

Sex, age, spleen size, and kidney fat of red deer relative to infection intensities of the lungworm *Elaphostrongylus cervi*

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Abstract We analyzed the relationships among spleen size, body condition (measured as kidney fat), and larval counts of the nematode *Elaphostrongylus cervi* in red deer (*Cervus elaphus*). The aim was to investigate the interaction between host body condition and intensity of infection with parasites. As red deer are highly polygynous, we also tested whether these relationships varied with sex and age of the hosts. Kidney fat and spleen size were positively correlated in subadults (2–3 years old) and adults (>3 years old), but not in calves (<1 year old) or yearlings (1–2 years old). Spleen size was negatively associated with nematode load in subadult females and in adult males. These two age classes are potentially the most nutritionally stressed, as subadult hinds are still growing and often engaging in rearing their first calf, and adult stags were sampled just after the rut, which is recognized as a substantial energy drain in this age–sex class, as they compete to hold females during the mating season. Body condition related negatively to parasite count only in adult males. In the context of red deer life history, these findings suggest that spleen size

is dependent on body condition and that it could be affected by variation in resource partitioning among immune defense, growth, and reproductive effort in red deer. For the first time in a wild mammal, the spleen mass is shown to be positively related to body condition and negatively related to parasite infection. We conclude that elucidating whether spleen mass reflects immune defense investment or a measure of general body condition should contribute to understanding topical issues in mammal ecology.

Keywords Body condition · Deer · Immune defense · Mating system · Polygyny · Spleen mass

Introduction

The extent to which an individual partitions resources into immune defense organs and the relationship this has with individual fitness has been the subject of much research in the last decade. The measurement of spleen size has contributed to understanding evolved strategies of immune investment (John 1994, 1995; Møller et al. 1998a,b; Shutler et al. 1999; Morand and Poulin 2000; Nunn 2002; Smith and Hunt 2004). The assumption is that parasite-infected individuals have larger spleen sizes because they are either producing more lymphocytes or are suffering from inflammation due the pathological changes (Møller et al. 1998a,b). However, there is some evidence that there is an interaction with nutrition such that animals in good condition can afford to invest more in immunity and, hence, will have large spleens (Møller et al. 1998a). Some studies on rodents have found a positive relationship between parasite loads or disease and spleen size (Vincent and Ash 1978; Garside et al. 1989; Watkins et al. 1991; Hōrak et al. 2006). While there is some evidence for this in birds (e.g., Smith and

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Hunt 2004) and rodent studies (mainly in laboratory experiments), there is little evidence from wild mammals, so the real significance of the size of the spleen remains a subject of debate. Furthermore, a smaller proportion of mammalian than avian spleens are involved in immune function (Reilly 1985).

Animals often face a trade-off between investment on antiparasite defenses and other activities related to self-maintenance, survival, and reproduction (Zuk and Stoehr 2002; Møller and Saino 2004). One of the most important components of life history that may drive resource allocation is host mating system (Møller et al. 1998b; Moore and Wilson 2002; Zuk and Stoehr 2002). For example, in polygynous species, males and females may differ in their allocation priorities, as females may obtain greater lifetime reproductive success by investing more in self-maintenance (such as growth or immunity) and rearing offspring (Landete-Castillejos et al. 2004), whereas males may tend to follow a “live hard, die young” strategy (Promislow and Harvey 1990; Carranza et al. 2004), allocating more resources to displaying and mate attraction than to immunity (Zuk and Stoehr 2002). This is expected to be particularly so in species where male reproductive competition is extreme, and males invest relatively little in offspring (Zuk 1990). Therefore, variation relative to sex and age should be taken into account when studying the immune response, although little information is available for ungulates (Mulvey and Aho 1993).

The trade-offs involving investment in immune capacity may be different in males and females in polygynous species like the red deer (*Cervus elaphus*) because changes in survival, reproductive effort, and reproductive expectancies differ between sexes and ages (Clutton-Brock et al. 1982). *Elaphostrongylus cervi* are nematode parasites found in the fascia and connective tissue around skeletal muscles of red deer (Handeland et al. 2000) and can cause damage to host neuromuscular and lung tissues (Watson 1983). The immunological basis for spleen involvement in the immune response to helminths includes development of lymphocyte (type 2 T help)-mediated immune response (Kopp 1990), which act as mediators of eosinophil responses (e.g., Bradley and Jackson 2004). B cells and immunoglobulins produced in the spleen also contribute to the immune response to nematode infection. In this context, we first analyzed the relationships between spleen size, body condition (measured as kidney fat), and counts of the nematode *E. cervi* to test the hypothesis that spleen mass is body-condition-dependent, which in red deer needs to be quantified before we can understand how spleen size affects the intensity of infection with parasites. Second, we tested if variation in the spleen–body condition relationships could be explained by differences in the sex and age of the hosts.

Materials and methods

Animals, study area, and sampling methods

Deer were sampled across the Ciudad Real province in south central Spain (from 37°13'48"N to 39°31'43"N, from 06°34'06"W to 2°25'54"W). Sampling took place in all years from 2000 to 2004 during the normal hunting season (November to January), which is after the September–October rut.

Postmortem study of each hunter-harvested deer ($n=521$) was performed in the field, and total body length to the nearest 0.1 cm and its sex were recorded. Age was determined from the teeth eruption pattern and/or tooth sections as described by Klevezal and Kleinenberg (1967). The spleens were collected, immediately transported to the laboratory at the IREC facilities (Ciudad Real), and the mass was measured to the nearest 0.1 g using an electronic digital scale. All the animals used in this study were shot and, therefore, presented bullet wounds. It is well established that in response to hemorrhage (as in a bullet track), the spleen ejects erythrocytes as a compensatory physiological response, minimizing the effect of blood content on spleen mass. Body condition was estimated using the kidney fat index (KFI), which gives a good estimation of body fat levels in cervids (Finger et al. 1981). KFI is defined as the weight of the fat that surrounds the kidney in relation to kidney weight expressed as a percentage.

The protostrongylid *E. cervi* is widespread in Spanish populations of red deer (Vicente and Gortazar 2001; Vicente et al. 2006). Red deer acquire the infection by accidentally ingesting the intermediate gastropod hosts that are infected with L3s. Adult *E. cervi* have a long life span (several years, Watson 1984). Female nematodes in the muscle fascias produce eggs that hatch into first stage larvae (L1) in the lungs. Red deer shed L1 in the feces (Handeland et al. 2000). The fecundity of adult extrapulmonary protostrongylids depends on the individual host immune status, and this is reflected in counts of larvae in feces (see Gaudernack et al. 1984 for humoral immunity in reindeer infected by *Elaphostrongylus rangiferi* and Pelletier et al. 2005 for bighorn sheep and *Protostrongylus* spp.). *E. cervi* larvae are counted in fresh fecal samples collected directly from the rectum during field necropsy. L1 larvae were extracted in less than 24 h from 8 g of feces (Forrester and Lankester 1997) and were expressed as number of larvae per gram of feces. *E. cervi* L1 were morphologically identified under a light microscope (Vicente and Gortazar 2001). Individual *E. cervi* counts can be compared because there is no temporal variation in *E. cervi* L1 counts during winter (Vicente et al. 2005). In our study area, 63% of male deer and 55% of female deer excreted *E. cervi* L1 at 2 years old (Vicente et al. 2006).

Statistical analysis

To test whether sex and age (as categorical explanatory variables) were associated with variation in *E. cervi* L1 counts, spleen size, and KFI condition (as response variables, respectively), we performed three generalized linear models (Crawley 1993). In the spleen mass model, body length was also included as an explanatory continuous variable. We selected the most parsimonious model, the final model having the lowest Akaike information criterion value (Burnham and Anderson 1998). We modeled with a Poisson error and an identity link function (Wilson and Grenfell 1997) because the spread in the residuals increased for the larger fitted values (variance increases with the mean). Overdispersion was controlled for by introducing a dispersion parameter when the overdispersion parameter of the model (deviance/df) was >1.

We used path analysis or causal modeling (Mitchell 1992) to understand comparative strengths of direct relationships and mediated pathways among our set of variables. In the path diagram, pathways represent hypotheses, and the goal is to build a path diagram that fits the data. Path analysis can test complex relationships between variables in one step (variables can have a causal effect on a set of variables and, at the same time, experience the effects of others), quantifying how well the data fit a set of hypothesized structured relationships between variables. Path diagrams consist of variables connected by arrows, representing directed relationships. These variables can be manifest (quantified and included explicitly in the model) or latent (proposed to exist and not quantified). Based upon the evidence detailed in the introduction, the proposed structure of pathways is outlined in Fig. 1. Spleen size and kidney fat are independently related to counts of the nematode *E. cervi*. Because we predicted sex and age variation in spleen size and kidney fat of red deer relative to

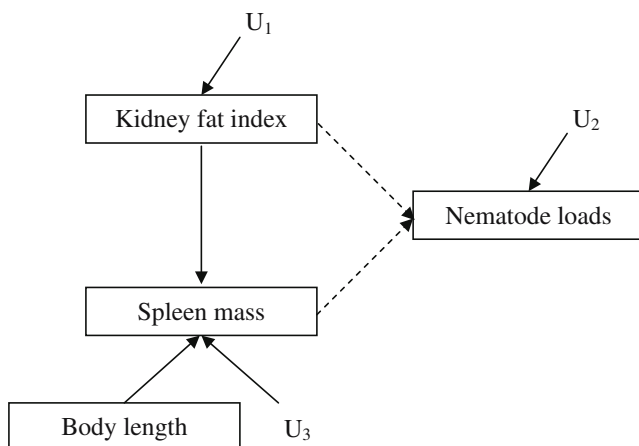


Fig. 1 Hypothesized path diagram. Lines denote positive effects, dashed lines denote negative effects. U represents latent variables (unexplained variance of the variable receiving the arrow)

infection intensities of the lungworm *E. cervi*, we analyzed each sex-by-age class separately to compare models as a function of this categorical variables. To test the goodness of fit of our models to the data, the chi-square test, the root mean square standardized residual (RMS), and the Bentler–Bonett tests were employed. Animals were grouped by age as follows: (1) calves (<1 year), n=58; (2) yearlings (individuals in their second calendar year), n=57, subadults (individuals in their third calendar year), n=95, and adults (≥ 4 years), n=311. These classes are of biological meaning in the red deer (Landete-Castillejos et al. 2004), as calves

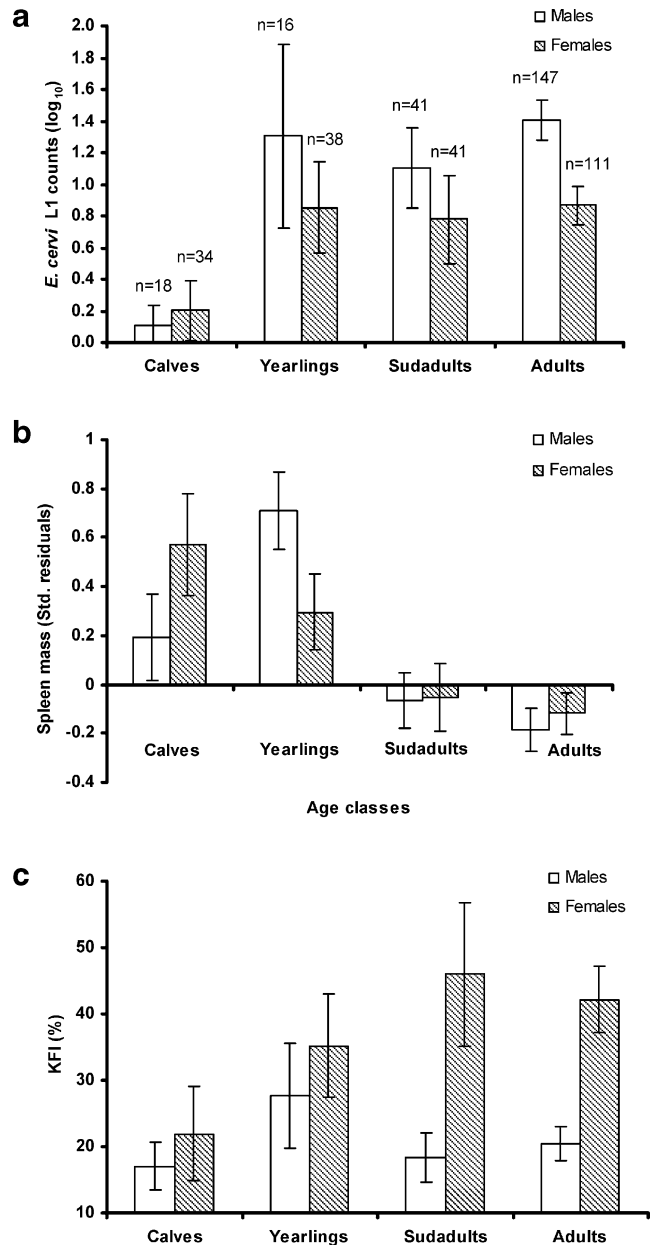


Fig. 2 Mean *E. cervi* larval counts (log₁₀ [E. cervi L1 count + 1]; a), spleen mass (as standardized residuals of the model; b), and body condition (KFI, %; c) across sex-by-age red deer specific classes. Statistics are provided in Table 1

are excluded from mating, yearlings and subadult classes are still growing and, in the case of males, are also usually excluded from mating. Only a small proportion of females breed as yearlings (Clutton-Brock et al. 1982, 1984). Mean abundance describes the mean number of parasites collected from all deer. Fecal *E. cervi* L1 counts +1 were \log_{10} transformed for the path analyses. The level of significance was established at 5%. Values for phenotypic traits and parasite infection rates are expressed as mean \pm 1.96 SE (i.e., 95% confidence intervals).

Results

Parasite counts differed significantly between age classes, and yearlings already had counts comparable to those of subadults and adults (Fig. 2a). Among yearlings, subadults, and adults, males had higher counts than females (significant interaction, sex-by-age). Spleen mass, after controlling for body size, was lower in subadults and adults than in calves (Fig. 2b). In females, spleen mass declined progressively from a maximum found in calves to low levels in adults. In males, spleen mass was highest in yearlings with lowest levels again in adults. Females exhibited statistically higher KFI scores than males (Fig. 2c), but a significant sex-by-age interaction indicated that, whereas KFI increased with age in females and stabilized at subadults, in subadults, a progressive decrease from 1 year of age in males was noted (and adult males had lower KFI than females, whereas there was no difference in KFI scores between the sexes in calves and yearlings).

Except for female calves (particularly for RMS and Bentler–Bonett tests), our models did not show a significant

lack of fit (Table 2). Therefore, the models proposed were consistent with the correlational structure of the data and well described the causal relationships hypothesized. The results showed that (1) There was a positive correlation between KFI and spleen size in subadults (marginally significant for males, $p=0.06$) and adults. (2) Spleen mass was negatively associated with nematode load in subadult females, whereas in males, this relationship was only evident in adults. (3) In addition, a direct negative effect of KFI on parasite counts was only found for adult males, but not in females at any age. (4) Allometric relationships were revealed by the significant and positive path coefficients from body size to spleen mass, except for subadult males.

Discussion

Our findings suggest that spleen size in red deer is dependent on body condition and could be affected by variation in resource partitioning between antiparasite defense and growth and that these relationships are sex and age dependent. This could be because sex differences and timing of the reproductive activities in red deer (rut in males and gestation/lactation in females) create differences in the allocation of resources for body condition vs that of immune defense investment.

Sex and age differences in spleen size, kidney fat, and *E. cervi* infection

There were sex-related statistical differences; males have higher *E. cervi* L1 counts than females at reproductive ages (adults, Fig. 2a). Previous studies have suggested sexual

Table 1 Generalized lineal models for *E. cervi* L1 counts (\log_{10} [*E. cervi* L1 count +1]), immune defense (spleen mass), and body condition (kidney fat index, referred as KFI)

	<i>E. cervi</i> counts				Spleen mass				Kidney fat index			
	df	Wald	p	E	df	Wald	p	E	df	Wald	p	E
Sex	1	12.9	<0.001	0.2	–	–	–	–	1	42.0	<0.001	–7.7
Age	3	237.1	<0.001	–0.7	3	41.3	<0.001	–15.4	3	20.9	<0.001	–9.1
				0.3				–134.6				2.8
				0.1				73.8				3.5
Length	–	–	–	–	1	909.8	<0.001	2.6	–	–	–	–
Sex \times age	3	23.3	<0.001	–0.2	3	40.4	<0.001	9.8	3	16.5	<0.001	5.3
				0.1				–9.6				2.8
				0.0				–2.3				3.5
Age \times length	–	–	–	–	3	44.6	<0.001	0.1	–	–	–	–
								0.9				
								–0.5				

We used a Poisson error and an identity link. Host sex is referred as sex and body length is referred as length. We display the degree of freedom (df) and the Wald statistic for significant test (Wald). Parameter estimates (E) for the different levels of fixed factors were calculated considering a reference value of zero for female level in the variable “sex” and a reference value of zero for adult level in the variable “age class”. From the top in each cell, parameter estimates refer to calves, yearlings, and subadults, respectively.

Table 2 Path diagram statistics

Directed relationships	Calves		Yearlings		Subadults		Adults	
	M	F	M	F	M	F	M	F
KFI→spleen	0.15	0.19	0.15	0.21	0.27****	0.27*	0.16*	0.09***
Spleen→ <i>E. cervi</i>	0.17	0.18	-0.05	-0.23	-0.17	-0.49**	-0.29***	0.10
KFI→ <i>E. cervi</i>	0.17	-0.32	-0.14	-0.21	0.13	0.12	-0.25***	0.10
Body Length→Spleen	0.15*	0.46**	0.56**	0.51***	0.12	0.44**	0.27***	0.09*
Chi ²	0.78	4.95	0.18	0.44	0.05	0.55	3.25	1.08
RMS	0.04	0.61	0.04	0.03	0.012	0.03	0.05	0.03
Bentler–Bonet	0.93	0.17	0.97	0.98	0.99	0.98	0.93	1.13

Goodness of fit of the models were tested by (1) the chi-square test, (2) the root mean square standardized residual (RMS), and (3) the Bentler–Bonett normed fit index (Bentler–Bonett). *p* values for chi-square test were always ≥ 0.05 ; thus, there are no significant differences between the real data and the models proposed. A model with an RMS value under 0.05 indicates that the path diagram proposed is consistent with the correlation structure of the data. Bentler–Bonett normed fit index approaches 1 in value as fit becomes perfect.

**p*<0.05 is the probability value for the directed relationship included in the path analysis and its statistical significance.

***p*<0.01 is the probability value for the directed relationship included in the path analysis and its statistical significance.

****p*<0.001 is the probability value for the directed relationship included in the path analysis and its statistical significance.

*****p*=0.06 is the probability value for the directed relationship included in the path analysis and its statistical significance.

dimorphism in susceptibility to parasitic infections and disease (e.g., Alexander and Stimson 1989; Zuk 1990; Schalk and Forbes 1997), males tending to exhibit higher rates of parasitism and disease and showing reduced immune responses compared with females (Moore and Wilson 2002). The immunocompetence handicap hypothesis postulates that it is due to the immunosuppressive effects of testosterone (Folstad and Karter 1992), but alternatively (or additionally), sex differences may be due to differences in the susceptibility to parasites caused by the different roles of males and females in activities related to sexual selection (Alexander and Stimson 1989; Zuk 1990). Furthermore, individuals of both sexes may simply differ in their exposure to parasites because of their different feeding habits or habitat use (Clutton-Brock et al. 1982), which could not be tested in our study.

In females, KFI was largest in adults and subadults, but in males, this was reversed (Fig. 2c). Body condition in polygynous ungulates responds to different selective pressures and has different effects on reproductive success in each sex (e.g., Festa-Bianchet 1998; Gaillard et al. 2000). Reproductive effort in female deer is related to weight and body condition, so reproductive age groups might be expected to be in better condition compared to younger growing animals that are partitioning resources into growth (Albon et al. 1983). For males, reproductive age classes might be expected to be in poorer condition after the rut than the non-reproductive age classes. During the energetically demanding period of rut, male reserves can be depleted up to starvation levels (Johns et al. 1984), as they invest resources mainly in male–male competition and hierarchy establishment during rut (which took place just before data collection).

However, both older male and female age classes had a lower spleen size than the younger animals (Fig. 2b). Yearling

females already experienced a decrease, whereas in males, this pattern was delayed (observed firstly in subadults), but the relative decrease in the spleen mass was more accentuated than in females. These patterns are coincident with specific age patterns of breeding effort in each sex, the higher the effort, the smaller the relative spleen mass. As previously mentioned, adult males (and to a lesser extent subadult males) are involved in fighting and holding harems of hinds during mating, whereas females, after 1 year of age allocate their breeding effort to pregnancy and lactation (Clutton-Brock et al. 1982).

Variation relative to sex and age in the relationships between spleen size, kidney fat, and *E. cervi* infection may reflect an evolved strategy of immune investment

We found a positive relationship between body condition and spleen size in subadult and adult classes. This finding may indicate a greater investment in immune defense by animals of high body condition, as immune defense is costly to mount and maintain (Møller et al. 1998a; Lochmiller and Deerenberg 2000). Individuals in prime body condition would invest more (or more efficiently) in antiparasite defenses, and parasite infection levels would be determined by the immune system working thorough the spleen. It is well known that the nutritional status of the host can influence the rate of acquisition of immunity to parasitic and other infections in man and many animal species, including ruminants (e.g., Coop and Kyriazakis 1999, 2001). Host nutrition (and subsequent improved body condition) can increase the ability of the host to cope with the adverse consequences of parasitism and also affects the development of the host immune response and the establishment and survival of parasite populations (Strain and

Stear 2001). The partitioning of available nutrients between immune responses and other body functions could, in part, explain the immune unresponsiveness to *E. cervi* infection and subsequent larval outputs. Therefore, parasite resistance of individuals may vary as the body condition does.

An optimum balance between the costs of mounting an immune response against the fitness benefits of preventing parasite effects should select a particular resource partitioning between immune defense, growth, and reproductive effort (Rantala and Roff 2005). Female deer engage relatively early in reproduction (as yearling age; Clutton-Brock et al. 1988; Landete-Castillejos et al. 2004) and are able to produce offspring across the entire life span with relatively low variability in reproductive success as compared to males. In contrast, males do not reproduce until they are able to compete with other grown males and need high investment to achieve high reproductive success during a reproductive period shorter than that of females (Clutton-Brock et al. 1982). This scenario may lead to sex-by-age differences in the relative investment in antiparasite defenses. The question that arises is how red deer differ in the strategy of antiparasite defense between sexes.

Energetically demanding periods should induce a trade-off between self-maintenance, parasitism, and breeding effort (Johns et al. 1984). Interestingly, only in adult males did we find a negative relationship between KFI scores and parasite counts. This suggests that depletion of resources during mating season reinforces this negative relationship between spleen mass and parasites, as effort is directed towards intrasexual competition (Clutton-Brock et al. 1982). The lack of a negative relationship between body condition and parasites in females could relate to the fact that females of polygynous species usually are more efficient “energy maximizers” than males are (Owensmith 1984; Wirtz and Oldekop 1991), and they prioritize storing body resources more than males during immunologically constrained periods. Subadult females were the only age class where spleen mass related negatively to parasite counts. In subadult females, the trade-off between immune defense and reproduction (late pregnancy and lactation requirements) may be reinforced by the needs to grow (Landete-Castillejos et al. 2004). Subadult females are still growing females that have just engaged with rearing of offspring (Clutton-Brock et al. 1984). In summary, subadults experienced the most costly period of breeding for a female deer during last gestation and early lactation (up to 95% could become pregnant, Landete-Castillejos et al. 2004), whereas yearlings were sampled during early–mid gestation. This could relate to the finding that yearlings showed higher spleen mass than subadults. A relaxation in immune defense against *E. cervi* at this age could be reflected in the negative relationship between spleen mass and parasite counts. This is so because fecundity of adult extrapulmonary protostrongylids (and therefore fecal larvae

counts) depends on the individual immune status that, in deer, could vary according to specific sex involvement in mating and breeding (Gaudernack et al. 1984; Pelletier et al. 2005).

To our knowledge, this is the first time in a wild mammal that spleen mass has been reported to be positively body-condition-dependent and negatively correlated to infection. Studies in mammals, mostly in rodents, have found a positive relationship between parasite loads or disease and spleen (Vincent and Ash 1978; Garside et al. 1989; Watkins et al. 1991; Hōrak et al. 2006), which suggests that a larger spleen size is a physiological response to parasite infection. Our negative relationship between spleen size and excretion of *E. cervi* larvae suggests that if any causal relationship exists, *E. cervi* larvae excretion would be determined (at least partially) by spleen mass and not vice versa (as may have been inferred if we had found a positive relationship) as a result of greater investment on immunity by healthier and/or higher quality individuals. Therefore, spleen mass could reflect immune capacity in red deer rather than immune system reactivation, and individuals with larger spleens are apparently more capable of maintaining lower parasite levels. In addition to an immune function, in mammal species, the spleen may serve as a reservoir for red blood cells (red pulp of the spleen; Reilly 1985). Therefore, although this research provided novel insights into the reliability of spleen mass as an index of investment in immune function in deer, more research on spleen mass as a measure of immune defense in mammals is needed.

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