Variations in immune parameters with age in a wild rodent population and links with survival

Coraline Bichet[1], Corinne Régis[2], Emmanuelle Gilet-Fromont[3], and Aurélie Cohas[2]

[1]CEBC
[2]LBBE
[3]VetAgro Sup

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Abstract

Recent findings suggest that immune functions do not unidirectionally deteriorate with age but that a potentially adaptive remodelling where functions of the immune system get down regulated while others get upregulated with age could also occur. Scarce in wild populations, longitudinal studies are yet necessary to properly understand the patterns and consequences of age variations of the immune system in the wild. In the meanwhile, it is challenging to understand if the observed variations in immune parameters with age are due to changes at the within-individual level or to selective (dis)appearance of individuals with peculiar immune phenotypes. Thanks to a long-term and longitudinal monitoring of a wild population of Alpine marmots, we aimed to understand within- and between-individual variation in the immune phenotype with age, in order to improve our knowledge about the occurrence and the evolutionary consequence of such age-variations in the wild. To do so, we recorded the age-specific leukocyte concentration and profile in repeatedly sampled dominant individuals. We then tested whether the potential changes with age were more likely due to within-individual variations and/or selective (dis)appearance. Finally, we investigated if the leukocyte concentration and profiles were correlated to the probability of death at a given age. The leukocyte concentration was stable with age, but the lymphocyte count decreased, while the neutrophil count increased, over the course of an individual's life. Moreover, between individuals of the same age, individuals with fewer lymphocytes but more neutrophils were more likely to die. Therefore, selective disappearance seems to play an important role in the age variations of the immune parameters in this population. Further investigations linking age variations in immune phenotype to individual fitness are needed to understand whether remodelling of the immune system with age could or could not be adaptive.

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Running title: Immune variations with age

Coraline Bichet[a,b], Corinne Regis[c], Emmanuelle Gilot-Fromont[c,d] and Aurélie Cohas[c,e]

[a]Centre d'Etudes Biologiques de Chizé, CNRS-La Rochelle Université, UMR-7372, Villiers-en-Bois, 79360-France

[b]Institut für Vogelforschung "Vogelwarte Helgoland" (Institute of Avian Research), Wilhelmshaven, Germany

[c]UMR-CNRS 5558, Laboratoire Biométrie et Biologie Évolutive, Université Claude Bernard Lyon 1, Villeurbanne, France

[d]Université de Lyon, VetAgro Sup, Marcy-l'Etoile, France

[e]Institut Universitaire de France (IUF)

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Author for correspondence:

C. Bichet

coraline.bichet@gmail.com

Abstract

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Thanks to a long-term and longitudinal monitoring of a wild Alpine marmot population, we aimed to understand within- and between-individual variation in the immune phenotype with age, in order to improve our knowledge about the occurrence and the evolutionary consequences of such age-variations in the wild. To do so, we recorded the age-specific leukocyte concentration and profile in repeatedly sampled dominant individuals. We then tested whether the potential changes with age were more likely due to within-individual variations and/or selective (dis)appearance. Finally, we investigated if the leukocyte concentration and profiles were correlated to the probability of death at a given age.

The leukocyte concentration was stable with age, but the relative number of lymphocytes decreased, while the relative number of neutrophils increased, over the course of an individual's life. Moreover, between individuals of the same age, individuals with fewer lymphocytes but more neutrophils were more likely to die. Therefore, selective disappearance seems to play a role in the age variations of the immune parameters in this population. Further investigations linking age variations in immune phenotype to individual fitness are needed to understand whether remodelling of the immune system with age could or could not be adaptive.

Keywords

Ageing, immunosenescence, immune cells, immune remodelling, survival, mammal, *Marmota marmota*

Introduction

The immune system is of primary importance to control diseases throughout an individual's life, and therefore crucial to its fitness. In vertebrates, the immune system involves different immune functions which are classically divided into innate and adaptive components (Hoebe et al., 2004); in close interaction with each other (Iwasaki & Medzhitov, 2010, 2015). The innate immune functions are the first defence against pathogens, involving phagocytic cells (e.g. neutrophils, macrophages and dendritic cells) and molecules such as cytokines, also able to activate other components of the immune system (Akira et al., 2006; Mantovani et al., 2011; Nathan, 2006; Vivier et al., 2011). The adaptive immune functions comprise a cell-mediated immune response, with the stimulation of T lymphocytes, and a humoral immune response, controlled by activated B lymphocytes that can produce immunoglobulins against specific antigens (Iwasaki & Medzhitov, 2010; Mantovani et al., 2011; Vivier et al., 2011).

Mounting an immune response carries costs (Graham et al., 2005; Lochmiller & Deerenberg, 2000; Maizels & Nussey, 2013) and trade-offs with other life-history traits are likely to emerge (Eraud et al., 2009; Graham et al., 2010; Hanssen et al., 2004; Lemaitre et al., 2015; Viney et al., 2005). Therefore, immunity is likely to change during an individual's life. Changes of immunity with age has been mainly studied in humans and laboratory animals (Bektas et al., 2017; Frasca et al., 2005; Gayoso et al., 2011; Larbi et al., 2008; Noreen et al., 2011; Solana et al., 2012), with the general pattern being a decline in adaptive immunity with age, while innate immunity remains unchanged and inflammatory markers increase (Bauer & De la Fuente, 2016; Franceschi, Bonafe, et al., 2000a; Franceschi, Bonafe, et al., 2000b; Franceschi et al., 2007; Frasca et al.,

2011; Panda et al., 2009; Shaw et al., 2013; Simon et al., 2015). In non-model organisms, a recent review found similar trends (Peters et al., 2019).

Some studies indicate that the decrease in the immune functions with age could impaired survival (e.g. Froy et al., 2019; Hanssen et al., 2004; Schneeberger et al., 2014). However, others suggest that variations in immune functions, characterised by changes in the proportion of the different cells involved in the immune response, could be adaptive (i.e. *immune remodelling*) and could fit with the different immune challenges faced throughout life (Fulop et al., 2018, p. 2018; Mueller et al., 2013; Nikolich-Zugich, 2018). It could even be a combination of both (Fulop et al., 2020).

Because the immune system is complex, involving many cell types and pathways, its characterization in non-model organisms is challenging, thus limiting the study of age-related variation of immunity in free-ranging animals (Boughton et al., 2011; Demas et al., 2011). Nevertheless, cross-sectional studies investigated the variations in the immune function with age (mammals: Abolins et al., 2018; Cheynel et al., 2017; Nussey et al., 2012; birds: Hill et al., 2016; Lecomte et al., 2010; Palacios et al., 2007; Saino et al., 2003; Vermeulen et al., 2017; reptiles: Massot et al., 2011; Ujvari & Madsen, 2011; Zimmerman et al., 2013; see Peters et al. 2019 for a review), and seem to confirm the pattern observed in humans and laboratory animals (see above). However, these studies cannot disentangle whether the observed variations arise from within-individual changes or from processes like selective disappearance, which supposedly eliminate individuals with poor (or unappropriate) immune defences from the population (van de Pol & Verhulst, 2006; van de Pol & Wright, 2009). Longitudinal studies investigating variations in immune functions with age exist, but are still very limited (to the best of our knowledge, seven studies: Beirne et al., 2016; Bichet et al., 2022; Froy et al., 2019; Graham et al., 2010; Roast et al., 2022; Schneeberger et al., 2014; Vermeulen et al., 2017). Therefore, we are far to understand the evolutionary consequences of such variations, and, more broadly how proximate mechanisms, like immunity, could explain (even partly) processes such as ageing (Bouwhuis & Vedder, 2017; Lemaitre et al., 2013; Peters et al., 2019).

In the present study, we recorded the age-specific leukocyte concentration and profile in 52 dominant individuals (i.e. fully grown and reproductive individuals) repeatedly sampled between 2011 and 2015 (for a total of 169 measurements) from a wild and long-term studied (1992-2018) population of Alpine marmots. We first tested whether leukocyte concentration and profile changed within individuals' ages (i.e. within-individual level). We then investigated if changes in these immune parameters could also be explained by selective (dis)appearance of individuals (i.e. among-individual level) with particular immune parameters, influencing their risk of death (survival analysis). Based on the previous studies, we expected the relative number of lymphocytes (mainly involved in acquired immunity) to decrease with age, while the others relative numbers of leukocytes (neutrophils, monocytes, eosinophils; mainly involved in innate immunity) to increase with age, at the within-individual level. We further expect both leukocyte concentration and leukocyte profile to compromise individual age-specific survival.

Material and methods

Studied species

Alpine marmots are territorial, socially monogamous and cooperatively breeding ground-dwelling squirrels (Allainé, 2000). They live in families of two to 16 individuals composed of a dominant pair monopolizing reproduction (Arnold & Dittami, 1997; Cohas et al., 2006; Hacklander et al., 2003), sexually mature ([?] 2 years) subordinates of both sexes, yearlings and pups of the year (Allaine, 2000). At sexual maturity, subordinates may keep their status, attempt to reach dominance in their natal groups or disperse to gain dominance in another territory (Lardy et al., 2012). Once an individual reaches dominance, it cannot reverse to subordinate status. Dominance is established for several years and lasts until the dominant individual is evicted or dies (Lardy et al., 2011). During the 23 years of study, only three males and one female lost their dominant status but established dominance in another territory (Lardy et al., 2011).

Field methods

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As part of a long-term study at La Grande Sassiere Nature Reserve (2,340 m a.s.l., French Alps, 45o29'N, 65o90'E, (see Cohas et al., 2008 for details), we captured marmots annually, from mid-April to mid-July using live traps placed close to the main burrows to assign trapped individuals to their family. Individuals were marked with a transponder and a numbered ear-tag, combined with a coloured plastic ear-tag for dominant individuals. At each capture, individuals were tranquilized by an intramuscular injection of Zoletil 100 (0.1 ml.kg^{-1}), sexed, aged, weighed and their social status was determined (large scrotum for dominant males and prominent teats for dominant females, characteristics of each sex all year round independently of reproduction). Social status was further confirmed by observations of scent-marking behaviour and territorial defence that are characteristics of dominants. Exact age was determined for the individuals born on the study site. For dominant immigrants (5 individuals), we assigned the age of three when they first reproduce, as marmots disperse at two years old and never reproduce before three years old. To determine individual fates, capture histories were combined with intensive observations (each family being observed on average 1 hour per day for a minimum of 30 hours per year, for details see Cohas et al., 2008). In our population the maximum lifespan observed over the 30 years of the study is 16 years, and the generation time is about 5 years (Devillard, unpublished data). At each capture, a blood sample (2 ml.kg^{-1} up to 5 ml per individual representing less than 5% of the total volume blood) was taken from the saphenous vein within 30 minutes after capture.

To ensure that all the individuals included in this study were in a comparable social status, and all fully grown and reproductive individuals, we restricted the subsequent analysis to the sole dominant marmots, (47 born on the study site and 5 immigrants, removing immigrants did not change qualitatively the results, nor their interpretations).

Leukocyte concentration

A 20μl blood filled capillary was released in 1ml of a kit solution (LEUKO-TIC "blue", Bioanalytic, Germany). This solution allows a microscopic count of leukocytes after the lysis of the erythrocytes and the fixation of the leukocyte nucleus stained in light blue. The counts were done at x100 enlargement using a Malassez counting chamber by a single observer (C.R.). Only the leukocytes entirely located inside the four 1mm^{2} corner squares (total volume of $4*1mm^{2}*0.2mm = 0.8μl$) were counted. All determinations of leukocyte concentration were done within 24 hours after blood collection.

Between 2013 and 2015, the leukocyte concentration was determined for 79 samples from 34 dominant individuals sampled between two and three times. 23 individuals had two samples in different years and 11 individuals had three. In this dataset, the age of the individuals varied from 3 to 12 years with an average age at 6.2 years (Figure S1A).

Leukocyte profile

Immediately upon blood collection, a drop of blood was smeared onto a slide, later stained with Giemsa stains using an aerospray (Aerospray Hematology Slide/Cytocentrifuge 7150, Wescor, France). Neutrophils, lymphocytes, monocytes, eosinophils and basophils were counted (observer: CR) up to 100 leukocytes, at 100x enlargement, according to Hawkey and Dennett's criteria (Hawkey & Dennett, 1989).

In Mammals, lymphocytes and neutrophils make up the majority (80%) of the leukocytes (Jain 1993). Lymphocytes play a central role in adaptive immunity: they are involved in immunoglobulin (antibodies) production, in the modulation of immune defence and in the production of memory cells (Jain, 1993; Roitt et al., 2001). Neutrophils are involved in the innate immune response as the primary phagocytic leukocytes, and circulating phagocytes proliferate in response to infections, inflammation and stress (Jain, 1993). Monocytes are long-lived phagocytic cells associated with innate defences against infections and bacteria (Roitt et al., 2001). They differentiate into macrophages in tissues and they are also involved in antigen presentation and cytokine production. Eosinophils play a role in the inflammation process and are associated with defence against internal parasites (Jain, 1993). Basophils, which are rare, play a key role against macroparasites and

are also involved in the inflammation process (Karasuyama et al., 2011). The relative number of basophils was low for nearly all individuals (min = 0, max = 49, median = 0) and therefore excluded in the subsequent analyses.

Between 2011 and 2015, the leukocyte profiles were determined for 169 blood smears from 52 dominant individuals sampled between two and five times. 18 individuals had two samples in different years, 12 had three, 13 had four, and 9 individuals had five. 33 of them were both measured for leukocyte concentration and leukocyte counts (which corresponds to 75 measurements). In this dataset, the age of the individuals varied from 3 to 12 years with an average age at 5.8 years (Figure S1B).

Statistical analyses

All statistical analyses were performed with R 3.6.1 (R Core Team, 2014).

Immune phenotype and variation with age

Within-individual variation in immune phenotype with age

To test whether the leukocyte concentration and profiles varied with age of an individual, we used the leukocyte concentration (log-transformed) as dependent variable in a Linear Mixed Model (LMM) and the relative numbers of lymphocytes, neutrophils, monocytes and eosinophils as dependent variables in four Generalised Linear Mixed Models (GLMMs) with a Poisson distribution (appropriate for the observed distribution of count data). The age was partitioned into "average age" and "delta age" components; where average age represents the among-individual age effect and delta age the within-individual age effect (van de Pol & Wright, 2009). The average age was calculated as the average of all ages at which an individual's leukocyte concentration or profile were measured. The delta age corresponds to the difference between the individual age at measurement and its average age (i.e. delta age = age - average age). To investigate the potential non-linear within-individual variations of the leukocyte concentration and profile with age, we followed the equation (3) recommended in Fay et al. (2022). This equation proposes to model a within-individual quadratic effect of age as age^2 - (average age)^2.

Body mass at capture, capture date, year of capture and the interaction between capture date and year of capture were further included as fixed effects. Because individuals were sampled several times over the years, we included individual's identities as random intercepts. Our models also included the interaction between "average age" ands sex as well as the interactions between sex and both the linear and quadratic effect of "delta age" to test for sex-differences in the leukocyte variation with age.

Among-individual variation in immune phenotype with age

Models including average and delta age cannot properly investigate the occurrence of selective appearance and disappearance of peculiar individual phenotypes from the population (Fay et al., 2022; van de Pol & Verhulst, 2006). For this purpose, we replaced the "average age" and "delta age" (linear and quadratic) effects in the previously selected within-individual models by the *'age at access to dominance'* , to assess selective appearance, and the *'age at last observation'* , to assess selective disappearance (Fay et al., 2022; van de Pol & Verhulst, 2006).

The functions "lmer" and "glmer" in the package "lme4" (Bates et al., 2015) were used to fit the models (Bolker et al., 2009). Final models were selected using a backward elimination procedure. We measured zero-inflation and variance inflation factors (VIFs) in all our models using the R package "performance" (Lüdecke et al., 2020). For all models, we checked *a posteriori* the distribution of the residuals to assess the fit of the models to the observed data. Since we observed moderate overdispersion (all dispersion ratios < 2.58) in some of our models (models for lymphocytes and neutrophils), we estimated all models' parameters using a Bayesian approach. From the final models, we used the "sim" function from the R-package "arm" to simulate values from the posterior distributions of the model parameters (Gelman & Yu-Sung, 2020). The

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95% credible intervals (CI) around the mean were obtained after 5000 effective simulations. Assessment of statistical support was obtained from the posterior distribution of each parameter. We considered a fixed effect to be important if zero was not included within the 95% CI.

Immune phenotype and survival probability

We tested whether the risk to die depended on leukocyte characteristics with mixed-effects Cox right-censored regression models (Nenko et al., 2018; Ripatti & Palmgren, 2000; Therneau et al., 2003). These models included leukocyte concentration or profiles as time-dependent covariates and survival as response variable using the "coxme" function in the "coxme" R package (Therneau, 2018). The age at first capture and the sex were also included as fixed effects. Individual identity and year of birth were added as random effects to take into account repeated measurements and cohort effects (Table 2). The data were encoded with a zero as starting point for all individuals and with the years to death, to the end of the study, or to the next capture (for individuals with repeated data) as right-censor stop points (Therneau, 2018). For the repeated data, the next interval started with the end of the previous interval. A '1' was assigned to the event variable, if the individual died during the interval. We assumed that an individual died if it was neither captured nor observed the following spring (monitored until 2018). A hazard ratio higher than one indicates that the corresponding explanatory variable is associated with an increased risk of death. All individuals were followed until death (n = 27 for leukocyte concentration and n = 43 for leukocyte counts) or still alive in 2018 (n = 4 for leukocyte concentration and n = 6 for leukocyte counts). Three individuals were excluded from this analysis because their fate (alive or dead) was uncertain, due to a capture permit forbidding the monitoring of their families in 2017 and 2018.

Results

The relative number of lymphocytes and neutrophils, as well as the relative number of neutrophils and monocytes, were negatively correlated, while the relative number of monocytes and eosinophils were positively correlated (Table S1).

Immune phenotype and variation with age

Over the course of an individual's life, the number of lymphocytes decreased with age (quadratic delta age: $\beta = 3.70.10^{-1}$, 95% CI = 0.26, 0.47; delta age: $\beta = -5.55.10^{-1}$, 95% CI = -0.71, -0.39; Table 1; Figure 1B). On the reverse, the number of neutrophils (quadratic delta age: $\beta = -0.25$, 95% CI = -0.32, -0.18; delta age: $\beta = 0.35$, 95% CI = 0.25, 0.45; Table 1; Figure 1C) and eosinophils (quadratic delta age: $\beta = -0.36$, 95% CI = -0.711, -0.019; delta age: $\beta = 0.262$, 95% CI = -0.62, 0.67; Table 1; Figure 1E) increased while an individual ages.

The age at access to dominance did not affect both lymphocytes and neutrophils relative numbers (Table 2). However, among-individuals, the higher the age at last observation increased, the higher the number of lymphocytes ($\beta = 0.27$, 95% CI = 0.15, 0.40) and the lower the number of neutrophils ($\beta = -0.15$, 95% CI = -0.21, -0.08) were. This indicates no selective appearance but selective disappearance for individuals with low number of lymphocytes and high number of neutrophils.

The leukocyte concentration (Figure 1A) and the number of monocytes (Figure 1D) did not vary with age (both among- and within-individual, linear or quadratic effects were not significant, Table 1) and were not affected by age at access to dominance nor by age at last observation (Table 2).

We did not find any difference in the age-trajectories between males and females (non-significant interactions between 'delta age' and 'sex' removed from the final models). We also did not observed sex differences in the average leukocyte concentration and profiles, except that males had fewer lymphocytes than females ($\beta = -0.22$, 95% CI = -0.41, -0.04, Table 1).

Immune phenotype and survival probability

We detected a positive correlation between the leukocyte concentration and the risk to die at a given age (Table 3). Moreover, we found that the relative number of neutrophils was positively correlated, while the relative number of lymphocytes was negatively correlated, with the risk of death (Table 3). The relative numbers of monocytes and eosinophils were not correlated with the risk to die (Table 3).

Discussion

The immune parameters (leukocyte concentration and leukocyte profile) measured on the marmots varied with their age. At the within-individual level, although the leukocyte concentration remains stable over the course of an individual life, the relative number of lymphocytes decreased, while the relative numbers of neutrophils and eosinophils increased with age. Lymphocytes play a central role in acquired immunity, being involved in immunoglobulin and memory cell production (Jain, 1993; Roitt et al., 2001). Neutrophils and eosinophils are both involved in the innate immune response, and more specifically in the inflammatory process (Jain, 1993). While neutrophils are the primary phagocytic leukocytes and eosinophils are often associated with defences against internal parasites (Jain, 1993).

The decrease in the number of lymphocytes with age is often interpreted as a consequence of the gradual decline over age in the generation, in the thymus, of new naïve T lymphocytes, responsible for generating new input in the immune memory. Such gradual decline is often suggested to lead to a decrease in the efficiency of the acquired immune system (Dowling & Hodgkin, 2009; Hakim & Gress, 2007; Shanley et al., 2009). Similarly, the observed increase in the relative number of neutrophils does not necessarily mean a higher performance of the innate immune system with age. Indeed, the phagocytic ability of neutrophils could decrease with age (Gomez et al., 2008) and an adaptive compensatory mechanism for such decline in neutrophil performance could lead to an increase in their relative number.

However, a decrease in lymphocytes, together with an increase in neutrophils (Cheynel et al., 2017; Kirk et al., 2010; and in Roast et al., 2022, even if not significant), and more broadly, a decrease in the acquired immune system combined with an increase (or upkeep) in the innate immune system, with age, has been observed in various vertebrate species (Franceschi et al., 2000a; Franceschi et al., 2000b; reviewed in Peters et al., 2019). Such modification of the acquired/innate immune balance with age (McDade et al., 2016), called *immune remodelling* , could be interpreted as an increased resource allocation strategy towards the production of cheaper immune components, at the expense of the more costly immune components (Klasing, 2004). Indeed, the acquired immune components are generally thought to be more expensive to maintain (Lee, 2006), and therefore, more prone to decline with age than the innate immune components (Peters et al., 2019). This could reflect that immune parameters are traded-off with other life history traits, and/or that terminal investment is unlikely to be turned to these parameters since their costs of production would outweigh their future benefit when age-related mortality becomes imminent. In such a scenario, given the lower probability to encounter new pathogens at old ages, downregulating the acquired immune system would not necessarily be the sign of any malfunction, but could be adaptive (Fulop et al., 2018). Consequently, immune systems should not be considered to undergo unidirectional deterioration with age (i.e.senescence) but would probably be better described by taking into account remodelling and reshaping of the immune functions with age (Fulop et al., 2018).

Apart from the hypotheses exposed above, age-related changes in the level of environmental stress endorsed by an individual could also affect the relative numbers of lymphocytes and neutrophils. Indeed, stress hormones such as glucorticoïds stimulate an influx of neutrophils from tissues into the blood; concomitantly, it causes a migration of lymphocytes from the blood circulation to other compartments (Dhabhar, 2002). Thus, a rise of plasma glucocorticoïds caused by stress increases the neutrophils to lymphocytes ratio over a time span of hours (Davis et al., 2008; Lopez-Olvera et al., 2007). Nevertheless, to date, no link has been clearly established between environmental stress, levels of glucocorticoids and patterns of age variations of lymphocytes and neutrophils (see for instance Roast et al., 2022; Watson et al., 2016).

We observed fewer lymphocytes for marmot males than for females. Various hypotheses such as sex-differences in resource allocation strategy, intra-sexual competition (Metcalf & Graham, 2018; Sheldon & Verhulst, 1996)

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or inhibition of the immune system by some steroid hormones were often suggested to induce differences between males and females (Gubbels Bupp et al., 2018; Klein & Flanagan, 2016; Taneja, 2018). However, we did not observe sex-specific differences in the variation of the immune phenotype with age. So far, studies of sex-specific variation on immune parameters with age remain equivocal: some suggested sex differences (e.g. Bichet et al., 2022; Gubbels Bupp et al., 2018; Tidière et al., 2020; van Lieshout et al., 2020), while others did not (e.g. Brooks & Garratt, 2017; Cheynel et al., 2017; Kelly et al., 2018; Peters et al., 2019). For instance, van Lieshout et al. (2020) found a decrease in the proportion of lymphocytes with age in male badgers (*Meles meles*), but not in females. The authors argued that this result could be explained by the high testosterone levels observed in male badgers, due to their polygynandrous mating system (Buesching et al., 2009), contrary to monogamous species (Sugianto et al., 2019) such as the Alpine marmot (Allainé, 2000; Cohas et al., 2006).

In our study, individuals with fewer lymphocytes but more neutrophils were more likely to die (Table 3), as also indicated by a significant selective disappearance of individuals with this phenotype (Table 2). Innate cellular response (involving neutrophils) can be costly in terms of energy, as well as autoimmune (Lee, 2006) and inflammatory damages (Franceschi et al., 2018; Goto, 2008). Individuals with neutrophil-oriented response may be unable to mount an appropriate immune response against challenges encountered at old ages (Froy et al., 2019), and/or may pay an excessive cost to this response and die (Pawelec, 2018). Studies investigating the potential links between age variation in immune phenotype and individual fitness are still scarce and show contrasting results (see also Froy et al., 2019). For instance, in the greater sac-winged Bat (*Saccopteryx bilineata*), the number of leukocytes decreased with age, both within- and among-individuals, while the immunoglobulin G concentration was higher in older individuals, but did not vary within individuals, and the bacterial killing capacity of the plasma did not vary with age, at both levels (Schneeberger et al., 2014). These variations with age also impacted the short-term survival probability (Schneeberger et al., 2014). However, in a study on purple-crowned fairy-wrens (*Malarus coronatus*), Roast et al. (2020) found no evidence that high levels of innate immune functions impaired short-term survival, nor any other fitness traits (annual reproduction and dominance acquisition). In our marmot population, more investigations on the link between fitness and immune variations with age are needed to better understand the evolutionary consequences of the within-individual age variations and the selective disappearance we observed.

More generally, to understand the complexity of age-related changes in immune functions, as well as their evolutionary causes and consequences, we must not only supplement the existing longitudinal studies focused on age-related pattern of immune parameters (to the best of our knowledge, seven studies: Beirne et al., 2016; Bichet et al., 2022; Froy et al., 2019; Graham et al., 2010; Roast et al., 2022; Schneeberger et al., 2014; Vermeulen et al., 2017), but also relate the observed patterns to individual fitness. Such studies are crucial to disentangle whether remodelling of the immune system with age could or could not be adaptive.

Data Accessibility

Data is available from the Dryad Digital Repository upon acceptance.

Competing Interests

The authors declare that they have no conflict of interest.

Author Contributions

C.B., E.G.-F. and A.C. designed the study; A.C. collected blood samples; C.R. and E.G.-F. did the laboratory work; C.B. analysed the data; C.B. and A.C. wrote the paper with contributions from all authors.

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Tables

Table 1: Parameter estimates and credible intervals at 95% (CI) for the selected models testing whether within-individual variation in leukocyte concentration or relative number of each type of leukocytes were explained by age. Parameters were obtained from the minimal adequate models. Significant effects (CI which do not overlap zero) are in bold. "- " means a parameter not retained in the model.

Dependant variable	Leukocyte concentration*	Leukocyte concentration*	Number of lymphocytes**	N
Fixed effects	Estimate	95% CI	Estimate	9
Intercept	**16.51**	**16.17, 16.86**	**3.27**	3
Average age	-0.06	-0.16, 0.05	-0.01	-
Delta age	-0.11	-0.31, 0.09	**-0.55**	-
Quadratic delta age	-	-	**0.37**	0
Sex (male)	-0.09	-0.30, 0.13	**-0.22**	-
Body mass	-0.05	-0.17, 0.08	**0.14**	0
Date	0.06	-0.08, 0.20	**0.09**	0
Year (2012)			0.09	-
Year (2013)			**0.45**	0
Year (2014)	**0.44**	**0.10, 0.78**	0.32	-
Year (2015)	0.49	-0.06, 1.04	0.35	-
Date:year 2012			**-0.27**	-
Date:year 2013			**-0.2**	-
Date:year 2014	-	-	-0.07	-
Date:year 2015	-	-	-0.07	-
Random ID (variance)	**0.03**	**0.02, 0.04**	**0.1**	0

*Between 2013 and 2015, the leukocyte concentration was determined for 79 samples from 34 individuals sampled between two and three times. 23 individuals had two samples in different years and 11 individuals had three.

**Between 2011 and 2015, the leukocyte counts were determined for 169 blood smears from 52 individuals sampled between two and five times. 18 individuals had two samples in different years, 12 had three, 13 had four, and 9 individuals had five. 33 individuals were both measured for leukocyte concentration and leukocyte counts (which corresponds to 75 measurements).

Table 2: Parameter estimates and credible intervals at 95% (CI) for the selected models testing whether variation in leukocyte concentration or relative number of each type of leukocytes were explained by age, age at access to dominance (AAD, i.e. selective appearance) and age at last observation (ALO, i.e. selective disappearance). Parameters were obtained from the minimal adequate models. Significant effects (CI which do not overlap zero) are in bold. "-" means a parameter not retained in the model.

Dependant variable	Leukocyte concentration*	Leukocyte concentration*	Leukocyte concentration*	N
Fixed effects	Estimate	95% CI	Estimate	Es
Intercept	**16.7**	**16.47, 16.92**	**3.51**	**3.**
Age	0.09	-0.11, 0.30	**-0.28**	**-0**
AAD	-0.12	-0.25, 0.00	-0.01	-0
ALO	-0.09	-0.28, 0.09	**0.27**	**0.**
Sex (male)	-0.09	-0.29, 0.12	**-0.3**	**-0**
Body mass	-0.05	-0.17, 0.07	**0.15**	**0.**
Date	0.06	-0.08, 0.20	**0.13**	**0.**
Year (2012)			-0.03	-0
Year (2013)			**0.26**	**0.**
Year (2014)	**0.28**	**0.02, 0.54**	0.09	0.
Year (2015)	0.14	-0.14, 0.43	0.08	0.
Date:year 2012			**-0.29**	**-0**
Date:year 2013			**-0.22**	**-0**
Date:year 2014	-	-	-0.13	-0
Date:year 2015	-	-	-0.1	-0
Random ID (variance)	0.02	0.01, 0.03	**0.08**	**0.**

*Between 2013 and 2015, the leukocyte concentration was determined for 79 samples from 34 individuals sampled between two and three times. 23 individuals had two samples in different years and 11 individuals had three.

**Between 2011 and 2015, the leukocyte counts were determined for 169 blood smears from 52 individuals sampled between two and five times. 18 individuals had two samples in different years, 12 had three, 13 had four, and 9 individuals had five. 33 individuals were both measured for leukocyte concentration and leukocyte counts (which corresponds to 75 measurements).

Table 3: Associations between immune phenotype and the risk of death. Significant effects are in bold.

Time-dependent covariate	Leukocyte concentration (N = 72, n = 27 events)	Leukocyte concentration (N = 72, n = 27 events)	Leukocyte concentration (N = 72, n = 27 events)	Number of lymphocytes (N = 163, n = 43 events)	Number of lymphocytes (N = 163, n = 43 events)	Number of lymphocytes (N = 163, n = 43 events)	Number of neutrophils (N = 163, n = 43 events)	Number of neutrophils (N = 163, n = 43 events)	Number of neutrophils (N = 163, n = 43 events)	Number of neutrophils (N = 163, n = 43 events)	Number of monocytes (N = 163, n = 43 events)	Number of monocytes (N = 163, n = 43 events)	Number of monocytes (N = 163, n = 43 events)	Number of monocytes (N = 163, n = 43 events)	Number of eo (N 16 n 43 ev

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|	Hazard ratio ± SE	Z value	p-value	Hazard ratio ± SE	Z value	p-value	p-value	Hazard ratio ± SE	Z value	p-value	p-value	Hazard ratio ± SE	Z value	p-value	p-va
Leukocyte variable	**1.00 ± 0.00**	**1.97**	**0.049**	**0.96 ± 0.01**	**-3.00**	**0.003**	**0.003**	**1.03 ± 0.01**	**2.80**	**0.005**	**0.005**	0.93 ± 0.07	-1.02	0.310	0.3
Age at first capture	**1.31 ± 0.10**	**2.68**	**0.007**	1.11 ± 0.09	1.21	0.230	0.230	1.12 ± 0.09	1.30	0.190	0.190	1.07 ± 0.09	0.78	0.440	0.4
Sex (male)	0.98 ± 0.40	-0.04	0.970	-0.53 ± 0.33	-1.90	0.058	0.058	0.61 ± 0.33	-1.54	0.120	0.120	0.72 ± 0.32	-1.02	0.310	0.3

Figures
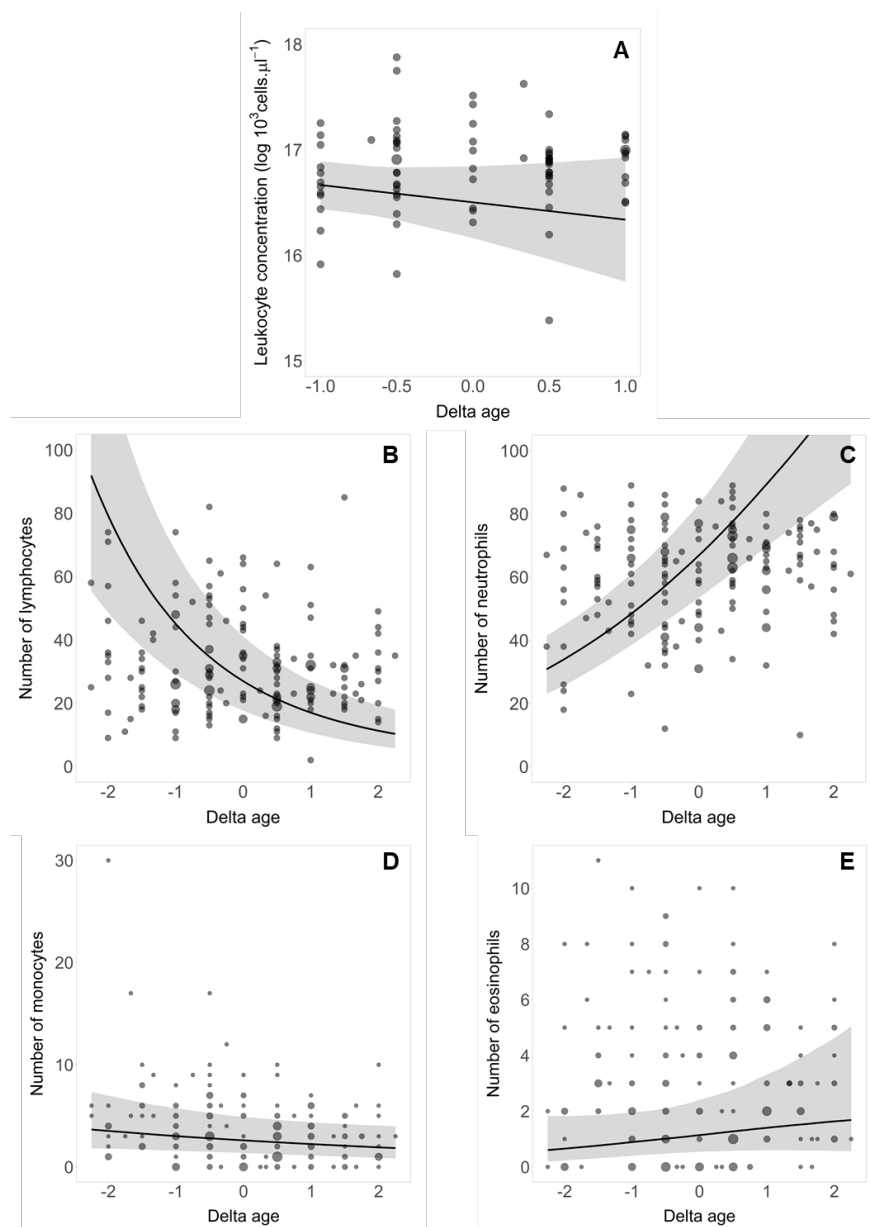
Figure 1: Within-individual age variations (delta age) of (A) leukocyte concentration and profiles for (B) the relative number of lymphocytes, (C) neutrophils, (D) monocytes and (E) eosinophils. Dots represent the raw data with size proportional to the sample size. Black solid lines represent model predictions with their 95% prediction interval (grey areas). Model predictions and their intervals were obtained over all samples for (A) and by fixing the continuous variables to their mean over all samples and the sex to female, the year to 2013 and the individual random effect to a given individual for (B, C, D, E).

Supporting Information

Table S1: Pearson coefficients of correlation between the leukocyte concentration and the lymphocytes, neutrophils, monocytes and eosinophils. Values in brackets represent the 95% confidence intervals. Significant correlations are in bold.

Figure S1: Histograms illustrating the age-distribution at sampling of the marmot individuals included in the study, for (A) leukocyte concentration and (B) leukocyte profile.