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Circulating white blood cell counts in captive and wild rodents are influenced by body mass rather than testes mass, a correlate of mating promiscuity

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Summary

1. Comparative studies of captive primates and carnivores have shown a positive correlation between total white blood cell (WBC) counts and the level of inferred mating promiscuity (e.g. using testes mass). This correlation has been interpreted to support the 'sexually transmitted diseases (STDs)' hypothesis, which states that differential spread of STDs is caused by variation in mating behaviour which shapes baseline aspects of the immune system in mammals.

2. In the present study, we tested the STDs hypothesis in rodents using 28 species from free-ranging and 9 species from captive populations. We compiled data set for the 9 studies of captive rodent populations from the International Species Information System (ISIS) and gathered 136 studies of wild populations from the literature.

3. Using phylogenetic generalized least-squares statistical models considering non-independence resulting from shared ancestry, we confirmed that species with greater adult body mass averaged across sexes had elevated total WBC and differential WBC (neutrophils and lymphocytes) counts and that captive animals presented higher lymphocyte counts than free-ranging ones.

4. However, we found that the total and differential WBC counts did not covary with the residual testes mass – a correlate of mating promiscuity. The results suggest that selection pressures caused by STDs may strongly vary among taxonomic groups. In order to determine the drivers of immunological variation among mammals, further comparative immunological studies including a wide range of taxonomic groups and socio-ecological variables should be performed and we recommend doing so by primarily focusing on free-ranging animals.

Key-words: phylogenetic comparative analysis, STDs hypothesis, testes mass, white blood cell (WBC)

Introduction

With the emergence of ecological immunology as a discipline (Sheldon & Verhulst 1996), a growing number of studies have attempted to explain differences in immune function of vertebrates from an evolutionary perspective (Nunn, Gittleman & Antonovics 2000; Cooper, Kamilar & Nunn 2012). One of the main challenges is to identify biotic and abiotic factors underlying variation in energy invested for the development and maintenance of the immune system (Lochmiller & Deerenberg 2000; Lazzaro & Little 2009; Cutrera *et al.* 2010). In mammals, this investment is generally estimated from total and differential white blood cell (WBC) counts (Nunn, Gittleman & Antonovics 2000; Semple, Cowloshaw & Bennett 2002; Schneeberger, Czirják & Voigt 2013). Studies of captive primates and carnivores have demonstrated a positive correlation between the total WBC counts and the level of mating promiscuity (Nunn, Gittleman & Antonovics 2000, 2003; Anderson, Hessel & Dixson 2004). It has been argued that the correlation results from selective pressure due to increased transmission of sexually transmitted pathogens in species exhibiting high levels of mating

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promiscuity (Smith & Dobson 1992; Thrall, Antonovics & Dobson 2000). Consequently, authors have proposed 'sexually transmitted diseases' (STDs) to be a main driver of the variation in immune investment among mammals in general, a proposition referred to as the STDs hypothesis (Nunn 2002; Nunn, Gittleman & Antonovics 2003).

However, some of the assumptions underlying the STDs hypothesis remain questionable (Read & Allen 2000). First of all, selection pressures caused by STDs are likely to vary among taxonomic groups (Lockhart, Thrall & Antonovics 1996). This is because the levels of STDs exposures are not only constrained by mating promiscuity but also by numerous environmental, demographical, behavioural and life-history parameters (Nesse & Foxman 2011). Additionally, while mating promiscuity may influence STDs, the latter can in turn shape the evolution of the mating system, which will then obscure the relationship between promiscuity and STDs. In particular, it has been predicted that different mating systems can evolve and coexist in the presence of STDs (Boots & Knell 2002). Animals are also known to adjust their behaviour and physiological traits to environmental conditions (Ghalambor *et al*. 2007; Mery & Burns 2010). Support for the STDs hypothesis is derived exclusively from captive animals, while the environment may differ substantially in free-living populations (Calisi & Bentley 2009). For example, free-ranging populations face high inter- and intraspecific competition compared to their captive counterparts, which may lead to relative immunosuppression due to stress (Mason 2010). Thus, the robustness of the STDs hypothesis, as a general explanation for the variation in basal immune investment among mammals, requires further testing including in taxonomic groups with data on wild populations (Nunn & Altizer 2004).

In this study, we tested the STDs hypothesis in rodents using a comparative analysis of 9 studies of captive and 136 studies of wild populations. Rodents constitute the most diverse group of mammals, accounting for over 40% of extant mammalian species and exhibiting a diversity of mating systems (Wolff & Sherman 2007). Similar to previous comparative immunological studies of mammals, we used the total WBC and differential WBC (neutrophils and lymphocytes, generally accounting for ca. 95% of WBC in rodents; Weiss & Wardrop 2010) counts as proxies for basal immune investment. To approximate mating promiscuity, we relied on residual testes mass (here controlled for body mass and phylogenetic relatedness), as this trait offers quantitative information, which has been shown to relate to levels of sperm competition and reproductive skew, and thus can be used as a correlate of promiscuity (Breed & Taylor 2000; Soulsbury 2010; Gómez Montoto *et al*. 2011; Winternitz *et al*. 2013). We are confident that our sampling was not particularly biased toward specific level of promiscuity for the reason that our species selection was driven by the availability of data on immune cell counts. Most of these immunological studies were conducted without focusing on any sexually related behaviour. We investigated the relationship between our proxies for basal immune investment and mating promiscuity using phylogenetic generalized least-squares (PGLS) statistical modelling (Garland & Ives 2000; Martins, Diniz-Filho & Housworth 2002) in order to account for the non-independence caused by phylogenetic relationships among species. We also accounted for the effect of adult body mass because previous studies of captive (e.g. primates, carnivores and ungulates) and free-ranging (e.g. bats and birds) populations showed a positive association between body mass and circulating immune cells (Cooper, Kamilar & Nunn 2012; Schneeberger, Czirják & Voigt 2013; Pap *et al*. 2015). Our results do not support the STDs hypothesis in rodents.

Materials and methods

DATA COMPILATION

For both captive and free-ranging rodents, we compiled the mean testes mass, mean adult body mass of males (male body mass), adult body mass averaged across sexes (adult body mass), and the mean and standard error of the following immune parameters: total WBC, neutrophil and lymphocyte. We obtained immune data for rodents raised in captivity from the International Species Information System (ISIS) (2002). Information on rodents captured in the wild was recovered by carrying out a literature search in the 'Web of Science', 'Google Scholar' and 'China National Knowledge Infrastructure (CNKI)' data base, using the following keywords: 'white blood cell', 'leucocyte' or 'haematology', and each of the 485 generic names describing all extant rodent species (Wilson & Reeder 2005). Additional primary sources were then identified within retrieved publications. The time span for selected publications ranged from 1972 to 2011. We excluded studies for which individuals were under known immunosuppressant status such as hibernation (Bouma, Carey & Kroese 2010) or that had been sampled in polluted areas (e.g. heavy metal contaminated areas, Adham, Al-Eisa & Farhood 2011). We additionally excluded the haematological data for captive capybaras (*Hydrochoerus hydrochaeris*) (ISIS 2002), as it was recently shown that this species present an unusual decrease in immune cell concentration in response to stress (Eberhardt *et al*. 2013). The values derived from this study were clear outliers in most of our analyses, and there was no way to determine the influence of stress as an additional value in the collection of the data. When immune parameters were provided for different age classes, we only considered those of adults. We assumed animals to be adult when no age information was provided.

We collected information about adult body mass from ISIS (2002) for captive animals. For free-ranging animals, adult body mass was obtained either directly from information provided in the references about immunity (if available), or from alternative contemporary publications for the species. Data on testes mass were obtained using additional publications (Table S1, Supporting information). When the weight of only one testis was available, testes mass were estimated by multiplying the testis mass by two; otherwise, we summed the mass of both testes. Male body mass information was also retrieved in order to remove the variation in testes mass explained by allometry during the statistical analysis. Overall, our data base included all relevant variables for 34 rodent species (28 wild and 9 captive species; Fig. 1; Table S1).

STATISTICAL ANALYSIS

All statistical procedures were performed with R 3.1.0 (R Development Core Team 2014). We analysed independently how each

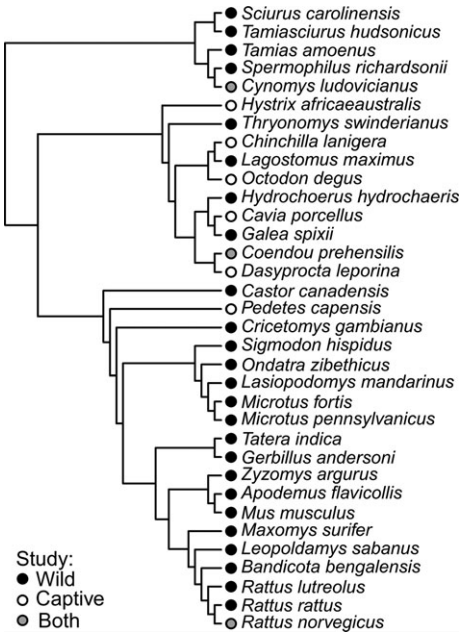

Fig. 1. Consensus phylogenetic tree on the 34 species of rodents included in this comparative analysis. This tree is reconstructed from 8 partial molecular phylogenies (see main text for details).

of the immune parameters (WBC, neutrophil, lymphocyte) relates to testes mass in rodents using phylogenetic generalized least-squares (PGLS) models. For each of the three linear models applied, the dependent variable was the number of immune cells per litre of blood and the covariates were the adult body mass, the residual testes mass (see below) and the captivity status (free-ranging or captive). In order to obtain a suitable distribution of residuals within each linear model, we natural-log-transformed immune cell counts and adult body mass in the analyses. We initially considered both a linear and a quadratic effect of adult body mass in the PGLS models, but found no support for a quadratic relationship. We therefore only presented models considering a linear effect of adult body mass for simplicity.

Body mass and testes mass are highly correlated across mammals, including rodents (Kenagy & Trombulak 1986), and this was indeed the case in our data set (Spearman correlation test, rho = 0·75, $P < 0{\cdot}001$). Therefore, considering both variables as covariates in the same regression model would impede the reliability of parameter estimates due to multicollinearity. We therefore removed the variation in testes mass caused by interspecific differences in body mass before considering the testes mass in the PGLS models (e.g. Breed & Taylor 2000; Gómez Montoto *et al.* 2011; Winternitz *et al.* 2013). To do so, we computed the normalized residual testes mass from another PGLS model predicting testes mass according to male body mass (Table S2). Both variables were expressed on a natural logarithmic scale in order to accurately model the allometric relationship between them (Kenagy & Trombulak 1986; Xiao *et al.* 2011).

In all PGLS models used in this study, including the one for testes mass, we accounted for the potential non-independence that results from shared ancestry between species (Felsenstein 1985; Grafen 1989; Garland, Bennett & Rezende 2005). To do so, PGLS models were fitted using the *gls* function from the package 'nlme' (Davidian & Giltinan 1995). This estimation procedure allows one to specify the dependence structure of the statistical errors and therefore to capture the effect of the phylogeny. Furthermore, this technique allowed us to account for the heteroscedasticity of statistical errors caused by within-study variability resulting from differences in sampling effort, measurement errors or natural processes, which is a common feature in comparative analysis (Paradis 2012). This was performed by weighting each study by the standard errors of the mean immune cell counts (Ives, Midford & Garland 2007). These standard errors did not significantly differ between captive and wild studies (performing simple Mann–Whitney *U*-test or PGLS analysis, data not shown).

To account for the non-independence between species that results from the phylogeny, we initially established the phylogenetic tree of our rodent species (Fig. 1) by building a consensus tree based on eight partial phylogenies (Herron, Castoe & Parkinson 2004; Jansa & Weksler 2004; Steppan, Adkins & Anderson 2004; Blanga-Kanfi *et al.* 2009; List *et al.* 2010; Geffen, Rowe & Yom-Tov 2011; Pérez & Pol 2012; Voss, Hubbard & Jansa 2013). We then compared different models of character evolution and identified the one that best accounts for the potential phylogenetic inertia in our analyses. Specifically, we fitted each PGLS model considering alternatively the Grafen's (1989) or the Pagel's (1999) covariance structure using the relevant functions provided by the package 'ape' (Paradis, Claude & Strimmer 2004). Because divergence times could not be retrieved between all species in our phylogeny, we assumed arbitrary branch lengths (Blomberg, Garland & Ives 2003). For the Grafen's (1989) covariance structure, we followed the author's original recommendations and calculated the branch length for each species as the number of descending taxa minus 1. For the Pagel's (1999) covariance structure, we assumed an initial fixed length of 1 for all branches in the phylogeny. For all regression models, the Grafen's (1989) covariance structure yielded a poorer fit than the Pagel model, as approximated by a large increase in the AIC (Akaike's information criterion) values. Therefore, we chose to retain only PGLS models fitted using the Pagel's (1999) covariance structure. This structure allowed us to quantify the strength of the phylogenetic signal according to the estimation of the lambda parameter (Münkemüller *et al.* 2012).

Results

We investigated the 'sexually transmitted diseases' (STDs) hypothesis in captive and free-ranging rodents using PGLS models accounting for potential phylogenetic inertia. The phylogenetic signal estimated by Pagel's lambda equated to 0·901, 0·899 and 0·993 for the model of WBC, neutrophils and lymphocytes, respectively. For the model used to compute residual testes size, the lambda value was 1·077. All lambda values were significantly higher than zero (likelihood ratio tests on restricted maximum likelihood fit, all likelihood ratios $\geq$13·236, df = 1, all $P < 0{\cdot}001$), rejecting the null hypothesis of the absence of phylogenetic inertia. For all models except those pertaining to neutrophils (likelihood ratio = 5·264, df = 1, $P = 0{\cdot}022$), lambda values did not significantly differ from 1 (all likelihood ratios $\leq$3·116, df = 1, all $P \geq 0{\cdot}078$), which is consistent with independent evolution of characters between species leading to a level of similarity between them proportional to shared evolutionary path lengths (i.e. Brownian motion, Felsenstein 1985; Freckleton, Harvey & Pagel 2002).

We found no support for the STDs hypothesis since the residual testes mass significantly covaried with neither the number of total WBC, nor the number of neutrophils or lymphocytes (likelihood ratio tests, all likelihood ratios $\leq$1·122, all $P \geq 0{\cdot}289$, Fig. 2, Table 1). This was true for

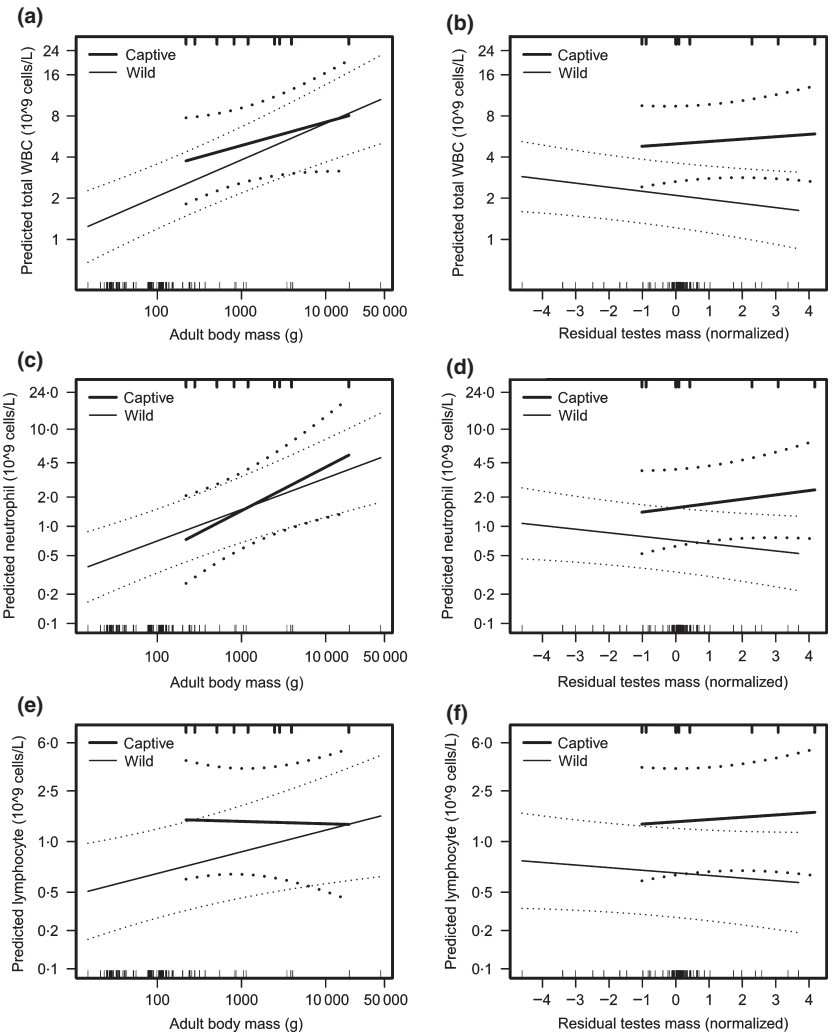

Fig. 2. Influence of body mass and residual testes mass on three types of circulating immune cells for captive and free-ranging rodents. Full lines depict mean effects predicted by the phylogenetic generalized least-squares models presented in Table 1. Dashed lines represent the 95% confidence interval on these mean effects. For plots showing the effect of the adult body mass (or residual testes mass), the residual testes mass (or the adult body mass) is set at the median value across studies with the corresponding captivity status. Upper and lower ticks indicate the x-values observed for captive and free-ranging species, respectively.

both captive and free-ranging populations (likelihood ratio tests of interactions between residual testes mass and captivity status: all likelihood ratios $\leq 2{\cdot}285$, all $P \geq 0{\cdot}131$). The number of total and differential WBC was significantly higher in species heavier than average (Table 1, Fig. 2). The relationship between adult body mass and total and differential WBC counts did not significantly differ between captive and free-ranging populations (likelihood ratio tests of interactions between adult body mass and captivity status: all likelihood ratios $\leq 1{\cdot}425$, all $P \geq 0{\cdot}233$). Independent of the effect of adult body mass, captive populations of rodents presented an amount of natural-log-transformed lymphocyte counts 1·85 SD higher than free-ranging populations (likelihood ratio = 9·577, df = 1, $P = 0{\cdot}002$). In contrast, the number of WBC and neutrophils did not significantly differ between captive and free-ranging populations (Table 1).

Discussion

The 'sexually transmitted diseases' (STDs) hypothesis posits that basal immune investment should increase with mating promiscuity in mammals (Nunn & Altizer 2004). Support for this hypothesis comes from primates and carnivores, and is thought to be generalized to other mammalian groups. However, this hypothesis has only been investigated in captive populations despite known physiological differences between captive and wild populations. Here, we assessed the effects of residual testes mass – a correlate of mating promiscuity – on both total and differential WBC counts – a proxy for immune investment – by performing a phylogenetic generalized least-squares (PGLS) comparative analysis using 145 studies on rodents, the most diverse existing mammalian group. Our sample encompasses a total of 2815 individuals and represents all five suborders of Rodentia, including both captive and free-ranging populations. We found that basal immune investment did not covary with mating promiscuity in captive and free-ranging populations, which does not support STDs as a main driver for rodent immunity.

This finding contrasts with the positive correlation between investment in cellular immune defence and the level of promiscuity previously reported for captive primates and carnivores (Nunn, Gittleman & Antonovics 2000, 2003; Anderson, Hessel & Dixson 2004). In the latter two taxonomic groups, other social or ecological factors

Table 1. Summary table of phylogenetic generalized least-squares models.

	Estimate	SE	LR	df	*P*
WBC					
Intercept	21·121	0·888	–	–	–
Adult body mass (ln)	0·171	0·120	22·816	1	**< 0·0001**
Residual testes mass (ln)	0·040	0·078	1·010	1	0·315
Captivity status	−0·901	0·826	3·641	1	0·056
Adult body mass:captivity status wild	0·096	0·118	0·701	1	0·402
Residual testes mass:captivity status wild	−0·109	0·087	1·603	1	0·205
Neutrophil					
Intercept	17·981	1·288	–	–	–
Adult body mass (ln)	0·449	0·174	17·907	1	**< 0·0001**
Residual testes mass (ln)	0·102	0·114	1·122	1	0·289
Captivity status	0·910	1·197	0·104	1	0·748
Adult body mass:captivity status wild	−0·126	0·172	0·487	1	0·485
Residual testes mass:captivity status wild	−0·188	0·126	2·285	1	0·131
Lymphocyte					
Intercept	21·215	1·251	–	–	–
Adult body mass (ln)	−0·019	0·167	4·433	1	**0·035**
Residual testes mass (ln)	0·041	0·104	0·007	1	0·932
Captivity status	−1·852	1·093	9·577	1	**0·002**
Adult body mass:captivity status wild	0·189	0·160	1·425	1	0·233
Residual testes mass:captivity status wild	−0·089	0·115	0·614	1	0·433

Significance was assessed by *sequentially* performing likelihood ratio tests. SE, standard error of the estimate; LR, likelihood ratio; df, degrees of freedom. Significance level is 0·05 (significant *P*-values in boldface).

revealed weak or lack of correlation with basal immune investment compared with mating promiscuity (Nunn 2002; Nunn, Gittleman & Antonovics 2003). In rodents, Bordes & Morand (2009) found that total WBC count increased with helminth infestation, and another study showed that sociality negatively covaried with species richness of directly transmitted ectoparasites (Bordes, Blumstein & Morand 2007). Moreover, the pathogens causing STDs in Rodentia were relatively less diverse than in primates and carnivores (Lockhart, Thrall & Antonovics 1996). Therefore, STDs may exert weaker selective pressures on immunity in rodents than in other mammalian taxa, when compared to other factors (e.g. helminth infestation, sociality).

Similar to previous studies, we found that the number of total and differential WBC was maximal for species of above average body mass (Cooper, Kamilar & Nunn 2012; Schneeberger, Czirják & Voigt 2013; Pap *et al.* 2015). One explanation could be that larger animals may host more pathogens than smaller ones. Indeed, several hypotheses have been proposed to account for such a relationship. For example, body mass could covary positively with parasite richness due to a larger exposure surface, slower life history or lower competition for resources among pathogens in large individuals (Kuris, Blaustein & Alio 1980; Tella, Scheuerlein & Ricklefs 2002; Johnson *et al.* 2012; Previtali *et al.* 2012). Although several comparative studies found support for a positive association between body mass (or body size) and parasite species richness in some taxa (Poulin 1997; Poulin & Morand 2004), little evidence for such a positive association has so far be found in rodents (Krasnov *et al.* 2004; Bordes, Morand & Krasnov 2011).

Previous research suggested that favourable environmental conditions experienced by captive animals (e.g. *ad libitum* food and water, hygiene: Calisi & Bentley 2009; Mason 2010) may lower the trade-off costs between immunity and other traits with high-energy investment requirements (French, DeNardo & Moore 2007; Speakman 2008; Ardia, Parmentier & Vogel 2011; but see Xu, Yang & Wang 2012) compared to their free-ranging counterparts. Here, we confirmed this effect for lymphocytes, which were more abundant in captive populations than in the wild, but we found no differences for neutrophils and total WBC counts. Neutrophils and lymphocytes are the cellular effectors of the innate and adaptive immunity, respectively, and target different pathogens. In general, neutrophils engulf invading parasites (e.g. bacteria or fungi), while lymphocytes neutralize the parasites (e.g. helminths) and pathogens (e.g. viruses) via specific antibodies or destroy pathogen-infected cells. Therefore, these two kinds of immune cells may require differential energetic investment (McDade 2003), which may explain our results. Other possibilities, such as difference in pathogen exposure or in stress associated with captivity status, are unlikely to account for the observed lymphocytosis in captive populations. Indeed, contrary to our observations, hygienic conditions are generally better in captivity than in the wild and chronic stress is known to induce a decrease rather than an increase, in lymphocytes (Dhabhar 2006; Calisi & Bentley 2009; Mason 2010).

Hence, our study did not support the 'sexually transmitted diseases' hypothesis in rodents despite sufficient statistical power to confirm influences of body mass and captivity status on immunity. This conclusion suggests that the role

of STDs in shaping the immune system may be weaker in some mammalian groups than in others. As a consequence, if selection pressures caused by STDs strongly vary among taxonomic groups, application of traditional PGLS approaches that assume all species to follow a same pattern of regression could be flawed. The current development of (generalized) linear mixed-effect models that can both consider complex covariance structure together with random slopes (Lee, Nelder & Pawitan 2006) may nonetheless offer a general solution to this problem.

In summary, our study highlights the need for further comparative analyses that include more taxonomic groups and more socio-ecological variables in order to determine reliably what drives variation in immune investment among mammals. We emphasize that such studies should primarily focus on free-ranging animals as both benefits (e.g. hygiene, *ad libitum* resources) and costs (e.g. stress) associated with the life in captivity may strongly influence relevant variation in immune function.

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Data accessibility

All data used in this manuscript are present in the manuscript and its supporting information.

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Supporting Information

Additional Supporting information may be found in the online version of this article:

Table S1. Data set.
Table S2. Summary table.