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## Research Article

## Age-related changes of oxidative status and immune function in a long-lived seabird

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Experimental studies in humans and laboratory species have shown that the decline of the immune system with age (immunosenescence) and the accumulation of oxidative damage to macromolecules are two key contributors to the onset and progression of the ageing process. Although laboratory models have provided important insights, the physiological basis of ageing in natural populations remains comparatively understudied, constraining our mechanistic understanding of the ageing process. The complexity of age-related physiological changes increases further in long-lived species, which appear to possess unique adaptations that mitigate immunosenescence and oxidative damage. However, studies investigating the underlying physiological mechanisms in long-lived birds have yielded contrasting results. In this study, we compared four markers of oxidative status and eight immune markers between younger and older breeders of a long-lived seabird, the Scopoli's shearwater *Calonectris diomedea*, to identify potential physiological signatures of ageing. Regardless of sex, older individuals exhibited higher levels of blood antioxidant enzymes, natural antibodies, and lymphocytes compared to younger birds, while levels of DNA damage and cellular effectors of innate immunity did not differ between age classes. These findings suggest that older shearwaters may upregulate antioxidant enzyme activity, possibly to cope with increased basal production of reactive oxygen species, in line with the oxidative stress theory of ageing. Alternatively, the higher antioxidant levels of older birds might reflect selective mortality of birds with reduced protection. In contrast to the oxidative status, the observed immune patterns do not support the immunosenescence hypothesis.

Keywords: ageing, antioxidants, immunity, oxidative stress, Scopoli's shearwater, seabirds



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## Introduction

Ageing in natural animal populations has historically been studied within an evolutionary framework, focusing on the two major components of fitness: survival probability (actuarial senescence) and reproduction (reproductive senescence) (Gaillard and Lemaître 2020). By contrast, the physiological underpinnings of ageing have received far less attention, limiting our mechanistic understanding of this complex biological process. Identifying the cellular and molecular changes responsible for the physiological decline of organisms over time is central to explaining the variation in ageing patterns observed both within and between species (Selman et al. 2012, Peters et al. 2019). Among the mechanisms proposed for physiological ageing, oxidative stress (Birch-Machin and Bowman 2016) and immunosenescence (Pawelec 2018) are supposed to play important roles in determining the onset and rate of this complex biological process. Oxidative stress results from an imbalance between the production and accumulation of reactive oxygen species (ROS) and antioxidant defences that cause oxidative damage to body constituents and loss of cellular homeostasis (Luo et al. 2020). Oxidative stress is expected to increase with age due to reduced investment in somatic maintenance, protection, and repair (e.g. oxidative stress theory of ageing; Metcalfe and Alonso-Alvarez 2010). On the other hand, immunosenescence refers to the decline of major immune functions with increasing age, which can be associated, among other effects, to a low-grade chronic inflammation known as ‘inflamm-ageing’ (Fulop et al. 2018). Adding to the complexity, age-related inflammation in turn may generate oxidative stress, further accelerating ageing in a multifaceted and interdependent process (oxidative–inflammatory theory of ageing; Romero Cabrera 2016).

Studying long-lived species is particularly relevant to research on physiological ageing, as their slow senescence may reflect unique physiological adaptations (Stenvinkel and Shiels 2019). Most of our current knowledge about the factors contributing to longevity comes from studies on humans (Bauer and De la Fuente 2014), short-lived species whose lifespan has been experimentally extended (Holtze et al. 2021), and comparative studies between long- and short-lived species (Delhaye et al. 2016, Marasco et al. 2017, Jové et al. 2023). The latter indicate that, in general, long-lived species experience lower levels of oxidative stress, exhibit higher antioxidant capacity, and show fewer signs of reproductive or immune decline, as they invest more in self-maintenance compared to short-lived species (Xia and Møller 2018, Peters et al. 2019).

Studies focusing on age-related variation in specific physiological markers within long-lived species yielded inconsistent results. For example, in long-lived birds, a decline in cell-mediated immunity has been reported for Leach’s storm-petrels *Oceanodroma leucorhoa* (Haussmann et al. 2005), while innate immune parameters (haemagglutination titer, haemolysis titer, and haptoglobin concentration) remain unchanged in common terns *Sterna hirundo* (Bichet et al. 2022). By contrast, European shags *Phalacrocorax aristotelis*

exhibit an increase in plasma oxidative damage with advancing age (Herborn et al. 2016), unlike budgerigars *Melopsittacus undulatus*, which retain exceptional oxidative damage resistance throughout their lifespan (Ogburn et al. 2001). The question of which set of hallmarks of ageing are truly more characteristic of long-lived species therefore remains open.

Here, we compared four markers of oxidative status and eight immune markers between younger and older adults of a long-lived seabird, the Scopoli’s shearwater *Calonectris diomedea*, to identify potential physiological signatures of ageing. We carried out the study during the laying season because it is a particularly energy-demanding period for this species, especially for females, which face higher energetic costs associated with egg production and investment (Becciu et al. 2012). As a result, signatures of ageing and sex-related differences are likely to be more evident than during other, less demanding stages of the life cycle.

Specifically, we expected older individuals to exhibit higher DNA damage and haptoglobin (inflammatory marker), and lower antioxidant defences and immune markers, both innate and adaptive, compared to younger individuals, reflecting a reduction in somatic function with age. We also expected that signatures of ageing would be stronger in females due to their higher investment into reproduction compared to males (Gomes et al. 2019).

## Material and methods

### Study birds and sampling

The Scopoli’s shearwaters investigated in this study breed on Linosa Island, in the south-western Mediterranean Sea (35°52’N, 12°52’E). Nests are located within rock cavities at ground level, making them easily accessible and allowing birds to be gently captured by hand. Moreover, the presence of an egg in the nest can be readily verified, ensuring that only breeding individuals were sampled. Blood samples were collected in May 2024, during the egg-laying period, from breeding individuals (20 males and 23 females) belonging to two age classes: younger (5–9 years old,  $n=21$ ) and older (17–36 years old,  $n=22$ ) (see the Supporting information for sample distribution by sex and age). All individuals were sampled after the onset of incubation. The younger category included birds from 5 to 9 years of age because shearwaters begin reproducing at 5–6 years of age and the cutoff for the prime age stage is 9 years (Berardi et al. 2025). The older age class spans a broader range because the ages of individuals older than 17 years are not evenly distributed in the population. In the sampling year, only two individuals were 36 years old, whereas most of the oldest individuals sampled were between 17 and 22 years of age. For these reasons, age classification should not be considered absolute, but rather a comparative grouping aimed at contrasting parameters between relatively younger and relatively older individuals. Sex was assigned based on the pronounced sexual dimorphism in body mass observed in adult Scopoli’s shearwaters, with males being heavier than females (Becciu et al. 2012). For each

individual, 400  $\mu$ l of blood was collected from the tarsal vein within 5 min of capture. Blood samples were transferred into heparinized Eppendorf tubes and kept in a cool box until centrifugation. An aliquot of 30  $\mu$ l of blood was preserved in 60  $\mu$ l of buffer (RPMI 1640 medium (Euroclone) supplemented with 20% fetal bovine serum (FBS, Euroclone) and 10% dimethyl sulfoxide (DMSO)) for subsequent Comet assay analyses. Additionally, a drop of blood from each sample was smeared onto a glass microscope slide for leukocyte counts. Samples were centrifuged for 5 min at  $1177 \times g$  within 2 h of collection to separate plasma from red blood cells and were then stored at  $-80^{\circ}\text{C}$  in laboratory facilities until analysis.

### Quantification of blood-based markers of oxidative status

To assess the oxidative status of birds, we relied on established methods (Costantini et al. 2013, Cheron et al. 2022). Briefly, we measured in haemolysates ( $n=37$ ) the activity of three antioxidant enzymes: superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). We quantified the levels of SOD using the RANSOD assay (Randox Laboratories). We added an aliquot of haemolysate to a buffer containing the substrate of the enzyme. Then, using a multi-channel pipette, we added xanthine oxidase to each well, and we measured the change of optical density in kinetics at 505 nm. The reaction between the substrate and the xanthine oxidase generates the superoxide free radical, which reacts with a chromogen generating a red colouration. The SOD present in the sample inhibits the reaction between the superoxide and the chromogen. We measured the levels of GPx using the RANSEL assay (Randox Laboratories). We added an aliquot of haemolysate to a reagent diluted in a phosphate buffer. Then, we added cumene hydroperoxide (substrate of GPx) to each well, and we measured the change of the optical density in kinetics at 340 nm. Finally, we measured the levels of catalase using the OxiSelect CAT Activity Assay (Euromedex). We added an aliquot of haemolysate to a reagent containing hydrogen peroxide, which is the substrate of catalase. In this first step of the assay, the catalase decomposes hydrogen peroxide into water and oxygen. In a second step of the assay, we added a chromogenic working solution that reacts with the remaining hydrogen peroxide in the reaction mixture. We measured the absorbance at 520 nm. To obtain reliable and precise measurements, we ran all analyses in duplicate, and we always included quality controls in each assay. The coefficient of variation, which indicates the precision of the instrumental analyses, was always  $< 10\%$ . We standardised levels of enzymes by the concentration of proteins in the haemolysates as quantified using the Bradford protein assay with albumin as reference standard (Sigma-Aldrich).

We also measured primary DNA damage, expressed as single-strand breaks ( $\mu$ m), using the Comet assay (single-cell gel electrophoresis). The assay was performed following previously published protocols (Giovani et al. 2022), with the only modification that, for shearwaters, 15  $\mu$ l of whole blood was mixed with 85  $\mu$ l of low-melting-point agarose (LMPA). The rationale for using this assay is that cells undergoing

oxidative stress during the ageing process can accumulate DNA strand breaks; therefore, this measure can be used as an additional marker of physiological ageing, as reported in previous studies on birds (Montoya et al. 2020, Domínguez-de-Barros et al. 2024).

Sample size differed among assays (both oxidative and immunological) due to field and laboratory constraints; however, this did not substantially change the proportion of males and females analyzed (Supporting information).

### Immunological assays

To analyze the immune function of birds, we relied on established methods that have been used in various species (Brust et al. 2022, Messina et al. 2025). Due to the commonality of the assays, we highlight the methodological details we adjusted to our species. Briefly, we determined 1) the plasma concentration of haptoglobin (in  $\text{mg ml}^{-1}$ ) ( $n=39$ ) using undiluted samples with the commercial kit 'PHASE'<sup>TM</sup> Haptoglobin Assay (Tridelta), 2) the plasma concentration of immunoglobulin Y (IgY, in OD) ( $n=39$ ) using an in-house sensitive ELISA with 1:4000 diluted samples and commercial anti-chicken antibodies (1:250 v/v), 3) the haemagglutination (HA) titer ( $n=39$ ) using the haemagglutination assay (using 15–15  $\mu$ l sample and rabbit erythrocytes (Davids Biotechnologie) as antigen; titres scored after 110 min), and 4) the number of different white blood cell types by fixing blood smears ( $n=34$ ) prepared while on the field with Wright-Giemsa Stain Solution (Reinoso-Pérez et al. 2025). Smears were examined under the microscope at 100 $\times$  magnification with oil immersion, and the relative number and types of leucocytes (lymphocytes, heterophils, basophils, eosinophils, and monocytes) were assessed by counting 100 leucocytes. The number of white blood cells of different types was expressed per  $10^4$  red blood cells (RBC) (Prüter et al. 2020). All haptoglobin, ELISA, and cell count measurements were performed in duplicate, and the mean of each duplicate was included in the statistical models. The coefficient of variation for the immune assays was always  $< 10\%$ . All assays were conducted blind to treatment and sampling group. Samples were randomly distributed across plates to avoid systematic plate effects.

### Statistical analyses

We fitted generalized linear models (GLM) to test the effect of age class on immune and oxidative status markers (R package 'lme4', ver. 1.1.36, Bates et al. 2015). In preliminary analyses, we also ran models entering age as a continuous variable. These models yielded similar results to those obtained with age class entered as a discrete variable (Supporting information). Thus, in each model, we entered age class (older versus younger) and sex as predictors, and the interaction between age and sex to examine whether the effect of age differed between females and males. Considering the non-normal distribution of the response variable and of the residuals, we ran GLMs with a Gamma family and log link function for the four oxidative status markers, haptoglobin, and IgY, and

negative binomial GLMs for the white blood cell counts. Non-significant interaction terms were removed to simplify the models, using  $\alpha=0.05$  as the significance threshold. Since body mass can influence different physiological processes (Ruhs et al. 2020), we performed Pearson's correlation tests between body mass and each analysed parameter separately for males and females prior to fitting the models. The results of these analyses (Supporting information) indicated

that the only significant associations were between male body mass and IgY ( $r=-0.507$ ,  $p=0.022$ ) and haptoglobin levels ( $r=-0.516$ ,  $p=0.020$ ). We therefore included body mass as a covariate in the models for age and these two markers in males; however, the results remained unchanged, indicating that age does not affect IgY and haptoglobin levels (Supporting information). In any case, the results for body mass are consistent with the fact that none of the sampled

Table 1. Outcomes of full and reduced models ( $\chi^2$  test on the deviance) fitted for four oxidative markers (SOD, GPX, catalase, and DNA damage) and eight immune markers (IgY, haptoglobin, haemagglutination titer, and eosinophils, basophils, heterophils, monocytes, and lymphocytes counts). Significant terms for each marker are shown in bold. df=degrees of freedom.

| Marker                  | Model term            | df | Deviance | Resid. Df | Resid. Dev | Pr(>Chi)          |
|-------------------------|-----------------------|----|----------|-----------|------------|-------------------|
| <i>Oxidative stress</i> |                       |    |          |           |            |                   |
| SOD                     | NULL                  | NA | NA       | 36        | 24.022     |                   |
|                         | <b>Ageclass</b>       | 1  | 3.499    | 35        | 20.523     | <b>0.014</b>      |
|                         | <b>Sex</b>            | 1  | 4.258    | 34        | 16.266     | <b>0.007</b>      |
|                         | Ageclass $\times$ sex | 1  | 1.133    | 33        | 15.133     | 0.149             |
| GPX                     | NULL                  | NA | NA       | 36        | 24.502     |                   |
|                         | <b>Ageclass</b>       | 1  | 5.880    | 35        | 18.622     | <b>0.003</b>      |
|                         | Sex                   | 1  | 0.287    | 34        | 18.335     | 0.511             |
|                         | Ageclass $\times$ sex | 1  | 1.098    | 33        | 17.237     | 0.198             |
| Catalase                | NULL                  | NA | NA       | 36        | 31.550     |                   |
|                         | <b>Ageclass</b>       | 1  | 5.461    | 35        | 26.089     | <b>0.007</b>      |
|                         | <b>Sex</b>            | 1  | 3.993    | 34        | 22.095     | <b>0.02</b>       |
|                         | Ageclass $\times$ sex | 1  | 0.003    | 33        | 4.246      | 0.893             |
| <i>Immunity</i>         |                       |    |          |           |            |                   |
| IgY                     | NULL                  | NA | NA       | 38        | 0.156      |                   |
|                         | Ageclass              | 1  | 0.001    | 37        | 0.156      | 0.72              |
|                         | Sex                   | 1  | 0.002    | 36        | 0.154      | 0.495             |
|                         | Ageclass $\times$ sex | 1  | 0.000    | 35        | 0.154      | 0.978             |
| Haptoglobin             | NULL                  | NA | NA       | 38        | 7.658      |                   |
|                         | Ageclass              | 1  | 0.001    | 37        | 7.657      | 0.943             |
|                         | Sex                   | 1  | 0.002    | 36        | 7.656      | 0.933             |
|                         | Ageclass $\times$ sex | 1  | 0.002    | 35        | 7.654      | 0.923             |
| Haemagglutination       | NULL                  | NA | NA       | 38        | 1.592      |                   |
|                         | <b>Ageclass</b>       | 1  | 0.159    | 37        | 1.433      | <b>0.045</b>      |
|                         | Sex                   | 1  | 0.007    | 36        | 1.426      | 0.665             |
|                         | Ageclass $\times$ sex | 1  | 0.015    | 35        | 1.411      | 0.541             |
| Eosinophils             | NULL                  | NA | NA       | 33        | 285.688    |                   |
|                         | Ageclass              | 1  | 1.495    | 32        | 284.194    | 0.691             |
|                         | Sex                   | 1  | 7.099    | 31        | 277.095    | 0.386             |
|                         | Ageclass $\times$ sex | 1  | 1.880    | 30        | 275.215    | 0.656             |
| Basophils               | NULL                  | NA | NA       | 33        | 179.323    |                   |
|                         | Ageclass              | 1  | 8.467    | 32        | 170.856    | 0.225             |
|                         | Sex                   | 1  | 4.383    | 31        | 166.473    | 0.383             |
|                         | Ageclass $\times$ sex | 1  | 19.547   | 30        | 146.925    | 0.065             |
| Heterophils             | NULL                  | NA | NA       | 33        | 311.282    |                   |
|                         | Ageclass              | 1  | 24.956   | 32        | 286.326    | 0.09              |
|                         | Sex                   | 1  | 2.025    | 31        | 284.301    | 0.629             |
|                         | Ageclass $\times$ sex | 1  | 18.011   | 30        | 266.290    | 0.15              |
| Monocytes               | NULL                  | NA | NA       | 33        | 194.948    |                   |
|                         | Ageclass              | 1  | 1.591    | 32        | 193.358    | 0.59              |
|                         | Sex                   | 1  | 0.143    | 31        | 193.214    | 0.871             |
|                         | Ageclass $\times$ sex | 1  | 7.270    | 30        | 185.944    | 0.249             |
| Lymphocytes             | NULL                  | NA | NA       | 33        | 162.509    |                   |
|                         | <b>Ageclass</b>       | 1  | 43.800   | 32        | 118.708    | <b>&lt; 0.001</b> |
|                         | Sex                   | 1  | 0.224    | 31        | 118.485    | 0.805             |
|                         | Ageclass $\times$ sex | 1  | 6.643    | 30        | 111.842    | 0.178             |

Table 2. Estimated marginal means ( $\pm$  SE) of each marker for the two age classes. Pairwise comparisons between age classes are reported with p-values. Degrees of freedom (df) are not shown for leukocyte counts, as they were obtained from quasi-Poisson models, which rely on different estimation procedures. Markers showing a significant effect of age are highlighted in bold.

| Marker                  | Young             | Old               | df | p-value      |
|-------------------------|-------------------|-------------------|----|--------------|
| <i>Oxidative stress</i> |                   |                   |    |              |
| SOD                     | 2.39 $\pm$ 0.396  | 4.31 $\pm$ 0.784  | 33 | <b>0.023</b> |
| GPX                     | 0.113 $\pm$ 0.021 | 0.256 $\pm$ 0.051 | 33 | <b>0.005</b> |
| Catalase                | 50.7 $\pm$ 9.73   | 111.5 $\pm$ 23.4  | 34 | <b>0.009</b> |
| DNA damage              | 11.03 $\pm$ 0.951 | 9.23 $\pm$ 0.877  | 33 | 0.174        |
| <i>Immunity</i>         |                   |                   |    |              |
| IgY                     | 0.266 $\pm$ 0.015 | 0.273 $\pm$ 0.015 | 35 | 0.735        |
| Haptoglobin             | 0.670 $\pm$ 0.072 | 0.663 $\pm$ 0.069 | 35 | 0.942        |
| Haemagglutination       | 5.37 $\pm$ 0.246  | 6.10 $\pm$ 0.272  | 35 | <b>0.054</b> |
| Eosinophils             | 11.5 $\pm$ 2.67   | 10.5 $\pm$ 2.42   |    | 0.775        |
| Basophils               | 2.366 $\pm$ 0.979 | 0.726 $\pm$ 0.647 |    | 0.229        |
| Heterophils             | 33.9 $\pm$ 4.56   | 27.2 $\pm$ 3.79   |    | 0.252        |
| Monocytes               | 9.99 $\pm$ 1.89   | 7.76 $\pm$ 1.70   |    | 0.382        |
| Lymphocytes             | 40.6 $\pm$ 3.12   | 54.7 $\pm$ 3.57   |    | <b>0.003</b> |

individuals showed values outside the normal range for the species in relation to the study period.

## Results

We found significant differences between younger and older individuals for SOD, GPx, CAT, haemagglutination titer, and lymphocyte counts (Table 1–2). Specifically, compared to younger individuals, older individuals had higher levels of SOD ( $4.31 \pm 0.784$  U mg<sup>-1</sup>,  $p = 0.023$ ) (Fig. 1a), GPx ( $0.256 \pm 0.051$  U mg<sup>-1</sup>,  $p = 0.005$ ) (Fig. 1b), CAT ( $111.5 \pm 23.4$ , U mg<sup>-1</sup>,  $p = 0.009$ ) (Fig. 1c), haemagglutination titer ( $6.10 \pm 0.272$ ,  $p = 0.054$ ) (Fig. 2a), and absolute number of lymphocytes ( $54.7 \pm 3.57$  cells/ $10^4$  RBC,  $p = 0.003$ ) (Fig. 2d).

Younger and older individuals had similar levels of all the other markers measured, for both females and males (Table 2; Supporting information). Finally, we found significant differences between females and males in CAT and SOD, with higher levels of both enzymes in males (Supporting information), independent of age.

## Discussion

Our study simultaneously assessed age- and sex-related variation in oxidative status and immune markers in a long-lived bird species. Contrary to our expectations, older shearwaters exhibited higher levels of antioxidant enzymes and similar levels of DNA damage compared to younger individuals. This

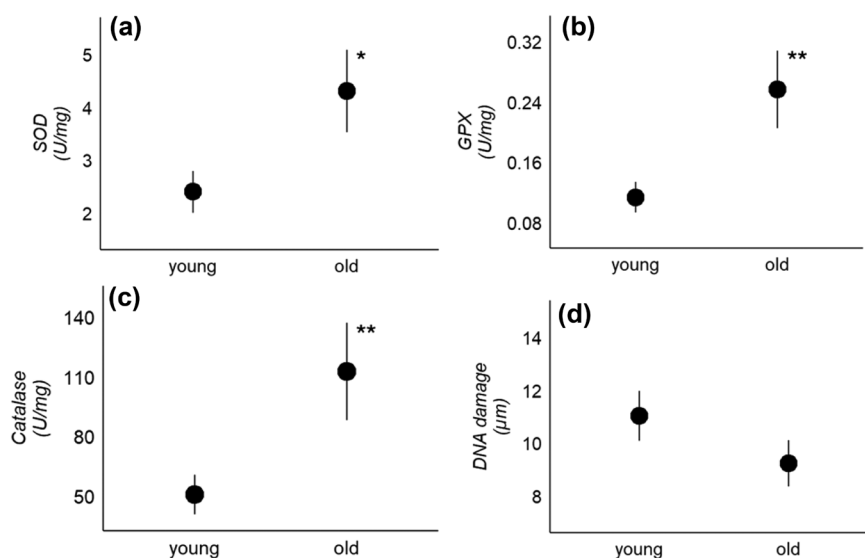


Figure 1. Activity levels of superoxide dismutase (SOD, expressed in Units mg<sup>-1</sup> of protein) (a), glutathione peroxidase (GPX, expressed in Units mg<sup>-1</sup> of protein) (b), catalase (expressed in Units mg<sup>-1</sup> of protein) (c), and quantification of DNA damage (expressed in  $\mu$ m) (d) in relation to the age class of shearwaters. Values are presented as estimated marginal means  $\pm$  SE. Significant differences between younger and older individuals are indicated (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

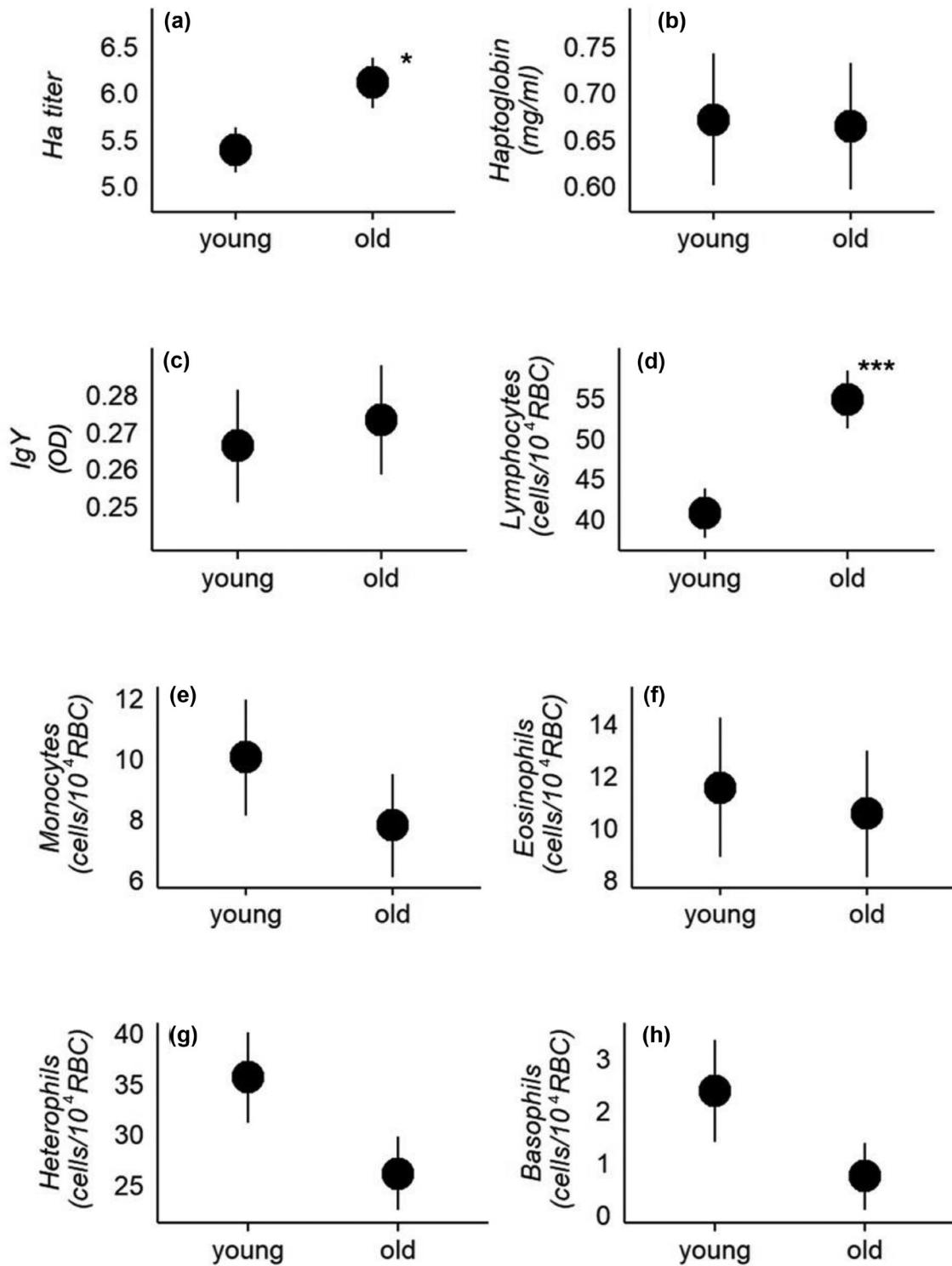


Figure 2. Haemagglutination titer (Ha) (a), levels of haptoglobin (expressed in  $\text{mg ml}^{-1}$ ) (b), and IgY (expressed in  $\mu\text{g ml}^{-1}$ ) (c), and number of lymphocytes (d), monocytes (e), eosinophils (f), heterophils (g) and basophils (h) (all expressed in number of cells/ $10^4$  red blood cells), in relation to the age class of shearwaters. Values are presented as estimated marginal means  $\pm$  SE. Significant differences between young and old individuals are indicated (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ). RBC=Red blood cells.

pattern may reflect an upregulation of antioxidant enzymes in older birds to cope with higher basal production of ROS, as predicted by the oxidative stress theory of ageing. Indeed, longitudinal and cross-sectional studies have shown age-related increases in oxidative stress, accompanied by a corresponding increase in antioxidant defences (Bize et al. 2014; Marasco et al. 2017; Costantini 2024). Moreover, investment in repair mechanisms varies according to environmental conditions and the life-history traits of different species, with longer-lived species generally exhibiting higher levels of antioxidants (Xia and Møller 2018). Alternatively, a selective mortality of individuals with lower antioxidant protection might occur, which would explain the higher antioxidant protection of older shearwaters. Increased antioxidant defences have been proposed to represent an adaptive response to the oxidative challenges associated with ageing and reproductive effort (Alonso-Alvarez et al. 2010). In a previous longitudinal study, we found that the decline in reproductive success in older breeders becomes pronounced only in the terminal stages of life (Berardi et al. 2025). Thus, it might be that protective physiological mechanisms are upregulated in older individuals to shield their offspring from the harmful effects of oxidative stress (Blount et al. 2015).

Regardless of age, males had higher levels of SOD and CAT than females, but similar levels of GPx and DNA damage. Although many species exhibit differences in immune and oxidative physiology (Costantini 2018), such patterns are not consistent across taxa (Vincze et al. 2022). The evolutionary drivers of sex-specific physiology are likely related to reproductive roles. For instance, sex hormones can differently affect cellular oxidative status (López-Arrabé et al. 2018). In our study, it is particularly important to consider sex-specific reproductive costs. Female shearwaters reach their lowest body mass during the egg-laying period (Becciu et al. 2012), indicating a high energetic demand associated with producing and laying the egg, which may come at a cost to their self-maintenance systems (Lin et al. 2022). Conversely, during the egg-laying period, males are in their best body condition compared to other phases of the life cycle, but they face multiple stressors related to nest attendance and prolonged fasting while waiting for the female to arrive and lay the egg. In addition, males typically take the first long incubation shift, fasting for 10–14 days while the female forages after egg-laying, relying on stored fat reserves. The higher antioxidant levels observed in males might be a consequence of the stimulatory effect that prolonged fasting may have on the individual physiology. It remains unclear whether sex differences in oxidative status markers would also be observed during less demanding phases of the life cycle. Further research is needed to address this point.

Current investigations into how immune function changes with age in natural animal populations have primarily reported declines in adaptive immune traits and increases or no change in innate immunity (Těšický et al. 2021, Bichet et al. 2022). Our results on adaptive immunity diverge from this general pattern. Indeed, we found an age-related increase in the number of lymphocytes and similar levels of the immunoglobulin Y (IgY) between younger and older individuals.

To our knowledge, this is the first documented case of an age-associated increase in lymphocytes in a wild bird population. Previous studies have examined lymphocytes mainly across developmental stages (e.g. chicks versus adults; Jakubas et al. 2015). We hypothesize that an efficient antigen-specific memory could be key to the survival of these birds, enabling only highly immunocompetent individuals to reach advanced age (Lavoie 2006, Minias 2019). An alternative explanation is that differences in lymphocyte counts reflect the history of exposure to pathogens and parasites, which should increase with age (Hill et al. 2016, Bichet et al. 2022).

Innate immunity in shearwaters, conversely, aligns with the general pattern described above for other bird species. Specifically, we found that haemagglutination titer – reflecting circulating levels of natural antibodies (NAbs) – increased with age, whereas the number of white blood cell types showed no age-related changes. Since NAbs can bind to and clear self-apoptotic and necrotic cells (Reyneveld et al. 2020), the observed increase in haemagglutination could potentially imply an anti-ageing or anti-inflammatory defence (Bichet et al. 2022). At the same time, the lack of age-related variations in white blood cells can be attributed to the crucial role of innate immunity as the first line of defence against pathogens (De Coster et al. 2010), coupled with its relatively low energetic maintenance cost (Nebel et al. 2013).

In conclusion, our study suggests a potential pattern in a long-lived bird species, where, regardless of sex and body mass, older individuals tend to exhibit higher levels of blood antioxidant enzymes, natural antibodies, and lymphocytes compared to younger individuals, while levels of DNA damage and other leukocytes appear similar across age classes. Furthermore, our results highlight the importance of simultaneously considering multiple physiological markers to address the complexity of biological processes occurring throughout ageing. We propose that maintaining high levels of antioxidants, natural antibodies, and lymphocytes into old age might be key to achieve a longer life and, possibly, a higher reproductive success due to the shielding of chicks against intergenerational transfer of oxidative damage or pathogens. Since this study is cross-sectional, longitudinal data are needed to disentangle the relative contributions of within- and between-individual changes to the underlying mechanisms of ageing in long-lived species.

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## Author contributions

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## Transparent peer review

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## Data availability statement

Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.63xsj3vgf> (Berardi et al. 2026).

## Supporting information

The Supporting information associated with this article is available with the online version.

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