Age related variation of health markers in Asian elephants

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Abstract

Although senescence is often observed in the wild, its underlying mechanistic causes can rarely be studied alongside its consequences, because data on health, molecular and physiological measures of senescence are rare. Documenting how different age-related changes in health accelerate ageing at a mechanistic level is key if we are to better understand the ageing process. Nevertheless, very few studies, particularly on natural populations of long-lived animals, have investigated age-related variation in biological markers of health and sex differences therein. Using blood samples collected from semi-captive Asian elephants, we show that pronounced differences in haematology, blood chemistry, immune, and liver functions amongst age classes are also evident under natural conditions in this extremely long-lived mammal. We provide strong support that overall health declined with age, with progressive declines in immune and liver functions similarly in both males and females. These changes parallel those mainly observed to-date in humans and laboratory mammals, and suggest a certain ubiquity in the ageing patterns.

Keywords. Ageing, long-lived mammal, immunosenescence, ecophysiology

1. Introduction

Most organisms experience senescence, a process by which individuals accumulate damage with increasing age, leading to loss of function and eventually death (Monaghan et al., 2008; Nussey et al., 2013). A large number of studies in humans, in model organisms, but also in wild vertebrates have now documented reproductive and actuarial senescence (Hawkes, 2003; Nussey et al., 2013). However, our knowledge on the molecular and physiological mechanisms underlying such senescence patterns comes largely from studies on model organisms, which are usually relatively short-lived, genetically homogenous, and maintained under standard conditions. In contrast, very little is known about the molecular and physiological parameters associated with increasing age in free-ranging animals, especially in long-lived species (Ma and Gladyshev, 2017). Limiting ageing research to model organisms might cause us to miss important factors that help explain key ageing processes in long-lived species like ourselves,

and leaves the wider evolutionary ecology significance of the detected age-related changes uncertain.

Until now, only few studies have explored immunological and haematological variation with age in wild mammals and even fewer investigated sex-differences in the age-related variation in health markers (Cheynel et al., 2017; Jego et al., 2014; Nussey et al., 2012). For instance, similarly in male and female roe deer (*Capreolus capreolus*), innate immunological functions declined for one of the two studied populations and inflammatory response increased with age while the adaptive response declined in late life (Cheynel et al., 2017). Nonetheless, to our knowledge none have investigated a wide range of health parameters simultaneously and considered multiple physiological functions (haematological, immune, kidney, liver) in populations living under natural conditions. However, such studies are needed, since ageing is a heterogeneous phenomenon, involving several mechanisms, and all biological functions might not decline at the same pace with age (Hayward et al., 2015) and in both sexes (but see (Cheynel et al., 2017)). Because data on long-lived animals living in natural habitats with this wide range of measures is rarely available, (1) these asynchronous patterns are still poorly understood (2) our knowledge of the mechanistic basis of such patterns in natural populations remains scarce; expanding our understanding of senescence to other biological systems is thus critical.

Here, we investigate age-related variation in health markers in a long-lived mammal, the Asian elephant (*Elephas maximus*). We take advantage of a unique health dataset of semi-captive Asian elephants (age range 4-72 years) for which physiological measures and exact age are known accurately, to investigate age-related differences in hepatic damage, muscle and immune functions in a long-lived mammal population living under natural conditions. The study population is described as “semi-captive” because it is comprised of state-owned, individually-marked Asian elephants in Myanmar that are used daily as draft and transport animals in the timber industry by the Myanma Timber Enterprise (MTE). Although elephants are managed as draft and transport animals by the MTE, they live largely under natural conditions (Lahdenperä et al., 2018), leading to mortality and reproductive patterns matching those of wild elephants (Clubb et al., 2008). To test the influence of life stages on health over three climatic seasons (between March 2016 and April 2018), we collected blood samples annually (three times per year, 586 measures on 181 elephants) to examine age related variation in blood haematology (haematocrit, haemoglobin), protein levels (globulins,

albumin, and total proteins), immune system (white blood cells counts), liver damage (aspartate aminotransferase-AST, alkaline phosphatase-ALKP), muscle damage (creatinine kinase), kidney functions (blood urea nitrogen-BUN, creatinine) and circulating fat (triglycerides) markers. To test whether and how health declines as these long-lived animals age, we determine the molecular and physiological parameters underlying the age-related declines in health by analysing variation in each trait separately, also testing for any sex differences in health deterioration with age. Second, we assess the overall variation in health across life stages using a multivariate mixed model framework that considers co-variation among parameters in a linear discriminant analysis (LDA). LDA allows assessing whether overall health changes from one life stage to the next as animals age. Survival, reproductive and body condition senescence have been already evidenced in this population (Hayward et al., 2014; Lalande et al., 2020; Robinson et al., 2012), calling for better understanding of the underlying mechanisms to design interventions and better care. Because as in many mammalian species, male elephants experience several years' shorter lifespan compared to females (Lahdenperä et al., 2018), we expect sex-differences in the age-related health patterns with males ageing faster than females.

2. Materials and Methods

2.1 Study population

Myanmar has the largest captive population of elephants worldwide, of around 5,000 individuals (Sukumar, 2006), 3000 of which are government-owned through the MTE for sustainable logging (Leimgruber et al., 2011). Government-owned timber elephants inhabit forest camps, distributed across Myanmar. MTE elephants are living in an environment which allows us to explore 'natural ageing': 1) they can display natural behaviours as they are released to the forest at night to forage, interact and socialise with conspecifics, 2) Breeding rates are natural with no reproductive management, 3) Timber elephants are never culled, and 4) Though elephants benefit from veterinary care, only basic healthcare was available during most of the study period. Importantly, the semi-captivity enables close monitoring of physiological health markers of animals with known birth dates, not possible in any wild population of a species this long-lived.

Reproductive females are given rest from mid-pregnancy (11 months into gestation) until the calves reach their first or second birthday. Mothers are then used for lighter work duties until

the calf reaches age four and is capable of foraging independently. Calves are separated from their mother and tamed/trained at around the age of four to five (Crawley et al., 2020), at which point they are assigned a rider, name, logbook and registration number. After the training period, elephants are used for light work duties until the age of around 20, at which point they enter the full workforce until retirement at around 50. The MTE imposes regulations on the daily and annual workload of elephants, which cannot be exceeded and are consistent for all individuals in the study population (Zaw, 1997). The work season lasts from mid-June to mid-February, with a rest period during October. This working season correspond to the monsoon (July-October) and cool (November-February) seasons, so that no work is done during the dry season (March-June) when temperature-related mortality is highest (Mumby et al., 2013). There are strict limits for the annual maximum tonnage of logs each elephant can move and also strict limits of weekly days and hours of work (in 2010; limits were set to a daily maximum of eight hours, with a break at noon, and five days of work in a week (Hayward et al., 2014)). Animal ethics was approved by MTE, Ministry of Natural Resources and Environmental Conservation in Myanmar and Turku University in Finland for manipulation and sample collection from these animals. The animals used in this study did not present signs of a clinical disease.

Each elephant is marked with a unique identification (ID) number and has important life-history information recorded in logbooks. Logbooks include individually-based information, such as the identification number and name of each animal, their birth origin (captive-born or wild-caught), date of birth, year of capture (if wild-captured), date of death or last known date alive. Birth dates are known precisely for captive-born individuals (72.8% of individuals included in this study), whereas the age at capture (and thus approximate birth year) of wild-caught individuals is estimated by comparing their height and body size with captive-born elephants of known age, and through morphological assessment (Lahdenperä et al., 2018; Mumby et al., 2015).

2.2 Elephant health's parameters

We measured a total of 17 health parameters in order to investigate age effects on several physiological responses from 2016 to 2018 over three seasons. Namely, we used a set of 12 parameters for the single-trait analyses, a set of five health parameters for the white blood cell multivariate analysis, and a set of 13 health parameters for the multivariate analysis (see

Statistical analysis's section for details). All elephants were measured and sampled in mornings on non-workdays. To investigate haematological and serum chemistry levels, blood was collected from an ear vein in three different tubes, namely EDTA, heparin and serum separator tubes by trained local veterinarians as part of their regular health monitoring of the animals, in accordance with the local and University of Turku ethical guidelines. The collected blood tubes were refrigerated for a maximum of 24 hours until analysis in the laboratory. For serum chemistry, the samples were centrifuged (RCF – 1320g) for 20 minutes and this process was done between 2 to 6 hours after collection, and sera were collected and frozen at -20ºC. These samples were stored between 6 and 316 days until analysis in a laboratory in Yangon using the IDEXX VetTest® (IDEXX, Westbrook 04092, USA). Several steps were taken to guarantee quality control in serum chemistry analysis, namely (i) the validity of every batch was always confirmed; (ii) some cartridges from the new batches were randomly selected and ran with a sample from the day before, with a maximum of 10% difference accepted; (iii) when a suspicious pattern was observed, a calibration run was performed and the samples reran thereafter; and (iv) once a month, a quality control was performed using pooled samples. These pooled samples were aliquoted and stored in −20◦C. Each quality control run was not expected to differ by more than 10% from the first run (Franco dos Santos et al., 2020). The blood samples collected in EDTA were used to perform a manual count of leucocytes using Turk's solution (Franco dos Santos et al., 2020). A 100-cell differential leucocyte counts were performed manually using a blood smear stained with Romanowsky stain (Franco dos Santos et al., 2020). We used VetScan i-Stat 1 with E3+ cartridges for measuring haematocrit and haemoglobin. This device is partially validated for Asian elephants (Tarbert et al., 2017).

2.2.1 Blood cells and haematology

We measured age-related variation in immune responses by counting total white blood cells (*TWBC*; 179 elephants) and each group of white blood cells (*lymphocytes*, *monocytes*, *heterophils*, *eosinophils* and *basophils*). The number of lymphocytes measured the adaptive immunity, the number of monocytes and heterophils measured the innate response, and the eosinophils and basophils measured the immunity against internal and macro-parasites, and the inflammatory response (Cheynel et al., 2017; Karasuyama et al., 2011). We measured the

percentage of red blood cells using haematocrit (178 elephants), and the oxygen carrying capacity using haemoglobin (161 elephants)(Fowler and Mikota, 2006).

2.2.2 Proteins and triglyceride levels

To investigate age effects on homeostasis, we measured the albumin, globulins levels, and the sum of albumin and globulins, which represents the total proteins (180 elephants). Albumin maintains the osmotic pressure and transport of several hormones, vitamins and haemoglobin. Globulins intervene in the immune and inflammatory responses (Fowler and Mikota, 2006). We also quantified age effects on the state of lipid storage using triglyceride levels (181 elephants) which are expected to decline as a result of senescence of body condition (Nussey et al., 2011).

2.2.3 Kidney function, liver and muscle damage

First, we investigated age effects on kidney function by measuring blood urea nitrogen (*BUN*; 181 elephants) and creatinine (178 elephants). As end products of protein and muscle metabolism, they are good indicators of age-related variation in kidney function (Fowler and Mikota, 2006). Second, we measured the age-related variation in enzyme activity in the liver by assessing aspartate transaminase, important in amino acid metabolism (*AST*; 181 elephants), and alkaline phosphatase working on energy metabolism (*ALKP*; 180 elephants)(Fowler and Mikota, 2006). To quantify age effects on enzyme activity in muscle, we measured creatinine kinase (*CK*; 177 elephants).

2.3 Statistical analysis

2.3.1 Procedure, outliers and repeatability

All analyses were conducted using R version 3.5.1 (Team, 2018). To investigate the age-related variation in health parameters, we used two complementary methods: a single-trait analysis and, a multivariate-health parameters analysis combined with a discriminant analysis. In the single-trait analysis, we analysed each health parameter separately in order to describe specific, potentially age-related changes in physiology. In the single-trait analysis and particularly for differential white blood cells, we used a multivariate-blood cells analysis using a Dirichlet distribution (see below in '*White blood cells: Multivariate generalized linear model*

framework' section). The multivariate analysis allowed us to capture an overall association of age with health by considering the covariation of health parameters.

To prevent outlier points driving our analyses, we removed outliers before conducting any statistical analysis using the Horn method that determines outliers in a Box-Cox transformed dataset using Tukey's interquartile (IQR) fences (see Table S1 for the details of datapoints removed). A point was considered as an outlier when it lied outside 1.5 * IQR from the 1st or 3rd quartile point. We used the function *horn.outliers* from the package "referenceIntervales" (Finnegan and Finnegan, 2015).

For all methods, age was included as a 4-level variable following elephants' life history: 1) taming young elephants to habituate the calves to humans (4-10 years old - calves); 2) training elephants used for light work tasks only (11-20 years old-juveniles); 3) adult elephants (21-50 years old--adults), with age 20 corresponding to the stop of growth and the average age of first reproduction (Lahdenperä et al., 2014) and peak fertility in females(Hayward et al., 2014); 4) retired elephants not subject to physical work (51-72 years old--seniors).

To assess within-individual consistency in the health parameters, we measured the repeatability of serological measures using the R package *rptR* (Stoffel et al., 2017) (see Methods in (Diogo J Franco dos Santos et al., 2020)). Most values were > 0.2 except for basophils equal to 0 (see table S2 for details in Supplementary information).

2.3.2 Single-trait analysis

We analysed the age-related variation in **immune function** (*TWBC*), in **haematology** (*haematocrit, haemoglobin*), **blood chemistry** (*globulins, albumin, and total proteins*), **fat storage** (*triglycerides*), **kidney function** (*BUN, creatinine*), **liver damage** (*AST, ALKP*), and **muscle function** (*creatinine kinase*), trait per trait. We first determined the probability distributions for each health parameter. To do so, we fitted univariate distributions to each parameter. We analysed the total white blood cells count, haematocrit, haemoglobin, total protein, globulins, albumin, and creatinine using a Gaussian distribution and AST, ALKP and CK using a Poisson error distribution with a log link function, and triglycerides and BUN using a Gamma distribution with a log link function. We used Wald tests with adjusted p-values for multiple testing to measure the contribution of the 4-level age factor. We included sex, captive or wild-caught origin of the elephant, season of sampling, and the elephant camps as fixed factors and the individual identity, the year of birth and, the year of sampling as random

factors. For health parameters linked to the serum chemistry (i.e. *total proteins*, *globulins, albumin*, *AST, ALKP*, *CK*, *triglycerides*, *BUN* and *creatinine*), we included the serum storage time as a covariate. When fitting models, singularities issues appeared because of low number of individuals for certain year of birth, we thus removed the year of birth as a random factor for TWBC, globulins and creatinine, and we removed the random factors for triglycerides. Moreover, as numerous studies have shown sex-specific senescence patterns (Maklakov and Lummaa, 2013), we also tested the interactive effect between age and sex using Wald tests. We used the function *wald.test* from the package "aod" (Lesnoff et al., 2010) - with the given p-value are adjusted for multiple testing using a Benjamini & Hochberg correction - *lmer* and *glmer* from the package "lme4" (Bates et al., 2015).

2.3.3 White blood cells: Multivariate generalized linear model framework

The blood cell count was performed using a manual differential approach where a fixed total of 100 cells was counted and thus, errors are necessarily correlated between the different cell types. Therefore, we specifically analysed the age-related variation in white blood cells (*lymphocytes, monocytes, heterophils, basophiles and eosinophils*) using a multivariate generalized linear model framework with a Dirichlet distribution, which allowed inclusion of multiple response variables. The Dirichlet distribution is the multivariate generalization of the univariate beta distribution. As the Dirichlet distribution is provided in GLMs but not GLMMs, we were not able to include random effects for this analysis. We used the function *DirichReg* from "DirichletReg" package (Maier, 2014).

2.3.4 Overall health: multivariate mixed model framework

To test whether and how overall health declines as these long-lived animals age, we assess the overall variation in health across the four life stages using a multivariate mixed model framework that considers co-variation among parameters in a linear discriminant analysis (LDA). In this analysis, we included 13 health variables: **haematocrit**, the **absolute white blood cells counts** (*lymphocyte, monocyte, heterophil, and eosinophil*), **protein levels** (*albumin, globulins*), **triglycerides**, **kidney function** (*BUN, creatinine*), **enzyme activity** in the liver (*ALKP, AST*) and in the **muscle** (*CK*). We did not include **total white blood cell counts** (*TWBC*) and **total proteins** (*TP*) because they are the sum of other health variables: TWBC= lymphocytes + heterophils + monocytes + eosinophils + basophils and TP= globulins + albumin. We did not

include basophils because of the absence of repeatability in the measure (Table S2). Haemoglobin was removed from the analysis because of convergence problems when fitting the model caused by a large number of missing values for this measure (180 NA's; absence of the device at the beginning of the study period). First, we used a Gaussian multivariate mixed model (MCMCglmm) which allows inclusion of multiple response variables, and from which we extracted residuals. We included the 13 health variables as multi-responses. We included elephant camp, season of sampling (hot, monsoon, cold), and wild-captive origin as fixed factors. We fitted the covariance between individuals by including individual as a random effect. We also checked whether our results were dominated by the priors (visual evaluations of the posterior distributions). We used a Gaussian error structure and identity link. MCMCglmm uses inverse-Wishart distributed priors for variances. We here specified proper priors with parameter "V" for the variances in **R** (matrix containing the residual covariances) and in **ID** (matrix denoting the between individual covariance) set at the repeatability for each trait. The parameter "nu" (degree of belief) was equal to the number of health parameters to be estimated in **R** and **ID** (Brommer et al., 2014)**.**

Second, we performed a Linear Discriminant Analysis (LDA) that projects the 13 health parameters into a lower-dimensional space with age class-separability; and provides a discriminant function between age groups based on a combination of health parameters. The discriminant functions represent axes that maximise the distance between means of age-category and minimize the variance of each age-category. As a result, the linear discriminant function maximizes the separation between age groups. We report the results of the two first discriminant functions called axis 1 and axis 2 which best maximize the variance between age groups. In a first LDA, we included the residuals of the multivariate mixed model and 4-level age category. In a second LDA, we included the residuals of the multivariate model and the interaction between 4-level age category and sex. We tested for the significance of the discriminant values (Eigenvalues) using a multivariate analysis of variance (MANOVA) with a Pillai test. We used the function *MCMCglmm* from "MCMCglmm" package (Hadfield et al., 2019), and the LDA was performed with "ade4" and the function *discrimin* (Dray and Dufour, 2007).

3. Results

3.1 Single health trait analysis

To determine whether and how health declines as long-lived animals such as elephants age and to identify the most important molecular and physiological parameters underlying the age-related declines in health, we first analysed age-related variation in each health trait separately and tested for sex differences. We found a significant change with life stage in haematology, blood chemistry, immune, and liver functions (fig.1), whilst the kidney (*BUN*, *creatinine*) function and muscle (*CK*) damage did not display statistically significant change with age. The haematocrit (4.5%), total proteins (7.4% g/dL) and globulins (10.9% g/dL) increased between calf and adult life stage. Serum chemistry (*albumin*: -3.9% g/dL) and circulating fat (*triglycerids*: -10.7% mg/dL) decreased between adult and senior life stage. Finally, immune function (*TWBC*: -11.7% cells/L), and liver functions (AST: -19.6% U/L; *ALKP*: -13.1% U/L) decreased between calf and senior life stage (see detailed results below and in SI).

3.1.1 Haematology

We found support that red blood cell levels varied with elephant life stage (haematocrit: χ^2= 11.2, df= 3, p-adjusted=0.02; haemoglobin: χ^2= 7.8, df= 3, p-adjusted=0.07, Table S3). We detected an increase in haematocrit between the calf and the juvenile life stages (β= 1.58 ± 0.59, t = 2.68), that remained constant thereafter (β= -0.08 ± 0.61, t = -0.13). We did not detect a significant difference between the sexes (β= -0.39 ± 0.35, t = -1.11) or an interaction between life stage and sex (χ^2= 4.9, df= 3, p-adjusted=0.31). Haemoglobin remained stable between calf and adult life stages (β= 0.38 ± 0.23, t = 1.63) and for seniors (β= 0.12 ± 0.24, t = 0.53). As for haematocrit, we did not detect any difference between the sexes (β= -0.21 ± 0.14, t = -1.48) or an interaction between sex and age (χ^2= 2.7, df= 3, p-adjusted=0.55). In contrast, we showed that TWBC strongly decreased over the life stages (TWBC; χ^2= 17.3, df= 3, p-adjusted<0.01; Table S3). The interaction between age and sex was not supported, confirming that such declines were similar for both males and females (χ^2= 0.9, df= 3, p-adjusted=0.83).

3.1.2 Protein levels

The three measures of protein levels varied strongly with life stage (*Total proteins*: χ2= 65.9, df= 3, p-adjusted<0.01; *Albumin*: χ2= 17, df= 3, p-adjusted<0.01; *Globulins*: χ2= 94.1, df= 3, p-adjusted<0.01,Table S4). Total proteins and globulins displayed similar age-related pattern to

each other, with an increase until adult stage ($\beta_{total\ proteins}$= 0.54±0.08, t-value= 6.95; $\beta_{globulins}$= 0.50±0.06, t-value= 8.33) which remained stable between adult and senior stage ($\beta_{total\ proteins}$= 0.06±0.09, t-value= 0.74; $\beta_{globulins}$= -0.05±0.07, t-value= -0.77). Albumin remained stable between calf and adult stage and decreased thereafter ($\beta_{adult\text{-}senior}$= -0.12±0.04, t-value= 2.96). The age-related variation in total proteins, globulins and albumin were similar both for males and females as we did not detect a significant interaction between sex and life stage on total proteins, albumin or globulins variation (*Total proteins*: χ2= 5.2, df= 3, p-adjusted=0.31; *Albumin*: χ2= 1.8, df= 3, p-adjusted=0.67; *Globulins*: χ2= 3.7, df= 3, p-adjusted=0.45).

3.1.3 Triglycerides levels

Triglycerides level varied with life stage (χ^2= 9.3, df= 3, p-adjusted=0.04, Table S5), remaining constant until adult stage ($\beta_{calves\text{-}adults}$= -0.05±0.15, t-value= -0.32) and declining thereafter (β= -0.32 ±0.16, t-value= -1.94). The age-related pattern remained the same between sexes (χ^2= 10.2, df= 3, p-adjusted=0.10).

3.1.4 Kidney activity

We showed that BUN did not vary with elephant's life stage (χ^2= 5.8, df= 3, p-adjusted=0.14; Table S6) in either sex (χ^2= 9.3, df= 3, p-adjusted=0.10; Table S6). Similarly, creatinine did not change with life stage (χ^2= 3.7, df= 3, p-adjusted=0.30) similarly for males and females (age x sex interaction: χ^2= 6.8, df= 3, p-adjusted=0.21).

3.1.5 Liver and muscular damage

Liver damage strongly varied with life stage (*AST*: χ^2= 10.3, df= 3, p-adjusted=0.03; *ALKP*: χ^2= 188.1, df= 3, p-adjusted<0.01; Table S7), though we did not find an influence of age on muscular damage (*CK*: χ^2= 3.3, df= 3, p-adjusted=0.27). We observed the same pattern of decline with age for both liver enzymes (Table S7). Such declines over life were similar for males and females (age x sex interaction for AST: χ^2= 10.1, df= 3, p-adjusted=0.10, for *ALKP*; p-adjusted=0.21 and for CK p-adjusted=0.55).

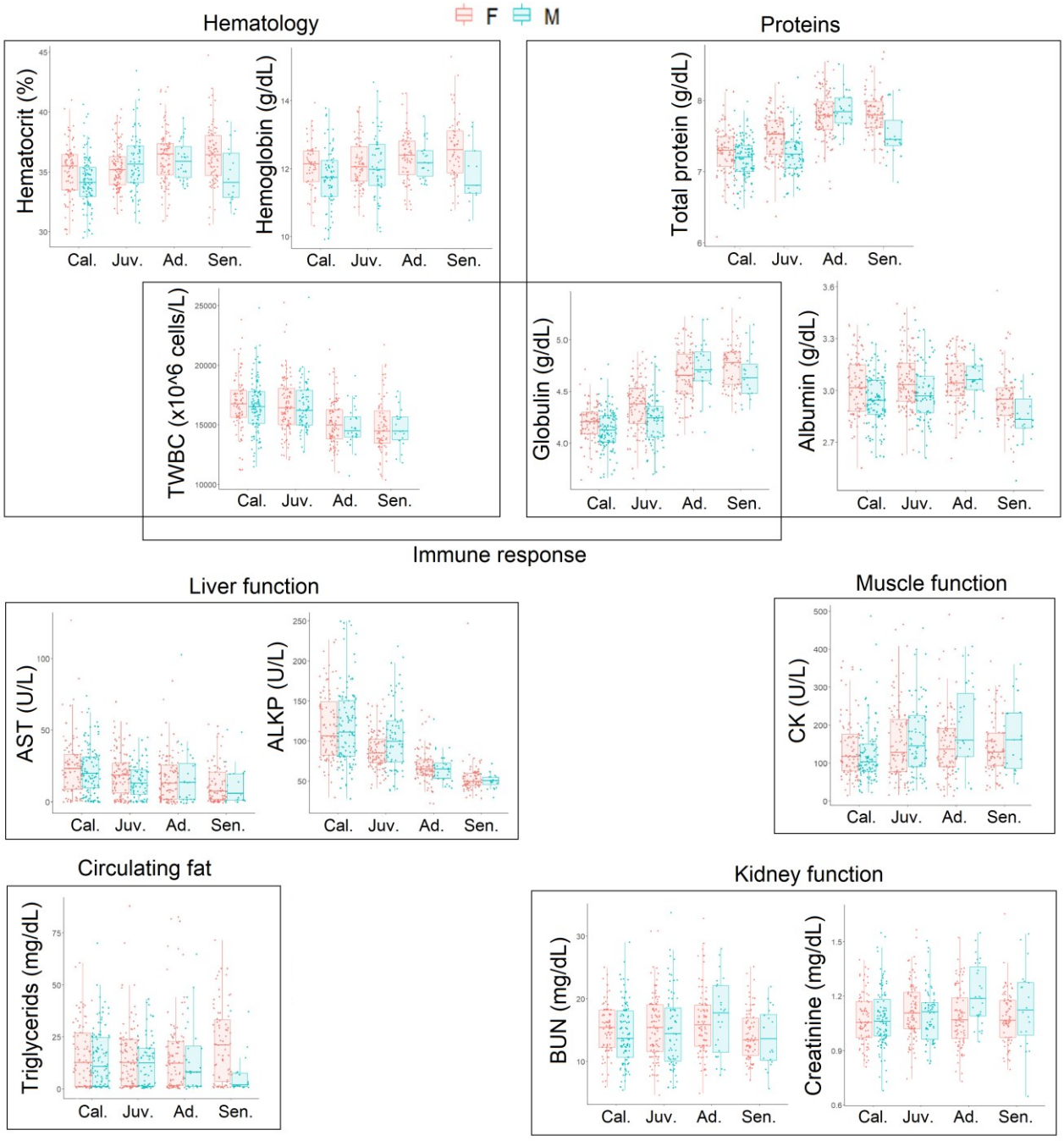

Figure 1. Age-related changes (calves, juveniles, adults, seniors) in health parameters for both sexes (red for females; blue for males). Solid circles represent the unscaled partial residual health traits which account for all main effects (see Appendix for the fixed and random confounding variables for each health trait) but not the effect of sex and age. The horizontal line within the box indicates the median of partial residuals, boundaries of the box indicate the 25th- and 75th -percentile, and the whiskers indicate the highest and lowest values of the partial residuals.

3.1.6 White blood cells

Although white blood cell counts declined with age overall, a more detailed analysis (see SI) reveals that the pattern varied depending on the cell type. Indeed, the decline of total white blood cells over life was due to the decline of lymphocytes, monocytes, and basophils with age (Fig.2). Such declines were similar for both males and females (χ^2= 18.3, df= 15, p=0.25).

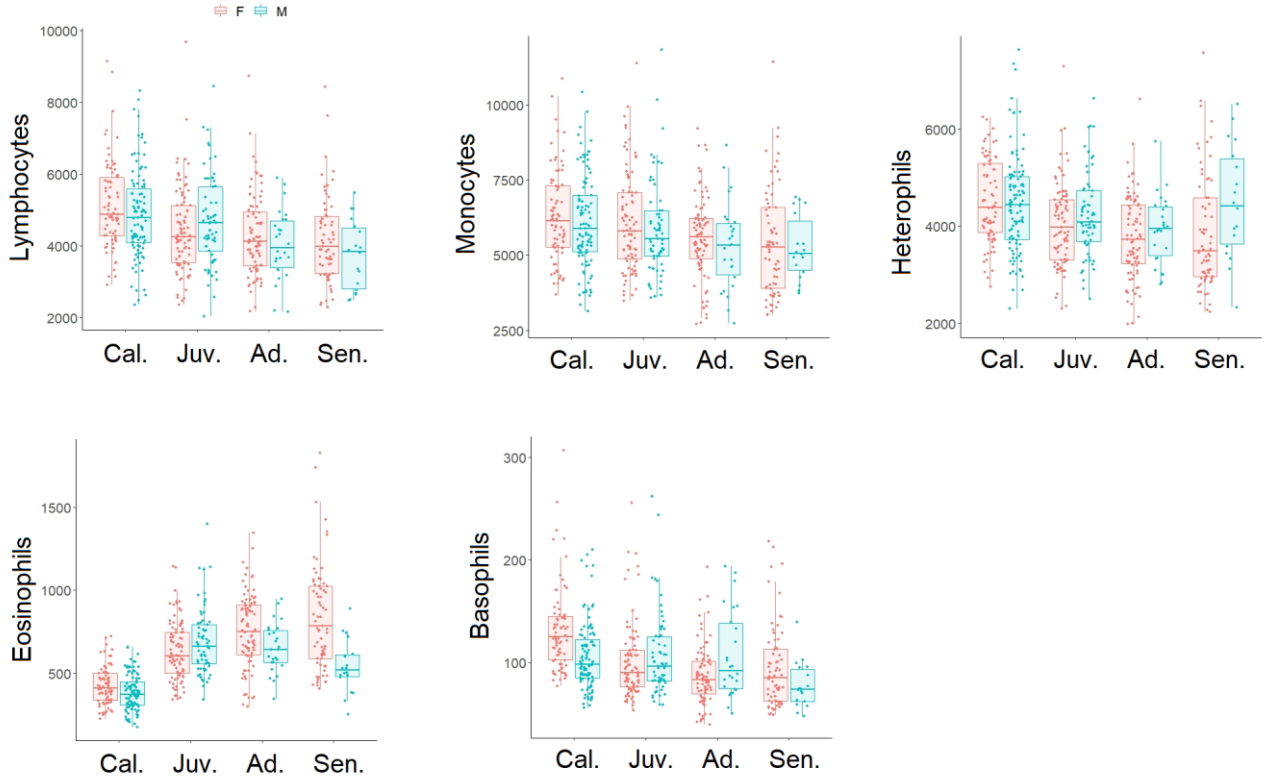

Figure 2. Age-related changes (calves, juveniles, adults, seniors) in white blood cells (x10^6 cells/L) for both sexes (red for females; blue for males). Solid circles represent the predicted values from the Dirichlet model which account for all main effects. The horizontal line within the box indicates the median of partial residuals, boundaries of the box indicate the 25th- and 75th -percentile, and the whiskers indicate the highest and lowest values of the partial residuals.

3.2 Overall health analysis

To confirm the observed patterns in health declines with age, we also assessed the overall variation in health across the four life stages using a multivariate mixed model framework that considers co-variation among parameters (Fig. 3 and see SI for details). Importantly, the multivariate analysis showed similar results as the single-trait analysis. We provide strong

support that overall health declined with age (df_{health}=413, Pillai=0.16, F=1.72, p<0.01) and while close to significance, we do not detect sex difference in the senescence pattern of overall health (sex x age interaction: df_{health}=409, Pillai=0.27, F=1.08, p=0.06). Indeed, age was a strong discriminating factor on the axis 1 of the LDA based on 13 health parameters (Fig. 3). The axis 2, which discriminated seniors from adults based on health, could represent an axis of ageing. Overall, age influenced mainly four health functions: immune function (*eosinophils*), liver damage (*alkp*), circulating fat (*triglyceride*s), and proteins level (*albumin*) as they showed the highest coefficients (called hereafter *loadings*; Table S9). In particular, the liver enzyme (*alkp*) had the highest loading on the axis 1 and the triglycerides, creatinine and albumin had the highest loadings on the axis 2.

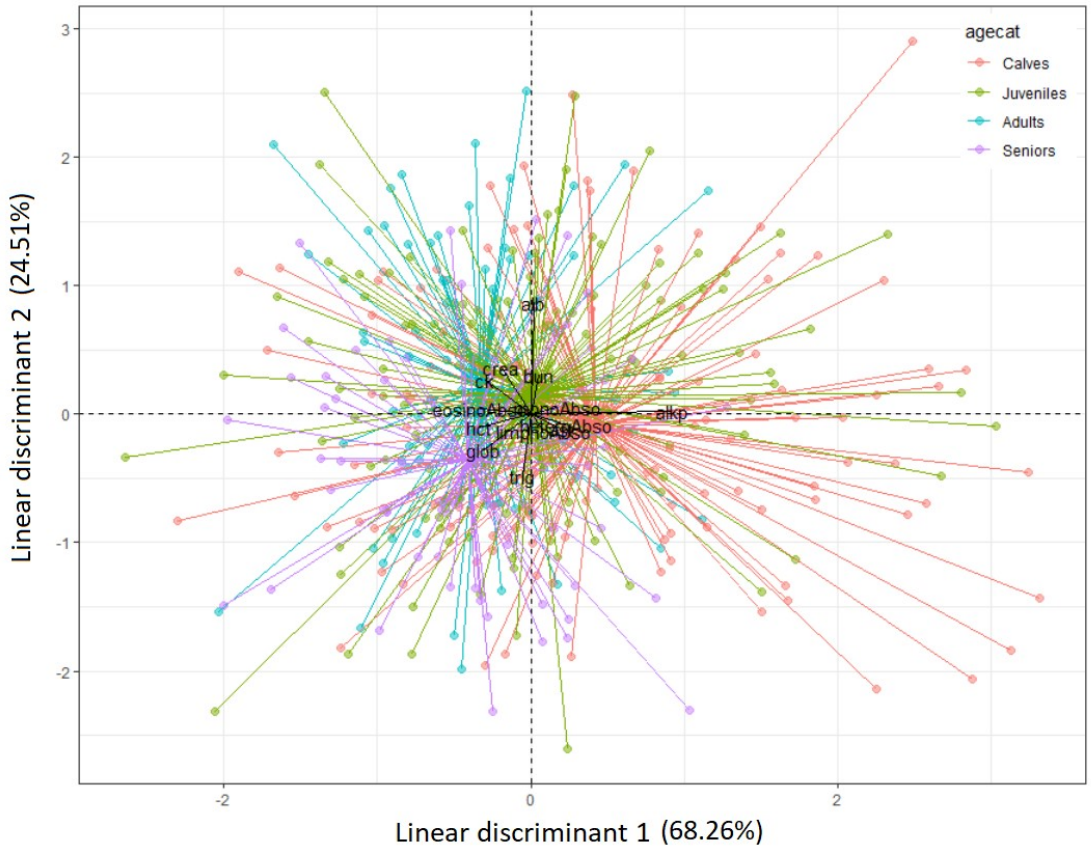

Figure 3. Biplot from the LDA showing clustering of "calves"-red, "juveniles"-green, "adults"-blue, and "seniors"-purple elephants across 13 health parameters. The figure combines the standardized coefficients of health in black (i.e. canonical weights of the linear discriminant functions on the two axes of the linear discriminant analysis), and the projection of the health samples with gravity centres of each life stage.

4. Discussion

Our results support that health parameters decline with age in long-lived mammals living in natural conditions. Up until recently, declines in molecular and physiological health functions associated with ageing were mainly documented in humans and laboratory mammals (Aunan et al., 2016; Partridge et al., 2018). Nonetheless, studies testing evolutionary predictions in natural populations could provide key insights into the causes of inter-individual variation in ageing patterns and into the mechanisms underlying such variation. By providing evidence of a decline in molecular and physiological health functions with age in semi-captive Asian elephants, our results complement the accumulating data that declines in health, reproduction and survival with age are observable in wild populations (Cheynel et al., 2017; Jones et al., 2014). Such detailed data could never have been obtained from the wild due to the need to collect blood repeatedly longitudinally over a long time period. The age-related patterns (i.e. lowest health at older ages) we document are unlikely driven by work schedules of our study population as during the study period logging was largely ceased, and the retired animals do not work: Elephants are retired at the age of 50, but are taken care of until the end of their life with similar feeding patterns (natural feeding). Hence, the decline in health observed in the retired group likely reflects senescence and physiological changes associated with ageing, such as accumulation of cellular damage that decrease physiological functions and lead to ageing and ill-health (Niedernhofer and Robbins, 2018; Paez‐Ribes et al., 2019). Interestingly, the age categories in which we observed the largest decline in the traits we measured correspond to the ones in which we also observe severe declines in reproduction and survival (Lahdenperä et al., 2014). However, our relatively "short-term", cross-sectional sampling relative to the extraordinarily long elephant lifespan precluded us from assigning causal links between the physiological parameters we measured and individual survival and/or reproductive senescence.

First, we determined the most important molecular and physiological parameters underlying the age-related declines in overall health by investigating each trait in a separate analysis, and found several parameters to co-vary strongly with the elephant's age, with a significant effect of age on the variation in haematology, blood chemistry, immunity, circulating fat and liver damage. Interestingly, for the kidney function and muscle damage, we did not detect any effect of age, as creatinine, BUN and creatinine kinase levels stayed

constant across all age groups. By contrast, as demonstrated in humans (Helman and Rubenstein, 1975), we showed that haematocrit and haemoglobin levels increased until the adult life stage and remained stable with age; and the serum chemistry (albumin), the lipid metabolite levels (triglycerides), the immune function (TWBC) and liver enzyme activity (ALKP) decreased sharply between the working and retired life stage. Although white blood cell counts overall declined with age, a more detailed analysis on the different white blood cells revealed that the pattern slightly varied depending on the cell type. While lymphocytes, monocytes, and basophils decreased with age, heterophils and eosinophils increased (see table S8 in SI). The same result has been observed in humans and reflects a dysfunction in the immune system that is a relevant marker for elderly disease prognosis (Cataudella et al., 2017). Both the decrease in total white blood cells and the increase in globulins with age are characteristics of decreased immune function with age, as previously shown in humans and few vertebrates in the wild (Franceschi et al., 2006; Nussey et al., 2012). This increase of globulins might suggest that a progressive dysregulation of the inflammatory response occurs at old ages, leading to an increase in the production of related inflammatory products, which could be responsible for chronic inflammation causing tissue degeneration. The declines in both the innate and the adaptive systems and the increase of globulins levels with age indicate the existence of immunosenescence in Asian elephants. In parallel, we also observe a decline in lipid metabolite levels, which often reflects an alteration of body condition with age (Jenni-Eiermann and Jenni, 1994; Toth and Tchernof, 2000). In addition, the decrease in AST and ALKP levels with age implies liver damage and/or a decline in liver function, which are symptomatic of an ageing hepatic function. Such age-related changes in health parameters were generally consistent in both males and females, because we did not detect significant interactions between sex and age for any of the health parameters.

To confirm the observed patterns in health declines with age, we also assessed the overall variation in health across the four life stages using a multivariate mixed model framework that considers co-variation among parameters. This discrimination of elephant's age according to health parameters allowed us to show that reduced fat levels and immunity were associated with senior elephants, change in liver function was associated to the youngest elephants, and proteins level changes were associated with adult elephants. Interestingly, we did not detect any significant sex difference in the senescence pattern of overall health. Lack of sex differences in these age-related variations in health parameters (see Supplementary

information) differs from the empirical evidence in the field (Roach and Carey, 2014), but might reflect species-specific patterns, and is consistent with higher age-specific mortality of males in our population leading the stronger selective disappearance of poor-health males as opposed to females from the oldest age classes (Lahdenperä et al., 2018).

To conclude, our results provide evidence of the underlying molecular and physiological health functions decreasing with age in a long-lived mammal, to-date mainly documented in humans and laboratory mammals. Although, our sampling schedule prevents us from directly linking the physiological parameters and survival and/or reproductive senescence, ageing effects on health were still observed when evaluating health globally but also in the majority of the individual traits, especially in immunity, circulating fat and liver damage. These findings match with the observed declines in survival and reproduction across taxa (Jones et al., 2014), and call for more studies that could create the link between those life-history traits and physiological mechanisms. By providing the first evidence for age-related differences in a broad range of physiological markers in a long-lived mammal living under natural conditions, our data suggest a certain ubiquity in the patterns previously observed (Cheynel et al., 2017; Nussey et al., 2012). Our results also suggest that such age-dependent differences in health markers might be a target for natural selection in wild populations and emphasise the need for more longitudinal research in wild populations to shed further light on the underlying mechanisms, the evolutionary causes and the consequences of their variation in ageing.

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