solution (see Houwen, 2001 for more details). The concentrations of each type of leukocyte were then determined as (WBC*parameter cells count/100). The concentrations of neutrophils, eosinophils, basophils and monocytes were considered as measures of innate immunity, while lymphocyte concentration was used as a measure of adaptive immunity as counts include both T and B cells, the latter being particularly involved in the production of antibodies and thus in adaptive responses (Demas et al. 2011).

2.4.2. Innate humoral immunity

Innate humoral immunity was assessed by measuring circulating levels of natural antibodies (NAbs) and complement activity of serum. The concentration of NAbs was measured by the hemagglutination test (HA) that measures NAbs ability to agglutinate exogenous cells (Matson et al., 2005). The complement is a group of proteins that acts in a chain reaction and causes lysis of exogenous cells in presence of an antigen–antibody complex. It is revealed by the ability of proteins to induce hemolysis (HL) (Matson et al., 2005). The HA/HL protocol developed by Matson et al. (2005) for birds has been previously adapted for roe deer (Gilot-Fromont et al., 2012). Positive and negative controls were present on each plate, as proposed by Matson et al. The positive control serum was issued from a rabbit experimentally immunized against chicken erythrocytes, with serum diluted 1/10.

2.4.3. Inflammatory markers

Inflammatory status was evaluated using levels of alpha-1, alpha-2, and beta globulins from serum samples (Petersen et al. 2004). The total concentrations of these proteins (in g/L) were quantified by a refractometer followed by an electrophoresis on agarose gel, using an automaton (HYDRASYS). Haptoglobin concentration (in mg/mL), which belongs to the alpha-2-globulin fraction and reflect infection or chronic inflammation, was also measured. Analyses were performed with a Konelab 30i PLC (Fisher Thermo Scientific) which operates on the principle of spectrophotometry. Due to project organization, haptoglobin was measured on the Trois-Fontaines and Chizé samples only.

2.4.4. Adaptive cellular immunity

Adaptive cellular immunity was assessed using lymphocyte concentration, determined by the leukocyte count described above, which includes both B and T lymphocytes.

2.4.5. Adaptive humoral immunity

The adaptive humoral immunity component was assessed using concentration of gamma globulins, obtained by the protein analysis described above for other globulins. Gamma globulin concentration has been used as an estimator of total antibodies, since they are essentially composed of circulating antibodies (Stockham and Scott, 2008).

2.5. Statistical analyses

In Aurignac, analyses were carried out on 144 to 188 observations, while they were conducted on 325 to 414 in Chizé, and 276 to 303 in Trois-Fontaines. Overall, we implemented 35 series of models, including 12 series for the populations of Trois-Fontaines and Chizé and 11 series for Aurignac (due to the absence of haptoglobin assays).

For each of the 12 immune parameters, we ran separate analyses for each of the three populations, using linear mixed effect models (LMMs). Each immune trait was taken as a response variable and some of them (concentrations of eosinophils, basophils, monocytes, lymphocytes, haptoglobin, alpha-1, alpha-2 and beta globulins) were transformed as $\log(x + 1)$ to ensure normality of model residuals. Model selection was carried out by adjusting a reference model (see below) that included all biologically relevant variables and interactions to test our hypotheses. We then compared this model with all its sub-models. Each continuous explanatory variable (except for age) was centred by population to obtain estimates corresponding to average values of the parameters in the corresponding population. The reference model included:

i) Individual variables: integrated cortisol level (FCMs), log-transformed and centred around the mean (mean value of the variable that is subtracted from every value), sex, body weight (in kg, centred), and age. In Aurignac, age was considered with two classes (juveniles and adults). In Chizé and Trois-Fontaines, age in years was considered to have either a linear effect, a quadratic function, or a threshold effect, based on a previous study carried out on these two populations (Cheynel et al., 2017). Body weight was used as an indicator of individual quality (sensu Wilson & Nussey, 2010), as Toïgo et al. (2006) demonstrated that it is the most relevant estimate of body condition in roe deer.

ii) Environmental variables: in Chizé and Aurignac, we considered the sector of capture as a marker of local resources quality and quantity. To account for temporal variations of resources among years, we used a centred year quality index. The quality of a given year was indexed using the average weight (in kg, centred) of juveniles caught the following winter (Pettorelli et al., 2003).

iii) Methodological variables: the time between capture and blood sampling is known to influence the level of certain immune parameters such as neutrophils and lymphocytes (Carbillet et al., 2019) and was thus taken into account (thereafter, delay, in minutes, centred). In addition, we included the Julian date of capture in our models to control for potential among-individual differences in immune parameters, body weight and FCMs due to the timing of sampling.

Several plausible interactions based on our hypotheses were also included in our reference model. First, the interaction between FCMs and age, to investigate a possible modification of the relationship with advancing age (prediction 3). Second, we considered interactions between FCMs and sector, and between FCMs and quality of the year, to account for a potential modulation of the relationship under poorer environments or years of poorer quality (prediction 2). The interaction between FCMs and sex was also included to control for a possible modification of the relationship according to sex, as females generally allocate much more to immunity than males in the wild (Metcalf et al., 2020). We also included an interaction between FCMs and body weight to control for a possible modification of the relationship in individuals with the poorest physical condition (Hau et al., 2016). Finally, an interaction between sex and age was also included in neutrophil models for the Trois-Fontaines and Chizé populations because a previous study on roe deer from these populations showed that neutrophil profile was affected by age in a sex-specific manner (Cheynel et al., 2017). This interaction was also included in the Aurignac population for all immune parameters, as no previous data was available on the sex-specific effect of age on other parameters in this population. Individual's identity and year of capture were included as random effects to avoid pseudo-replication problems (Hurlbert, 1984) and to control for unexplained variance due to among-individual differences and inter-annual variation. During the study, the average number of capture events per individual was 1.13, 1.64, 1.37, for Aurignac, Chizé and Trois-Fontaines, respectively. Specifically, individuals were captured between 1 and 5 times in Chizé and Trois-Fontaines, and between 1 and 3 times in Aurignac. For the Chizé and Trois-Fontaines populations, the birth cohort is known and was also included as a random effect to account for potential differences between individuals in the environmental conditions they experienced early in life, which may have persistent effects on their phenotype (Douhard et al., 2014).

As a result, our (general) reference models read as follow:

Immune parameter = f(FCMs + year quality + sector + sex + age + weight + delay + julian date + FCMs*sector + FCMs*year quality + FCMs*sex + FCMs*age (either linear, quadratic or threshold) + FCMs*weight + 1|individual + 1|year of capture + 1|birth cohort).

To select the best models describing variation of each immune parameter, each reference model was compared to all its sub-models (N = 358 models overall) using a model selection method based on the second-order Akaike's information criteria (AICc, Burnham and Anderson, 2003). Models with a difference in AICc (ΔAICc) > 2 units from the best model were considered to have less support, following Burnham and Anderson (2003). In addition, we removed models within two AICc units of the top model that differed from a higher-ranking model by the addition of one or more parameters. These were rejected as uninformative, as recommended by Arnold (2010) and Richards (2008). Using the selected models, we then applied a conditional model averaging procedure to estimate parameters. We calculated AICc weights (AICcw) to measure the relative likelihood that a given model was the best among the set of fitted models and goodness-of-fit was assessed by calculating marginal (R2m; variance explained only by fixed effects) and conditional (R2c; variance explained by the entire model) variance using the r.squaredGLMM function of the MuMIn package (Barton, 2016). We used p-value only for interpretation of the F-test conducted in order to test prediction 1 of a relationship between FCMs and immunity. This was necessary only when interactions between FCMs and age, year quality, sectors, body weight, or sex were retained by the model selection procedure and interaction parameters did not overlap 0. In this case, the marginal effect of each variable is not estimated by the model and must be tested separately. For each parameter in each population, the normality of residuals was tested (Shapiro-Wilk test) and visually assessed. All analyses were performed using R version 3.5.1 (R Development Core Team 2018) and using the lmer function (lme4 package, Bates et al., 2014) and the dredge function in order to generate a model selection table of models (MuMIn package, Barton, 2016).

3. Results

The text below describes the covariations between FCMs and immune parameters, and their variations according to the availability and quantity of food resources, age, sex, and body weight of roe deer (Table 1, 2 & 3). The full results of the model selection procedure are given in Tables S1, S2 and S3. A summary of these results is provided in Table S4. Correlation matrix between each immune parameters for each population are provided in Table S5.

3.1. Innate cellular immunity

As expected from prediction 1, negative covariations between FCMs and cellular concentrations were observed for neutrophils in Aurignac (slope of −0.51; CI = [−0.94; −0.08]; Fig. 1A) with a similar trend in

Table 1
Parameters of linear mixed-effect models selected for each immune parameters of the Aurignac population. R2m and R2c correspond respectively to the marginal and conditional variance explained by the model, CI corresponds to the upper and lower limits of the 95% confidence interval, and n represents the number of observations per analysis. Variables in bold correspond to variable of primary interest in our study. W + stands for importance scores of variables and is calculated by summing the Akaike weights across all the selected models including the target variable. See the material and methods section for a full definition of model sets and explanation regarding the difference in the number of observations.

Immune trait	Parameter	Estimate	CI	W+
Innate immunity				
Neutrophils (n = 188) $R^2m = 0.07$; $R^2c = 0.71$	Intercept	6.07	5.75 to 6.39	
	Delay	0.006	0.002 to 0.01	1.00
	FCMs	**−0.51**	**−0.94 to −0.08**	**1.00**
Eosinophils (n = 185) $R^2m = 0.12$; $R^2c = 0.46$	Intercept	0.05	0.04 to 0.07	
	Age (juvenile)	−0.03	−0.06 to −0.0001	0.43
	Julian date	0.001	0.0003 to 0.002	1.00
	Delay	−0.0002	−0.0003 to −0.00002	1.00
	Weight	−0.005	−0.009 to −0.0008	0.43
	Year quality	−0.05	−0.08 to −0.01	1.00
	Sex (male)	−0.02	−0.04 to 0.003	0.33
Basophils (n = 188) $R^2m = 0.04$; $R^2c = 0.32$	Intercept	0.05	0.03 to 0.07	
	Delay	−0.0001	−0.003 to 0.00001	0.75
	Sector (2)	0.01	−0.01 to 0.04	0.41
	Sector (3)	0.03	0.003 to 0.06	
Monocytes (n = 187) $R^2m = 0.01$; $R^2c = 0.01$	Intercept	0.11	0.10 to 0.12	
	Year quality	−0.04	−0.09 to 0.007	0.59
Hemagglutination (n = 168) $R^2m = 0.12$; $R^2c = 0.74$	Intercept	3.64	3.15 to 4.16	
	FCMs	**−0.15**	**−0.37 to 0.08**	**0.64**
	Year quality	−1.54	−3.49 to 0.39	0.64
	FCMs*Year quality	**0.96**	**0.07 to 1.86**	**0.64**
Hemolysis (n = 170) $R^2m = 0.31$; $R^2c = 0.66$	Intercept	2.89	2.20 to 3.58	
	FCMs	**−0.19**	**−0.43 to 0.05**	**0.54**
	Year quality	−3.12	−5.84 to −0.41	1.00
Inflammatory markers				
Alpha-1 globulins (n = 146) $R^2m = 0.35$; $R^2c = 0.38$	Intercept	1.40	1.36 to 1.44	
	Julian date	0.002	0.0003 to 0.003	1.00
	FCMs	**0.03**	**−0.01 to 0.07**	**1.00**
	Sector (2)	−0.03	−0.08 to 0.01	1.00
	Sector (3)	−0.07	−0.12 to −0.02	
	Weight	−0.02	−0.03 to −0.01	1.00
	Sex (Male)	0.07	0.03 to 0.11	1.00
	FCMs*Sector (2)	**0.06**	**−0.01 to 0.13**	**0.63**
	FCMs*Sector (3)	**−0.03**	**−0.11 to 0.04**	
	FCMs*Weight	**−0.006**	**−0.01 to 0.002**	**0.40**
	FCMs*Sex (male)	**−0.05**	**−0.11 to 0.01**	**0.21**
Alpha-2 globulins (n = 144) $R^2m = 0.35$; $R^2c = 0.38$	Intercept	1.67	1.64 to 1.70	
	Julian date	0.0008	−0.0002 to 0.002	0.55
Beta globulins (n = 147) $R^2m = 0.10$; $R^2c = 0.13$	Intercept	1.95	1.91 to 1.99	
	Age (juvenile)	−0.16	−0.25 to −0.07	1.00
	FCMs	**0.03**	**−0.009 to 0.08**	**0.52**
	Weight	−0.02	−0.03 to −0.004	1.00
Adaptive immunity				
Gamma globulins (n = 147) $R^2m = 0.24$; $R^2c = 0.72$	Intercept	13.16	12.05 to 14.28	
	Delay	−0.006	−0.01 to 0.0003	0.34
	Age (juvenile)	−3.47	−5.12 to −1.81	1.00
	FCMs	**1.58**	**0.40 to 2.76**	**1.00**
	Sector (2)	−1.13	−2.31 to 0.05	1.00
	Sector (3)	−0.52	−1.88 to 0.85	
	Weight	−0.33	−0.56 to −0.10	1.00
	Year quality	1.75	0.20 to 3.30	0.66
	Sex (male)	0.47	−0.62 to 1.55	1.00
	FCMs*Sector (2)	**−2.68**	**−4.38 to −0.98**	**1.00**
	FCMs*Sector (3)	**−2.11**	**−3.87 to −0.35**	
	FCMs*Sex (male)	**−2.10**	**−3.62 to −0.57**	**1.00**
Lymphocytes (n = 188) $R^2m = 0.24$; $R^2c = 0.72$	Intercept	1.28	1.21 to 1.35	
	Weight	−0.02	−0.03 to −0.01	1.00
	Sex	−0.07	−0.14 to −0.002	0.73

Chizé (slope of −0.26; CI = [−0.54; 0.03]). A trend for a negative covariation between FCMs and monocytes was also observed in Chizé (F = 2.92; p = 0.09; slope of −0.04 for average quality food resources) and in Trois-Fontaines (slope of −0.06; CI = [−0.13; 0.009]). However, in Chizé only, the negative covariation was buffered in years offering better quality food resources (slope of 0.06; CI = [0.003; 0.11]; Table 2; Fig. 2A), as expected from prediction 2. In Chizé, whereas basophil concentration was negatively related to FCMs levels in general (F = 6.71; p = 0.01), this particularly applied to areas of intermediate (sector 2: slope of −0.02; CI = [−0.04; −0.0002]) and poor quality (sector 3: slope of −0.03; CI = [−0.06; −0.004]), while the covariation was close to 0 in the area of best quality (Table 2; Fig. 3A; slope of −0.002; CI = [−0.01; 0.01]). We found no significant association between eosinophil concentrations and FCMs in any of the populations, and no interaction between FCMs and age (prediction 3) on any of the innate immune parameters measured (Table 1, 2, 3).

3.2. Innate humoral immunity

In Aurignac, the overall covariation between FCMs and hemagglutination titers was not significant (F = 1.69; p = 0.20) but a negative covariation appeared when annual food quality resources decreased, as expected from prediction 2 (slope of 0.96; CI = [0.07; 1.86]; Table 1; Fig. 2B). In Chizé, while hemagglutination titers and FCMs were overall positively related (F = 5.80; p = 0.02; slope of 2.57 for animals aged <8 years old), the covariation became negative as age of roe deer increased (-0.33 per year; CI = [−0.59; −0.06]; Table 2; Fig. 4A). In addition, in Aurignac, FCMs were retained in the model selection to explain hemolysis titers, with a negative trend (slope of −0.19; CI = [−0.43; 0.05];

Table 2
Parameters of linear mixed-effect models selected for each immune parameters of the Chizé population. R2m and R2c correspond respectively to the marginal and conditional variance explained by the model, CI corresponds to the upper and lower limits of the 95% confidence interval, and n represents the number of observations per analysis. Variables in bold correspond to variable of primary interest in our study. W + stands for importance scores of variables and is calculated by summing the Akaike weights across all the selected models including the target variable. See the material and methods section for a full definition of model sets.

Immune trait	Parameter	Estimate	CI	W+
Innate immunity				
Neutrophils (n = 390) R^2m = 0.10; R^2c = 0.44	Intercept	4.49	3.95 to 5.03	
	Delay	0.003	0.0001 to 0.005	1.00
	Age (linear)	0.13	0.06 to 0.20	0.43
	I(Age^2)	0.008	0.001 to 0.01	0.57
	Julian date	0.01	0.000002 to 0.02	0.86
	FCMs	**−0.26**	**−0.54 to 0.03**	**0.67**
	Year quality	−0.32	−0.67 to 0.04	0.55
	Weight	0.06	0.004 to 0.11	0.57
Eosinophils (n = 390) R^2m = 0.14; R^2c = 0.19	Intercept	0.10	0.06 to 0.14	
	Delay	−0.0003	−0.0004 to −0.0002	1.00
	Age (linear)	−0.01	−0.03 to 0.004	0.24
	I(Age^2)	0.0007	−0.0006 to 0.002	0.82
	Sector (2)	0.02	−0.007 to 0.05	1.00
	Sector (3)	−0.03	−0.06 to 0.005	
	Weight	0.005	0.0006 to 0.008	1.00
	Year quality	−0.02	−0.04 to −0.005	1.00
	Sex (male)	0.01	−0.02 to 0.04	0.37
	I(Age^2)*Sex (male)	−0.001	−0.002 to −0.0001	0.37
Basophils (n = 390) R^2m = 0.04; R^2c = 0.32	Intercept	0.04	0.01 to 0.07	
	FCMs	**−0.002**	**−0.01 to 0.01**	**0.55**
	Age (linear)	0.002	0.0004 to 0.004	0.42
	Sector (2)	0.01	−0.004 to 0.02	0.55
	Sector (3)	0.004	−0.01 to 0.02	
	Weight	0.002	0.0003 to 0.003	0.58
	FCMs*Sector (2)	**−0.02**	**−0.04 to −0.0002**	**0.55**
	FCMs*Sector (3)	**−0.03**	**−0.06 to −0.004**	
Monocytes (n = 389) R^2m = 0.07; R^2c = 0.55	Intercept	0.67	0.54 to 0.81	
	Age (linear)	−0.06	−0.08 to −0.05	1.00
	FCMs	**−0.04**	**−0.07 to −0.007**	**1.00**
	Year quality	−0.06	−0.09 to −0.03	1.00
	Sex (male)	−0.05	−0.09 to −0.009	1.00
	FCMs*Year quality	**0.06**	**0.003 to 0.11**	**0.73**
Hemagglutination (n = 325) R^2m = 0.07; R^2c = 0.59	Intercept	4.72	2.77 to 6.66	
	Age (threshold: 8 years)	−0.08	−0.29 to 0.13	1.00
	Julian date	−0.007	−0.01 to 0.0009	0.60
	FCMs	**2.57**	**0.37 to 4.77**	**1.00**
	Weight	−0.07	−0.11 to −0.04	1.00
	FCMs*Age (threshold)	**−0.33**	**−0.59 to −0.06**	**1.00**
Hemolysis (n = 325) R^2m = 0.02; R^2c = 0.65	Intercept	3.88	−0.05 to 7.82	
	Age (threshold: 10 years)	−0.30	−0.61 to 0.02	0.65
	Julian date	−0.01	−0.02 to −0.004	1.00
Inflammatory markers				
Alpha-1 globulins (n = 414) R^2m = 0.22; R^2c = 0.42	Intercept	1.43	1.39 to 1.47	
	Delay	0.0002	0.00003 to 0.0003	1.00
	FCMs	**−0.03**	**−0.05 to −0.007**	**1.00**
	Weight	−0.01	−0.02 to −0.01	1.00
	Sex (male)	0.04	0.02 to 0.07	1.00
Alpha-2 globulins (n = 414) R^2m = 0.14; R^2c = 0.24	Intercept	1.93	1.90 to 1.95	
	Julian date	−0.001	−0.002 to −0.00006	0.79
	Delay	0.0002	−0.00002 to 0.0003	0.56
	FCMs	**−0.004**	**−0.03 to 0.04**	**1.00**
	Weight	−0.004	−0.008 to 0.0002	0.70
	Year quality	0.06	0.03 to 0.09	1.00
	Sector (2)	−0.01	−0.05 to 0.03	1.00
	Sector (3)	−0.05	−0.01 to 0.005	
	FCMs*Year quality	**−0.06**	**−0.01 to −0.005**	**1.00**
	FCMs*Sector (2)	**−0.11**	**−0.17 to −0.05**	**1.00**
	FCMs*Sector (3)	**−0.06**	**−0.13 to 0.007**	
Beta globulins (n = 412) R^2m = 0.24; R^2c = 0.51	Intercept	1.88	1.81 to 1.95	
	Age (linear)	0.07	0.05 to 0.10	1.00
	I(Age^2)	−0.004	−0.006 to −0.002	1.00
	Julian date	0.001	0.0004 to 0.002	1.00
	FCMs	**−0.02**	**−0.05 to 0.01**	**0.71**
	Weight	−0.007	−0.01 to −0.0008	1.00
	Sector (2)	0.0001	−0.04 to 0.04	0.71
	Sector (3)	−0.02	−0.07 to 0.03	
	Sex (male)	0.05	0.02 to 0.09	1.00
	FCMs*Sector (2)	**0.03**	**−0.02 to 0.09**	**0.71**
	FCMs*Sector (3)	**0.11**	**0.04 to 0.17**	
Haptoglobin (n = 406) R^2m = 0.13; R^2c = 0.28	Intercept	0.25	0.19 to 0.33	
	Julian date	0.002	0.0001 to 0.004	1.00
	Year quality	−0.15	−0.23 to −0.08	1.00
	Sex (male)	0.11	0.04 to 0.19	1.00
Adaptive immunity				
Gamma globulins (n = 414) R^2m = 0.07; R^2c = 0.67	Intercept	18.59	16.37 to 20.81	
	Age (threshold: 4 years)	0.43	0.18 to 0.69	1.00
	Delay	0.004	−0.0001 to 0.008	0.67
	FCMs	**1.13**	**−0.57 to 2.83**	**1.00**
	Sector (2)	0.15	−0.94 to 1.25	1.00
	Sector (3)	−1.45	−2.82 to −0.08	
	FCMs*Age (threshold)	**−0.30**	**−0.56 to −0.05**	**1.00**
	FCMs*Sector (2)	**−0.78**	**−2.12 to 0.55**	**1.00**
	FCMs*Sector (3)	**1.77**	**0.04 to 3.51**	
Lymphocytes (n = 390) R^2m = 0.24; R^2c = 0.72	Intercept	1.14	1.01 to 1.27	
	Age (linear)	−0.06	−0.09 to −0.03	1.00

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Table 2 (*continued*)

Immune trait	Parameter	Estimate	CI	W+
	I(Age^2)	0.003	0.0007 to 0.006	1.00
	Delay	−0.0004	−0.0007 to −0.0002	1.00
	FCMs	**−0.00005**	**−0.04 to 0.04**	**0.50**
	Year quality	0.006	−0.13 to 0.14	0.50
	Sex (male)	−0.02	−0.09 to 0.05	1.00
	I(Age^2)*Sex (male)	−0.003	−0.005 to −0.0006	1.00
	FCMs*Year quality	**−0.09**	**−0.17 to −0.02**	**0.50**

Table 1).

3.3. Inflammatory markers

In Aurignac, while alpha-1 globulin concentration was not related to FCM levels overall ($F = 1.45$; $p = 0.23$), a trend for a negative covariation appeared in area of poor quality (sector 3: slope of −0.03; CI = [−0.11; 0.002]; Table 1), as expected from prediction 2. In addition, there was also a trend for a negative covariation between alpha-1 globulins and FCMs in males compared to females (slope of −0.05; CI = [−0.11; 0.01]), and in heavier individuals (slope of −0.006; CI = [−0.01; 0.002]), while beta-globulins showed a trend for a light positive link with FCMs (slope of 0.03; CI = [−0.009; 0.08]). In Chizé, alpha-1 globulins were negatively related to FCMs (slope of −0.03; CI = [−0.05; −0.007]; Table 2; Fig. 1B), as expected from prediction 1. Still in Chizé, the covariation between FCMs and alpha-2 globulins was overall negative ($F = 11.9$; $p < 0.01$), as expected from prediction 1. However, this negative covariation only held in the areas of intermediate (sector 2: slope of −0.11; CI = [−0.17; −0.05]; Table2; Fig. 3B) and poor (sector 3: slope of −0.06; CI = [−0.13; −0.007]; Table2; Fig. 3B) quality. In addition, and unexpectedly, the covariation between FCMs and alpha2-globulins was more pronounced when the average fawn body mass decreased (slope of −0.06; CI = [−0.01; −0.005]). This result suggests that the FCM-alpha2 globulins relationship was stronger when the annual quality of food resources decreased, which is in contradiction with prediction 2 (Table 2; Fig. 2C). In Chizé again, prediction 2 was not supported by results on beta-globulin concentrations, as a positive covariation with FCMs was found in area of poor quality (sector 3: slope of 0.11; CI = [0.04; 0.17]), with a similar trend in the area of intermediate quality (sector 2: slope of 0.03; CI = [−0.02; 0.09]), while no covariation was detected in the area of best quality (sectors 1, Table 2; Fig. 3C). In Trois-Fontaines, a slightly negative covariation between haptoglobin and FCM levels was observed in youngest animals but was greatest in old ones, in accordance with prediction 3 (slope of −0.08 per year; CI = [−0.17; −0.01]; Table 2; Fig. 4B).

3.4. Adaptive cellular immunity

Consistent with prediction 1, we observed no covariation between lymphocyte concentrations and FCM levels in any of the 3 populations. However, in Chizé, and contrarily to prediction 2, a positive covariation between FCMs and lymphocytes appeared during the worse quality years (slope of −0.09; CI = [−0.17; −0.02]; Table 2; Fig. 2D). In Trois-Fontaines, a negative covariation between FCMs and lymphocytes appeared only in older roe deer (slope of −0.03; CI = [−0.06; −0.007]; Table 3; Fig. 4C), in accordance with prediction 3.

3.5. Adaptive humoral immunity

Gamma-globulin concentrations were overall positively related to FCM levels in Aurignac ($F = 2.62$; $p < 0.01$), but the covariation differed

Table 3
Parameters of linear mixed-effect models selected for each immune parameters of the Trois-Fontaines population. R2m and R2c correspond respectively to the marginal and conditional variance explained by the model, CI corresponds to the upper and lower limits of the 95% confidence interval, and n represents the number of observations per analysis. Variables in bold correspond to variable of primary interest in our study. W + stands for importance scores of variables and is calculated by summing the Akaike weights across all the selected models including the target variable. See the material and methods section for a full definition of model sets.

Immune trait	Parameter	Estimate	CI	W+
Innate immunity				
Neutrophils (n = 293) R^2m = 0.06; R^2c = 0.42	Intercept	5.83	5.28 to 6.38	
	Age (I^2)	0.02	0.007 to 0.03	1.00
	Delay	0.003	0.00007 to 0.005	0.72
	Sex (male)	−0.06	−0.66 to 0.54	1.00
	Age (I^2)*Sex (male)	−0.02	−0.03 to −0.003	1.00
Eosinophils (n = 292) R^2m = 0.09; R^2c = 0.34	Intercept	0.07	0.06 to 0.09	
	Delay	−0.0003	−0.0004 to −0.0002	1.00
	Weight	0.004	0.001 to 0.006	1.00
Basophils (n = 291) R^2m = 0.01; R^2c = 0.37	Intercept	0.04	0.01 to 0.07	
	Delay	−0.00005	−0.0001 to 0.00002	0.42
	Weight	0.001	0.00001 to 0.003	0.82
Monocytes (n = 293) R^2m = 0.05; R^2c = 0.46	Intercept	0.27	0.12 to 0.42	
	Age (linear)	−0.01	−0.02 to 0.0005	0.34
	Julian date	−0.003	−0.005 to −0.0007	1.00
	Delay	−0.0004	−0.0007 to −0.00005	1.00
	FCMs	**−0.06**	**−0.13 to 0.009**	**0.27**
	Weight	−0.005	−0.01 to 0.001	0.20
Hemagglutination (n = 290) R^2m = 0.02; R^2c = 0.26	Intercept	1.54	1.40 to 1.68	
	Julian date	−0.003	−0.005 to −0.0003	1.00
Hemolysis (n = 276) R^2m = 0.00; R^2c = 0.29	Intercept	2.32	1.72 to 2.90	
Inflammatory markers				
Alpha-1 globulins (n = 288) R^2m = 0.19; R^2c = 0.69	Intercept	1.47	1.36 to 1.58	
	Age (linear)	−0.03	−0.05 to −0.004	1.00
	Age (I^2)	0.002	0.0006 to 0.004	1.00
	Weight	−0.007	−0.01 to −0.002	1.00
	Julian date	−0.003	−0.004 to −0.002	1.00
Alpha-2 globulins (n = 287) R^2m = 0.02; R^2c = 0.28	Intercept	1.92	1.81 to 2.03	
	Delay	0.0002	−0.00004 to 0.0005	0.68
	Sex (male)	−0.05	−0.10 to 0.004	0.73
Beta globulins (n = 288) R^2m = 0.22; R^2c = 0.56	Intercept	1.82	1.71 to 1.93	
	Age (linear)	0.03	0.02 to 0.03	1.00
	Julian date	−0.004	−0.005 to −0.002	1.00
Haptoglobin (n = 303) R^2m = 0.15; R^2c = 0.60	Intercept	−0.38	−0.74 to −0.009	
	Age (threshold: 9 years)	0.06	0.02 to 0.10	1.00
	FCMs	**0.50**	**−0.42 to 1.42**	**0.73**
	Year quality	−0.08	−0.16 to 0.005	0.63
	Sex (male)	0.02	−0.005 to 0.05	0.68
	FCMs*Age (threshold)	**−0.08**	**−0.17 to 0.01**	**0.54**

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Table 3 (*continued*)

Immune trait	Parameter	Estimate	CI	W+
Adaptive immunity				
Gamma globulins (n = 288)	Intercept	11.60	10.03 to 13.44	
$R^2m = 0.12$; $R^2c = 0.56$	Age (threshold: 4 years)	0.46	0.29 to 0.65	1.00
	Julian date	−0.04	−0.07 to −0.02	1.00
Lymphocytes (n = 293) R^2m = 0.08; R^2c = 0.60	Intercept	1.18	1.10 to 1.25	
	Age (linear)	0.007	−0.008 to 0.02	0.44
	Julian date	−0.002	−0.003 to 0.0004	0.43
	Delay	−0.0003	−0.0006 to 0.00006	0.59
	FCMs	**0.07**	**−0.04 to 0.17**	**0.44**
	Weight	−0.02	−0.02 to −0.007	1.00
	FCMs*Age (linear)	**−0.03**	**−0.06 to −0.007**	**0.44**

among areas of different qualities, consistent with prediction 2. Precisely, the positive covariation was only present in the area of best quality (sector 1: slope of 1.58; CI = [0.40; 2.76]), whereas negative covariations appeared in areas of intermediate (sector 2: slope of −2.68; CI = [−4.38; −0.98]) and poor (sector 3: slope of −2.11; CI = [−3.87; −0.35]) quality (Table 1; Fig. 3D). Furthermore, in the Aurignac population, the covariation between gamma-globulins and FCMs differed between females and males, with females showing a positive link, while males had a negative covariation between FCMs and gamma-globulins (slope of −2.10; CI = [−3.62; −0.57]). In Chizé, a similar overall slight positive covariation was observed between gamma-globulins and FCMs ($F = 3.18$; $p < 0.01$), but the covariation became negative as age increased (slope of −0.30 per year; CI = [−0,56; −0.05]; Table 2; Fig. 4D). As in Aurignac, the positive covariation between gamma-globulin concentrations and FCMs also varied depending on the sector. However, contrary to Aurignac and to prediction 2, in Chizé, the covariation was positive only in the area of poor quality (slope of 1.77; CI = [0.04; 3.51]; Table 2; Fig. 3E). No significant correlation with FCMs was observed in the Trois-Fontaines population.

Taking all our results together, it is worth noting that all fixed effects, including FCMs concentrations, did not explain the same amount of variation in each of the studied immune parameters, as shown by the R2m values (Table 1, 2,3). Precisely basophils, monocytes, hemagglutination in Chizé, monocytes in Trois-Fontaines, and neutrophils in Aurignac had R2m values < 0.10.

4. Discussion

Overall, our results highlight clear associations between integrated glucocorticoid levels and immune parameters and show that the strength and direction of these associations differ between the three studied populations. Of the 12 measured immune parameters, only three (monocytes, lymphocytes and haptoglobin) showed significant co-variations with FCMs in Trois-Fontaines, and six (neutrophils, hemagglutination, hemolysis, alpha-1, beta and gamma-globulins) in Aurignac. The co-variation between FCMs and immunological parameters was particularly marked in Chizé (nine parameters), a site where the population faces harsh environmental conditions (Gaillard et al., 1993; Gaudry et al., 2018). Taken together, our results constitute one of the rare pieces of evidence that negative relationships between glucocorticoid levels and immune functions also occur in the wild.

Furthermore, and as expected, we found that covariations between FCMs and immune parameters differed between immunity components and that these covariations were affected by individual and environmental factors. Our results support the idea that immune system components are differentially linked to glucocorticoid levels (Bourgeon and Raclot, 2006). As levels of FCMs increased, we observed an overall decline in cellular (neutrophils, monocytes, basophils) and humoral (hemolysis) innate immune functions, in accordance with prediction 1 and previous reports. For instance, in the Belding's ground squirrel (*Urocitellus beldingi*), experimental chronic elevation of cortisol levels reduced serum bactericidal competence, a component of the constitutive innate immune response, compared to a control group (Brooks and Mateo, 2013). The most plausible explanation for this negative covariation between FCMs and innate immune parameters relies on the high energy cost of this immune system component. Indeed, the maintenance of immune defences requires energy and nutrients (Lochmiller and Deerenberg, 2000), and the cost of maintenance and activation is higher for innate than for adaptive immunity (McDade et al., 2016). Consequently, long-term elevation of glucocorticoid levels may selectively redirect energy away from the innate immunity towards other energy demanding functions (Lee, 2006; Martin, 2009). This process could be seen as a physiological adjustment for energy savings to maximize investment in reproduction and long-term survival through mechanisms other than innate immunity.

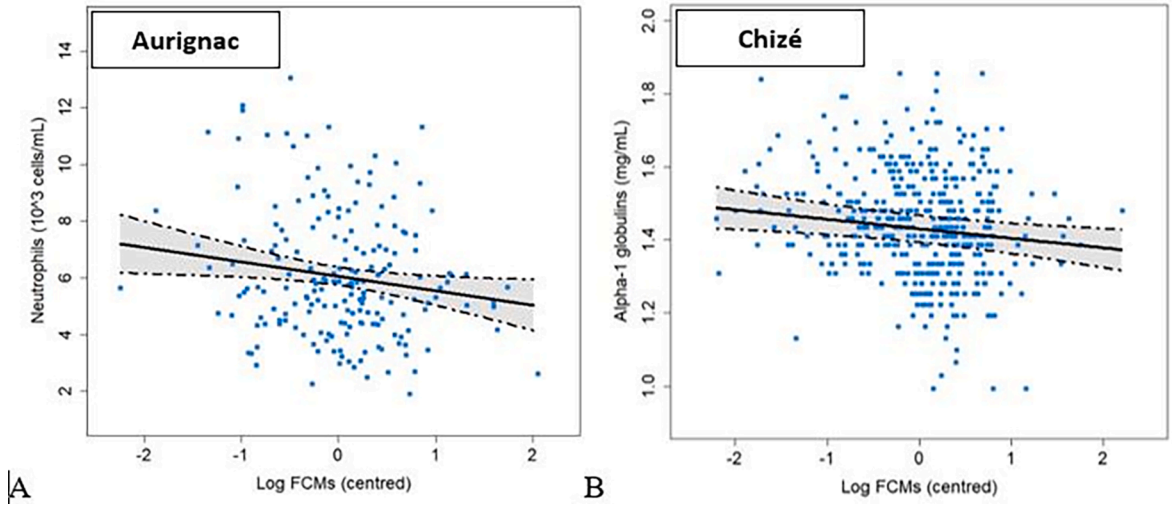

Fig. 1. Relationship between faecal cortisol metabolites (FCMs) and immune parameters in roe deer populations (prediction (i)): A) neutrophils in Aurignac; B) alpha-1 globulins in Chizé. Points represent observed values. Solid black lines represent model predictions, dashed lines and shaded area represent 95% confidence intervals. FCMs values are centred around the mean (mean value of the variable that is subtracted from every value).

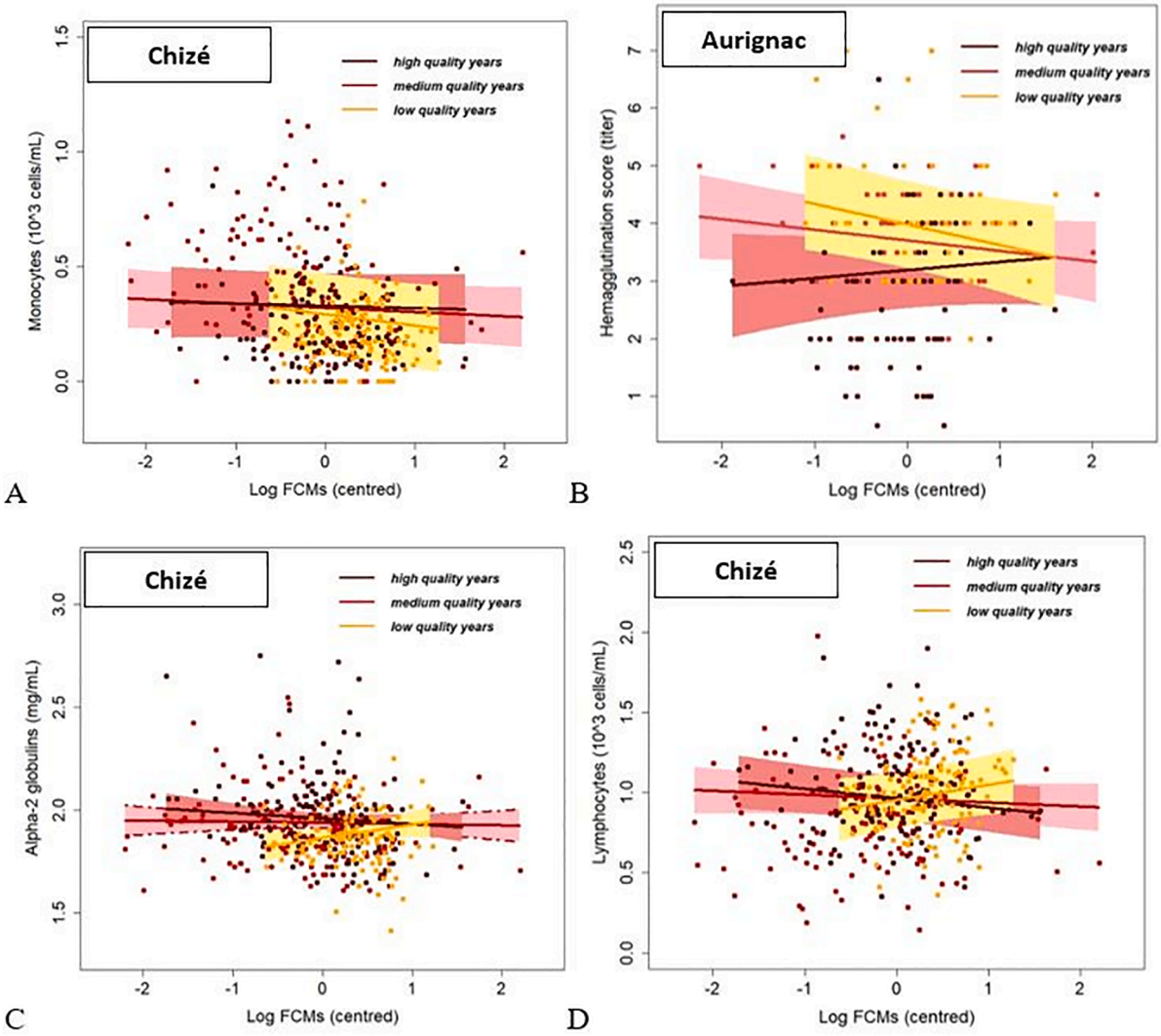

Fig. 2. Relationship between faecal cortisol metabolites (FCMs) and immune parameters in roe deer populations at varying year quality (prediction (ii)). Year quality was indexed using the population average body mass of juveniles (in kg) captured during the following winter: A) monocytes in Chizé; B) hemaglutination score in Aurignac; C) alpha-2 globulins in Chizé; D) lymphocytes in Chizé. Points represent observed values. Solid lines represent model predictions and shaded areas represent the 95% confidence interval. FCMs values are centred around the mean (mean value of the variable that is subtracted from every value).

Our results show that, of the four inflammatory markers measured, three (alpha-1, alpha-2 and haptoglobin) were negatively associated with FCMs in line with our predictions. Indeed, negative covariations between FCMs and inflammatory markers are consistent with the energy trade-off hypothesis, and likely due to their particularly high costs (McDade et al., 2016). However, we also found weak positive covariations between FGMs and beta-globulins. Why the relationship was positive for one of the inflammatory markers while it was negative for the others remains unanswered. First, beta-globulins, like alpha-1 and alpha-2 globulins, are a complex group of proteins, that may each have distinct relationship with stress level. Previous studies also pointed out that the relationship between glucocorticoids and immune parameters of the same arm may be different, such that some antigen responses in chickens have been shown to be affected by stressors, while others were not (El-Lethey et al., 2003). A possible explanation for these differences could be an insensitivity to glucocorticoids for certain immune parameters as suggested by El-Lethey and colleagues (2003), and as supported by a study on mice (Stark et al. 2001). More information would need to be gathered to support this hypothesis. The discrepancy between markers of the same arm of immunity highlights an important aspect of our work: the complexity of the relationship between immunity and glucocorticoids calls for great caution in the choice of the immune markers and in their interpretations.

Our results also partially supported our prediction of a moderate or absence of covariation between FCMs and cellular (lymphocytes) or humoral (gamma-globulins) adaptive immune parameters, which we observed in the three studied populations. Since the activation costs of adaptive immunity are lower than those of innate immunity (McDade et al., 2016), adaptive immune functions are expected to be less prone to energy trade-off. However, we essentially measured constitutive immunity unrelated to specific challenge, thus costs are linked to the development and maintenance of immunity. In species where a trade-off between growth and immune development has been showed, whether it concerns more innate or adaptive immunity remains to be established (Van der Most et al., 2011). However, in roe deer, such a trade-off is not present (Cheynel et al., 2019). An alternative non-exclusive explanation to this lack of link, or positive covariation (in the case of gamma-globulins in Aurignac in the high-quality sector), between FCMs and adaptive immune parameters may be that individuals repeatedly or chronically exposed to stressors may actually allocate more in adaptive immunity, especially if major stressors are pathogens, in order to maximize long-term survival. This might be particularly true in long-lived species such as roe deer, which repeatedly face the same pathogens in their environment, and are expected to exhibit stronger

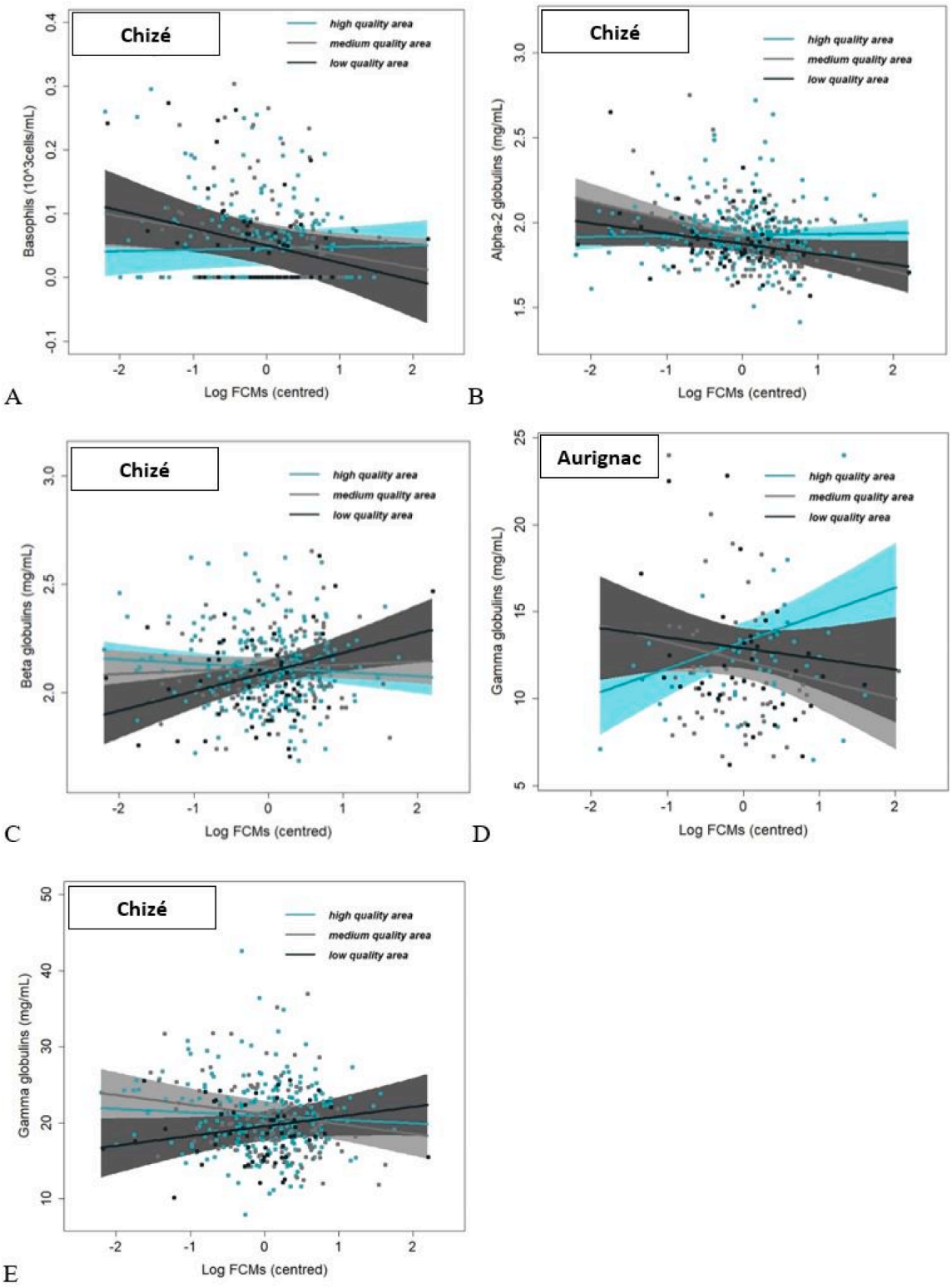

Fig. 3. Relationship between faecal cortisol metabolites (FCMs) and immune parameters in roe deer populations, at varying area quality (prediction (ii)). Areas quality differed among the three sectors described in the material and methods section: A) basophil concentrations in Chizé; B) alpha-2 globulins in Chizé; C) beta-globulins in Chizé; D) gamma-globulins in Aurignac; E) gamma-globulins in Chizé. Points represent observed values. Solid lines represent model predictions and shaded areas represent the 95% confidence interval. FCMs values are centred around the mean (mean value of the variable that is subtracted from every value).

allocation in adaptive, and particularly antibody-mediated immunity, compared to innate immunity (Lee, 2006). In all the three studied populations it has been shown that roe deer are exposed to several parasites such as gastrointestinal nematodes, protostrongylids and coccidia (Cheynel et al., 2017; Beaumelle et al., 2021), hard ticks (Bariod et al., 2022), protozoan parasites such as Toxoplasma gondii (Gotteland et al., 2014), abortive bacteria such as Chlamydia abortus (Sevila et al., 2014) and viruses such as Schmallenberg virus (Rossi et al.,

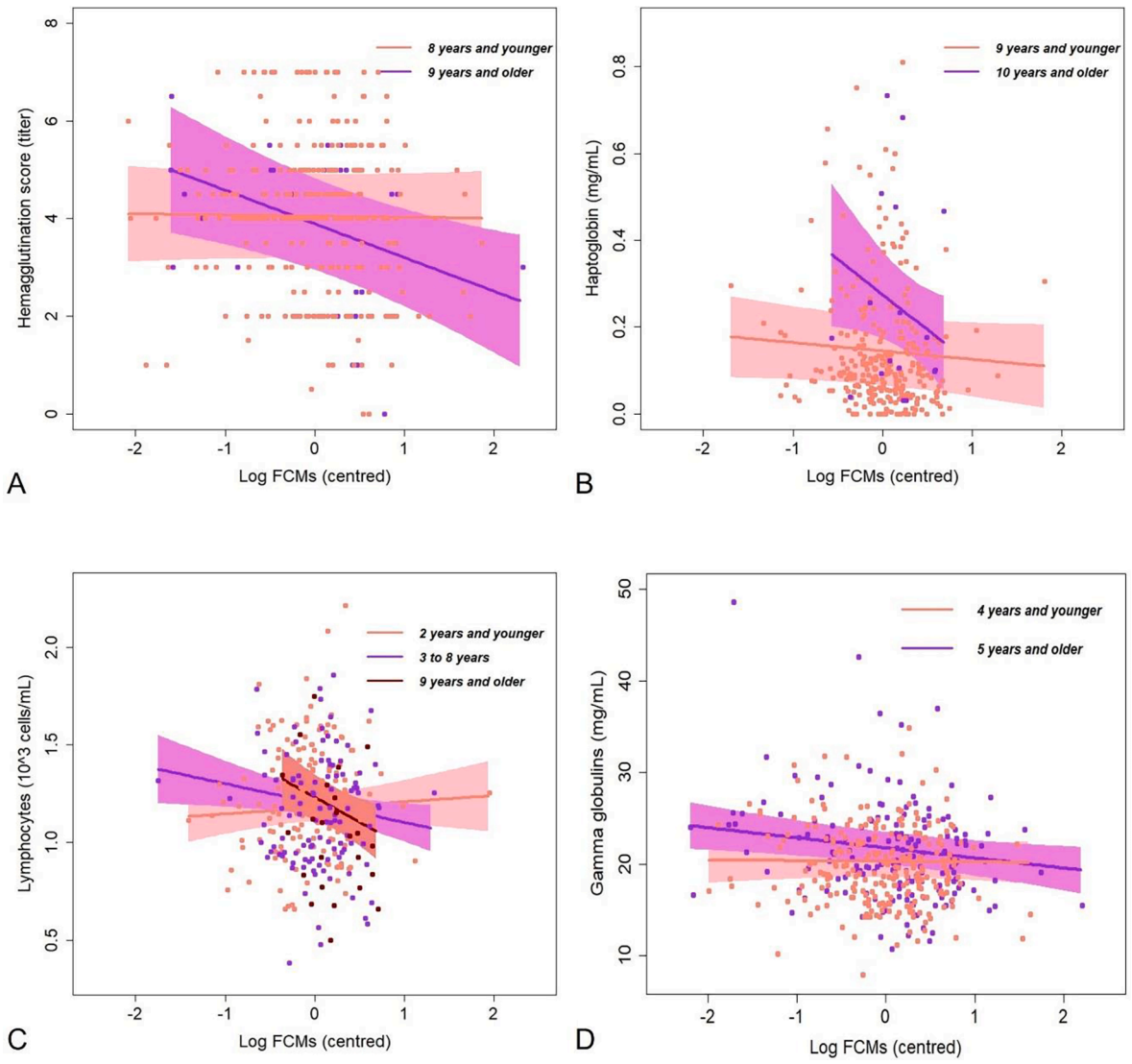

Fig. 4. Relationship between faecal cortisol metabolites (FCMs) and immune parameters at varying ages in roe deer populations (prediction (iii)). Age was considered either as linear or with a threshold function (determined from a previous study, see main text for details), in which case the graphical representation compares before/after threshold age: A) hemagglutination in Chizé (threshold: 8 years old), B) haptoglobin in Trois-Fontaines (threshold: 9 years old); C) lymphocytes in Trois Fontaines (linear, but for the graphical display, three age classes were constituted on the basis of sample size: up to 2 years, 3–8 years, 9 years and older); D) gamma-globulins in Chizé (threshold: 4 years old). Points represent observed values. Solid lines represent model predictions and shaded areas represent the 95% confidence interval. FCMs values are centred around the mean (mean value of the variable that is subtracted from every value).

2017) are also present.

Besides the difficulty of making general assumptions about the immunity-glucocorticoid relationship due to the above-mentioned differences between components, we found that other factors may modulate the observed relationships. In particular, the age of individuals as well as the spatial and annual heterogeneity of food resources influenced most of the covariations between FCMs and innate, inflammatory, and adaptive markers of immunity in the three studied populations. As predicted in 2, we found that during years providing less and/or lower-quality food resources a negative covariation between innate immunity (monocytes and hemagglutination) and integrated glucocorticoid levels appeared, contrary to better years. The same observation was made for the spatial heterogeneity in food resources. In the Chizé and Aurignac populations, roe deer from areas with low quality food resources showed negative covariations between some immune markers (basophils, alpha-1, alpha-2 and gamma globulins) and FCM levels, while no or positive covariations were observed in areas of high quality. High levels of food resources have been shown to be associated with reduced glucocorticoid levels (Fokidis et al., 2012; Carbillet et al., 2020) but may also provide sufficient energy and nutrients to sustain the cost of innate immune response, as previously suggested in by Strandin and colleagues (2018). In their review, these authors showed that food provisioning in field studies tended to increase both innate and adaptive immunity, whereas food restriction frequently impaired immunity. However, in contrast to those results, our data also suggested that negative covariations may, in some cases, appear between immunity and integrated glucocorticoid levels in areas and years of high food resources. Alpha-2 globulins and lymphocytes were positively associated with FCM levels when annual food resources were scarce, and there were positive covariations with FCMs for beta-globulins and gamma globulins in poor food resources areas, while no association was observed in areas providing better food resources. At first glance, these results might appear counter-intuitive. However, these observations were all made in the population of Chizé, which has the lowest availability in food resources compared to the two other populations, even in the best sector (Gaillard et al., 1993; Gaudry et al., 2018). These results thus seem to match with an alternative theoretical framework proposed by Davis and Maney (2018), who suggested that in poor quality habitats, glucocorticoid secretion should be

downregulated to prevent impairment of immune functions. Therefore, in Chizé, during the worst years, or in the worst sector, energy allocation to immune function could be prioritised and independent of glucocorticoid levels, which should be minimised with low among-individual variations. Consistent with this hypothesis, variance in FCMs appeared to be lower during years of low food availability than during years of high food availability in Chizé (Fig. 2). However, this hypothesis is not supported for all immune parameters studied, suggesting that not all immune parameters would be independent of glucocorticoid levels in poor quality habitats within the framework proposed by Davis and Maney (2018). In addition, pathogen load, and thus immune challenge, may covary with food resources availability across time. In particular, variations in population abundance may determine both resource availability and pathogen exposure: high population density leading to both limited resources and high exposure to directly or indirectly transmitted pathogens. In this case, high pathogen pressure could act as a stressor on roe deer and lead to an elevation of FCMs, while stimulating immune defences to cope with the threat.

Finally, age influenced the relationship between immunity and integrated glucocorticoid levels. In accordance with our predictions 3, negative relationships between FCMs and immune parameters (hemagglutination, haptoglobin, gamma globulins and lymphocytes) only appeared, or were steeper, in older individuals. In roe deer, the ingestion capacity is known to be less efficient in old individuals (Gaillard et al., 1993), leading to a diminution in available energy and nutrients, and individuals suffer from a loss of condition, as documented through a senescence in body mass (Douhard et al., 2017) and various biological markers of ageing (Cheynel et al., 2017, Wilbourn et al., 2017; Lemaître et al., 2022). Consequently, older individuals exhibiting high levels of integrated glucocorticoids might have poorer abilities to increase their allocation toward the immune functions. Moreover, in line with previous evidence that stress hormones might accelerate the aging process through an increased telomere shortening in roe deer (Lemaître et al., 2021), our results suggest that they might also accelerate the previously documented immunosenescence (Cheynel et al., 2017). Long-term or repeated exposure to stressors may therefore constitute a major selective pressure, especially on old individuals that may not be able to maintain an overall efficient immune response and would therefore be exposed to higher risks of diseases, contributing to the reduction in survival and reproductive success throughout life.

To conclude, our results show that the immunity of wild ungulates is significantly linked to glucocorticoid levels, but that this relationship differs between innate, adaptive and inflammatory markers of immunity. While glucocorticoids are overall negatively correlated with innate and inflammatory immunity, they appear to be less or even positively linked to adaptive immunity. In addition, spatial and temporal availability in food resources appear to shape the relationship between glucocorticoids and immunity with non-linear patterns, while negative covariations between FCMs and immunity is strongest in old individuals. Our work highlights the need to consider a multi-marker approach for future studies investigating the effect of stress hormones on immune functions in wild animals. Such an approach, combined with consideration of the environmental context and individual phenotype, could help improve our understanding of the mechanisms underlying among-individual differences in immunity and susceptibility to diseases in the wild.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

I have shared the link to data and codes in the Data accessibility section of the manuscript

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Authors' contributions.

JC, EGF and HV conceived and designed the study. JC, EGF, HV, BR, MP, JD, SP, FD, JM, JFL performed fieldwork. EGF, AG and CR performed immunological analyses. RP and JC ran FCM assays. JC and MH performed the statistical analysis, wrote the first draft of the paper, and then received input from other co-authors. All authors approved the final version of the manuscript and agree to be held accountable for the content therein.

Data accessibility

Data used in this study are available at https://github.com/JeffreyCarbillet/CarbilletHollainEtal2022.

Competing interests

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2022.114141.

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Further readings

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