Social effects on age-related and sex-specific immune cell profiles in a wild mammal

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Abstract

Evidence for age-related changes in innate and adaptive immune responses is increasing in wild populations. Such changes have been linked to fitness, and understanding the factors driving variation in immune responses is important for the evolution of immunity and senescence. Age-related changes in immune profiles may be due to sex-specific behaviour, physiology and responses to environmental conditions. Social conditions may also contribute to variation in immunological responses, for example, through transmission of pathogens and stress from resource and mate competition. Yet, the impact of the social environment on age-related changes in immune cell profile requires further investigation in the wild. Here, we tested the relationship between leukocyte cell composition (agranulocyte proportion, i.e. adaptive and innate immunity) and age, sex, and group size in a wild population of European badgers (*Meles meles*). We found that the proportion of agranulocytes decreased with age only in males living in small groups. In contrast, females in larger groups exhibited a greater age-related decline in the proportion of agranulocytes compared to females in smaller

groups. Our results provide evidence for age-related changes in immune cell profiles in a wild mammal, which are influenced by both the sex of the individual and their social environment.

Keywords (3 to 6): European badger, social effects, innate immunity, adaptive immunity, leukocytes

1. Introduction

The immune system involves multiple mechanisms that protect the host against pathogens [1]. The functioning of the immune system is related to sex [2, 3] and changes throughout life [4-9]. Since age-related changes in immune responses have been linked to mortality in the wild [9], understanding the factors driving differences in immune responses can provide insight into the evolution of immunity and senescence.

The immune system comprises two components: innate and adaptive immunity [1]. The innate immune response is the first defence against pathogens, involving phagocytic cells (e.g. neutrophils, macrophages and dendritic cells) to detect antigens and produce cytokines that trigger other parts of the immune system [10-14]. The activation of adaptive immunity includes the cell-mediated immune response, with the stimulation of T lymphocytes and humoral immunity, which is controlled by activated B lymphocytes that can differentiate to produce immunoglobulins against specific antigens [13, 15]. The relative components of adaptive and innate immunity are therefore reflected in agranulocytes (i.e. lymphocytes and monocytes) and granulocytes (i.e. neutrophils, eosinophils and basophils), respectively [16-19].

The adaptive immune system generally undergoes an age-related decline in performance, i.e. immunosenescence, and evidence for this process has been emerging in wild populations [4-9]. In contrast, the innate immune response is usually maintained, or even enhanced with age [4-9]. This enhanced innate immune response can be a consequence of overstimulation of the immune system, due to a reduced T cell repertoire and bias towards CD8+ effector memory cells, leading to chronic inflammation and accelerated immunosenescence, as seen in humans [20, 21].

53 The innate and adaptive immune responses, mediated by genes and hormones, are sex-
54 specific [2, 3]. For example, in the human innate immune response, males have higher frequencies of
55 natural killer cells and higher phagocytic activity of neutrophils and macrophages than females [22,
56 23], whereas in the adaptive immune response, females have stronger antibody responses and have
57 higher basal immunoglobulin levels and B cell numbers than males [22, 24]. Such sex differences in
58 immune responses may be exacerbated with age [3, 25]. For example, male Soay sheep (*Ovis aries*)
59 exhibit steeper sex-specific changes in leukocyte cell composition with age [26]. However, such
60 changes may be species-specific since no sex differences in the rate of change in leukocyte cell
61 composition with age were detected in roe deer (*Capreolus capreolus*; [5]).
62 Social stress is emerging as a potential driver of variation in immune responses in the wild [27-
63 29], where gregarious species often experience greater stress due to social interactions or increased
64 mate competition [28, 30, 31]. For instance, polygynous males have more circulating testosterone
65 than conspecific females or monogamous males, which has a suppressive effect on the immune
66 system [32, 33], indicating a potential role for the social system and the environment in sex-specific
67 immune cell profiles. Moreover, social species may experience the costs of increased pathogen
68 exposure due to group-living compared with solitary species [29]. For example, greater early-life
69 exposure to pathogen variety and intensity within social groups could prime the immune system and
70 result in enhanced later-life immunity with the risk of late-life auto-immunity [34, 35]. However, to
71 date, there has been no clear evidence for the effects of the social environment on sex-specific
72 immune cell profiles and their age-related changes.
73 Here, we use blood samples collected across 2017 and 2018 from a wild population of
74 European badgers (*Meles meles*; hereafter 'badger') to explore longitudinal changes in sex-specific
75 immune cell profiles and how this relates to social conditions. We quantify the relative components
76 in the immune system through the proportion of agranulocytes out of the total number of leukocytes,
77 which reflect the relative balance between adaptive and innate immunity [16-19]. Specifically, we test

78 whether the proportion of agranulocytes: (i) changes with age, (ii) exhibits sex differences, and (iii) is
79 linked to group size.
80
81 **2. Methods**
82 **(a) Study species and data collection**
83 We conducted this study in Wytham Woods, Oxfordshire, UK (51°46'24"N, 1°20'04"W), a 424 ha semi-
84 natural woodland surrounded by mixed arable pasture [36]. The resident high-density badger
85 population (mean±SE = 36±3 badgers/km^2; [37]) is segregated into large mixed-sex social groups
86 (mean group size = 11, range = 2–29; [38]). Badgers have a polygynandrous mating system with high
87 extra-group paternity [39, 40], where males exhibit seasonal peaks in testosterone levels [41, 42].
88 Badgers are exposed to pathogens such as coccidia which negatively impacts development and causes
89 juvenile mortality [43-45].
90 Trapping was undertaken three times per year, for three consecutive days per social group.
91 Trapped badgers were anaesthetised using an intra-muscular injection of 0.2 ml ketamine
92 hydrochloride per kg body weight [46]. Individuals were identified by a unique tattoo number on the
93 left inguinal region, with capture date, social group affiliation and sex recorded. Age was determined
94 as the difference between capture date and the 14th of February in the respective birth years. Badgers
95 first caught as adults were aged through tooth wear [47], where a tooth wear score of 2 typically
96 indicates a 1-year old adult. Blood was collected through jugular venipuncture into vacutainers with
97 EDTA anticoagulant. Badgers were released at their setts, after full recovery from anaesthesia.
98 Additionally, bait-marking was conducted periodically to delimit social group range sizes [48] and
99 calculate group sizes using appropriate dispersal rules (see supporting information).
100 Immediately after blood collection, one drop of blood from the vacutainers was smeared on
101 a glass microscope slide. Slides were air-dried for one hour and subsequently stained using a Kwik-Diff
102 staining kit (Thermo Scientific, Manchester, UK) according to the manufacturer's protocol. Leukocyte
103 cell counts were conducted by the same observer (blind to group size and sex) by counting 100 cells

per slide (4 repeats per slide, not consecutively to avoid bias; *n* = 82 slides, 23 individuals; 9 females, 14 males), at 40x magnification using the 'battlement technique' [49]. Cells were identified as neutrophils, eosinophils and basophils (i.e. granulocytes) or lymphocytes and monocytes (i.e. agranulocytes; [50]). From these data, we calculated the proportion of agranulocytes out of the total number of leukocytes. Slides containing less than 100 white blood cells were turned into proportions (*n* = 7 repeats, 5 slides).

(b) Statistical analyses

Statistical analyses were conducted in R 3.3.1 [51], using a log-likelihood ratio test to determine significance of predictors, set at $p < 0.05$, in *lme4* 1.1-14 [52]. The mixed model had a binomial error distribution (link = logit) with the proportion of agranulocytes in the leukocytes as the response variable. We first tested which age transformation (linear or logarithmic) best fitted these data using AICc values, where the relationship between the proportion of agranulocytes and age followed a negative logarithmic pattern (ΔAICc = 2.9). Logarithmic age was included in the mixed model along with sex, group size, and the interactions between the three. Season was included as a fixed factor and body condition index ($\log_{10}$weight/$\log_{10}$body length; [42, 53]) as a fixed covariate since body size and season may affect immune cell concentrations [54-56]. Body condition index can be interpreted as body-size adjusted body condition [57]. Cohort, social group, and slide nested within individual ID were included as random effects. We used parametric bootstrapping (*n* = 5000) to obtain 95% confidence intervals.

3. Results

There was an interaction between age, group size and sex on the proportion of agranulocytes (Table 1). In males, the strength of the logarithmic decrease in the proportion of agranulocytes with age depended on group size: males living in smaller groups had a higher proportion of agranulocytes in early-life which declined with age, whereas there was no clear change with age in males living in larger

groups (Figure 1). In contrast, in females the proportion of agranulocytes in early-life was similar when living in smaller and larger groups, but with a stronger decrease with age for females living in larger groups (Figure 1).

4. Discussion

We found a relative decrease in the proportion of agranulocytes with age. This may have arisen due to there being quantitatively fewer acquired immunity cells, or because of a greater number of innate cells being produced. In humans, this pattern has been associated with age-related reduction in thymus size [58, 59], reducing the number of naïve T cells [60] and $CD4^+$ T and $CD8^+$ subpopulations with age, which has detrimental implications for effective immune responses to new antigens [10, 61-65]. Alternatively, innate immune mechanisms may become more active with age through increased production of pro-inflammatory cytokines [66]. Such low-grade chronic inflammation in older individuals has detrimental effects on health and contributes to biological ageing and the development of age-related pathologies [21]. While we cannot provide direct evidence of immunosenescence due to the relative nature of the proportion of agranulocytes, the relative decrease in adaptive immune cells and increase in innate immune cells with age accords with previous studies in the wild [4-6]. Furthermore, understanding changes in immune cell profiles with age in badgers is important for the interpretation of leukocyte telomere dynamics [47]. Since granulocytes have longer telomeres than agranulocytes in humans and baboons [67, 68], any change in telomere length with age in mammals could be due to a change in leukocyte cell composition, or selective loss of leukocytes, with age, and lead to spurious inferences on telomere shortening.

We also provide evidence that social conditions (i.e. group size) have sex-specific effects on changes in individual immune cell profiles with age. In larger groups, early-life exposure to a greater diversity, or higher intensity, of pathogens or greater stress associated with resource or mate competition led to a stronger bias toward innate over adaptive immune cell ratios by age. According to the 'hygiene-hypothesis' [27, 29, 34, 35, 43, 69], this could subsequently alleviate the detrimental

consequences of such pathogens in later-life and thus slow age-related changes in immune cell profiles. In smaller groups, lower exposure to pathogens in early-life can have the opposite effect [70, 71], accelerating changes in immune cell profiles with age. Indeed, we found that the proportion of agranulocytes in early-life was greater in male badgers living in smaller social groups. Moreover, if fewer conspecifics share the pathogen burden, this could lead to a stronger pressure on the immune response and rapid changes in the proportion of agranulocytes.

Even though female badgers exhibited a relative decrease in the proportion of agranulocytes with age, this was not as strong as in males. Possibly, females develop a stronger immune response against pathogens in early-life (i.e. smaller change in the proportion of agranulocytes with age), which would corroborate previous findings in Soay sheep (*Ovis aries*), where males had a steeper decline in agranulocyte proportion with age than did females [26]. Males, given the polygynandrous mating system of badgers, have high levels of testosterone, particularly compared to other species [42], leading to immunosuppression and stronger decreases in adaptive immunity (i.e. agranulocytes) with age [32, 33]. This accords with sex-specific responses to environmental conditions and associated sex differences in immune responses seen in other species [2, 3].

While males showed stronger relative decreases in the proportion of agranulocytes with age in smaller groups, for females this effect was stronger in larger groups. Since badgers exhibit high levels of extra-group paternity (48%), increasing in proportion to a deficit of within-group candidate fathers, males in smaller groups may be exposed to higher extra-group competition and higher pathogen diversity [39, 40]. In contrast, females compete for resources with other females within their social group [72], which could lead to detrimental effects of larger group sizes on the proportion of agranulocytes. We were, however, unable to sample individuals until at least three months of age, due to welfare legislation (Protection of Badgers Act, 1992), and thus we cannot rule out the possibility of selective disappearance of individuals with poor innate immune responses. Nonetheless, our results indicate that age-related changes in immune profiles are associated with the social environment and these effects differ between the sexes.

Ethics

All work was approved by the University of Oxford's Animal Welfare and Ethical Review Board, ratified by the University of Leeds, and carried out under Natural England Licenses, currently 2017-27589-SCI-SCI and Home Office Licence (Animals, Scientific Procedures, Act, 1986) PPL: 30/3379.

Data accessibility

The data are available on Dryad upon acceptance.

Author contributions

The study was conceived by S.H.J.v.L. and H.L.D., and developed by E.P.B., M.W.T.M., C.N., C.D.B. and D.W.M.; Slides were prepared by S.H.J.v.L., and analysed by M.W.T.M.; Statistical analyses were conducted by S.H.J.v.L. with input from E.P.B. and H.L.D.; The paper was written by S.H.J.v.L., E.P.B. and H.L.D. and all authors gave final approval for publication.

Competing interests

We have no competing interests

Funding

S.H.J.v.L was funded by a Leeds Anniversary Research Scholarship from the University of Leeds with support from a Heredity Fieldwork Grant from the Genetics Society and a Priestley Centre Climate Bursary from the University of Leeds. H.L.D. was supported by a Royal Society Research Grant (RG170425).

Acknowledgements

We thank all members of the Wytham badger team, past and present, for collecting data. We also thank Alexandra Sparks for comments on an earlier draft of this manuscript.

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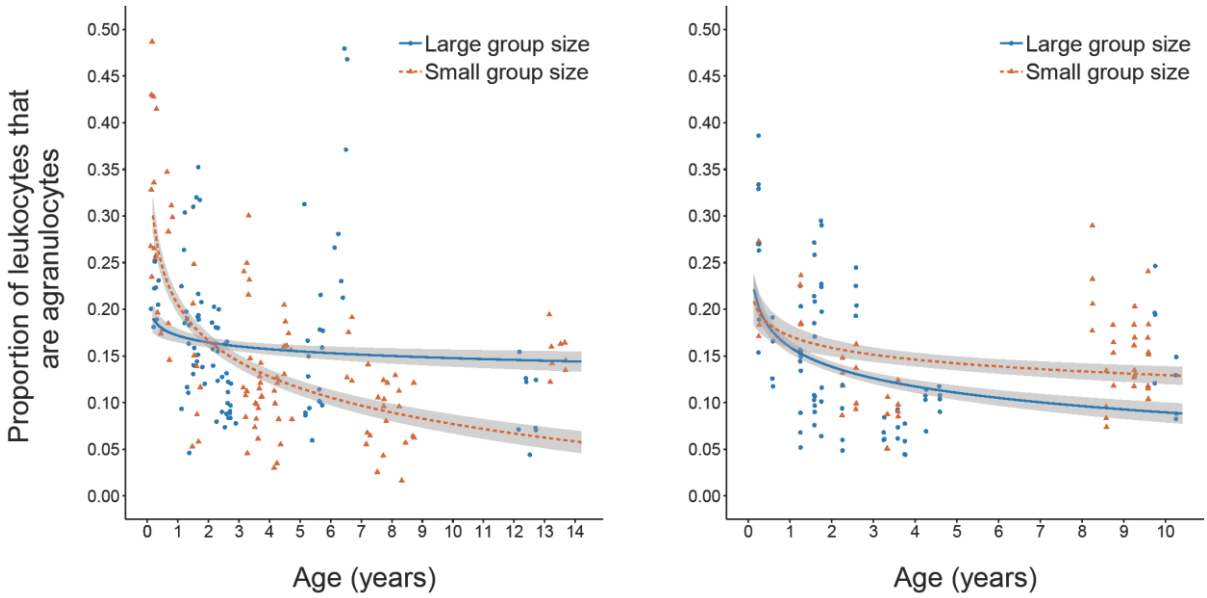
Tables and Figures

Table 1: Parameter estimates and 95% confidence intervals of fixed effects from a mixed model testing age, sex and group size effects on the proportion of agranulocytes in European badgers. β = direction and magnitude of effect, S.E. = standard error, 95% CI = 95% confidence interval from parametric bootstrapping, χ^2 = chi-squared value with associated p-value; reference terms in brackets = reference level for factors; * = interaction. Significant parameters ($p < 0.05$) are in bold.

Parameter (reference level)	β	S.E.	95% CI	χ^2	p-value
Intercept	-1.892	0.098	-2.087 to -1.703		
Log age	-0.031	0.097	-0.218 to 0.156	0.143	0.741
Sex (female)	0.099	0.104	-0.111 to 0.313	0.873	0.350
Group size	-0.047	0.086	-0.218 to 0.124	0.300	0.584
Season (Spring)				5.341	0.069
Summer	0.027	0.099	-0.163 to 0.219		
Autumn	0.346	0.154	0.042 to 0.651		
Body condition index	**-0.246**	**0.074**	**-0.388 to -0.102**	**9.831**	**0.002**
Log age * Sex (female)	-0.014	0.102	-0.211 to 0.185	0.019	0.889
Log age * Group size	-0.052	0.091	-0.230 to 0.119	0.312	0.556
Sex (female) * Group size	**0.255**	**0.117**	**0.036 to 0.472**	**4.176**	**0.041**
Log age * Sex (female) * Group size	**0.225**	**0.104**	**0.027 to 0.430**	**4.380**	**0.036**

Random effect estimates (variance): Individual ID ($1.169*10^{-2}$), Slide nested in individual ID ($1.249*10^{-1}$), Social group ($<1.000*10^{-12}$), Cohort ($5.026*10^{-3}$)



411

412 **Figure 1:** The interplay between age and group size on the proportion of leukocytes that are
413 agranulocytes for males and females. Raw data points are shown. Group size was modelled as a
414 continuous variable in the mixed model, but for visualisation is shown for males in small (range = 1 –
415 9; *n* = 99 repeats; 25 slides; 9 individuals; brown triangles and dashed line) and large (range = 10 – 16;
416 *n* = 96 repeats; 24 slides; 8 individuals; blue circles and solid line) groups, and for females in small
417 (range = 1 – 9; *n* = 52 repeats; 13 slides; 4 individuals; brown triangles and dashed line) and large
418 (range = 10 – 16; *n* = 79 repeats; 20 slides; 6 individuals; blue circles and solid line) groups. X-axis scales
419 differ between plots. Fitted lines represent the model prediction for age interacting with sex and group
420 size, with associated 95% confidence intervals as shaded areas.

421

Supporting information

Social effects on age-related and sex-specific immune cell profiles in a wild mammal

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Group size estimation:

Group sizes were determined by the number of individuals (cubs and adults) that were present in a social group in a given year. Given high natal philopatry (75.8%), low permanent dispersal rates (19.1%), and high levels of inter-group movements leading to extra-group paternity in badgers [73], individuals (*n* = 1726) were assigned as a resident of a social group each year, according to the following rules adapted from [40, 73]:

1. Badgers first caught as cubs (*n* = 1241) were considered resident in the social group they were first caught, until they subsequently satisfied dispersal rules or were considered dead.
2. Badgers first caught as adults (*n* = 490) were assigned to their lifetime modal social group, until dispersal rules applied. If an individual was captured equally between two groups (*n* = 29), they were assigned to the social group they were initially captured in until dispersal rules applied.
3. Dispersal rules were satisfied when the two most recent captures of an individual (>30 days apart), as well as 1 of 2 captures before, were made in a different social group than the current residential social group. Individuals were resident in the new social group until dispersal rules applied again.

The number of individuals per social group were then calculated as the sum of individuals present in the social group in a given year.