Does baseline innate immunity change with age? A multi-year study in great tits

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Abstract

Throughout their life animals progressively accumulate mostly detrimental changes in cells, tissues and their functions, causing a decrease in individual performance and ultimately an increased risk of death. The latter may be amplified if it also leads to a deterioration of the immune system which forms the most important protection against the permanent threat of pathogens and infectious diseases. Here, we investigated how four baseline innate immune parameters (natural antibodies, complement activity, concentrations of haptoglobin and concentrations of nitric oxide) changed with age in free-living great tits (*Parus major*). We applied both cross-sectional and longitudinal approaches as birds were sampled for up to three years of their lives. Three out of the four selected innate immune parameters were affected by age. However, the shape of the response curves differed strongly among the innate immune parameters. Natural antibody levels increased during early life until mid-age to decrease thereafter when birds aged. Complement activity was highest in young birds, while levels slightly decreased with increasing age. Haptoglobin levels on the other hand, showed a linear, but highly variable increase with age, while nitric oxide concentrations were unaffected by age. The observed differences among the four studied innate immune traits not only indicate the importance of considering several immune traits at the same time, but also highlight the complexity of innate immunity. Unraveling the functional significance of the observed changes in innate immunity is thus a challenging next step.

Keywords: Immunosenescence, innate immunity, natural antibodies, complement system, haptoglobin, nitric oxide

1. Introduction

The immune system enables animals to fight off invading pathogens and established infections (Sheldon and Verhulst 1996). It can be divided in innate immunity, which serves as a non-specific first line of defense, and adaptive immunity, which provides specific protection and immunological memory (Abbas and Lichtman 2009; Grogan et al. 2008). The immune system has a high fitness value because it reduces the risk of disease-related mortality. But it is also costly and may thus be traded-off against other life history functions (Sheldon and Verhulst 1996). Trade-offs such as between the investment in reproduction and self-maintenance likely vary across the lifespan causing variation in immune function with age (e.g. Palacios et al. 2007). In addition, animals accumulate mostly detrimental changes in cells, tissues and their functions in the course of their life, causing a decrease in individual performance and ultimately an increased risk of death. Such a progressive decline in the overall performance of an ageing organism (=senescence) (Torroba and Zapata 2003; Lavoie 2005; Tosato et al. 2007; Holmes and Martin 2009; Noreen et al. 2011; Hammers et al. 2015) can have significant negative effects on survival and central life-history traits such as reproductive performance in many wild animal species ranging from birds to mammals (Catchpole et al. 2000; Wilson et al. 2007; Descamps et al. 2008; McCleery et al. 2008; Sharp and Clutton-Brock 2010; Bouwhuis et al. 2012). A deterioration of physiological functions with age may also affect the immune system, despite the fact that it forms the most important protection against the permanent threat of pathogens and infectious diseases (Palacios et al. 2007, Effros 2003). Evidence for ageing effects on immunity has been shown in a number of animal species, ranging from invertebrates to vertebrates (reviewed in Müller et al. 2013). Nevertheless, most knowledge about (immuno)senescence still derives from human studies or studies using laboratory animals, while studies using free-living animals remain rather rare (Cichon et al. 2003; Holmes and Martin 2009; Beirne et al. 2016). Ageing affects both arms of the immune system, leading for example to a diminished proliferative capacity of T cells, reduced B-cell responses, loss of lymphocyte subgroups and a qualitative deficiency of B-lymphocytes (Malaguarnera et al. 2001). In the case of innate

immunity, ageing has been shown to cause a decrease in natural killer cell activity, altered cytokine production and a decreased ability of macrophages to destroy bacteria (Plackett et al. 2004; Aw et al. 2007). Still, most studies investigating immunosenescence focus on cell-mediated and humoral immunity, while only recently the research field is extending to innate immunity (Aw et al. 2007; Palacios et al. 2007).

However, age-related changes are not only occurring towards later life stages (Lavoie et al. 2007; Møller and Haussy 2007). Young animals still have to develop their immune system and measures of immunity will thus increase during early life phases (Klasing and Leshchinsky 1999). As a consequence, immune traits are expected to show a bell-shaped relationship with age (Cichon et al. 2003; Saino et al. 2003; Lavoie et al. 2007; Møller and Haussy 2007; Noreen et al. 2011). Similar patterns have also been shown for traits associated with fitness (e.g. survival probability, reproduction and song consistency), while here early improvements are thought to relate to an increase in experience followed by a decline due to a functional deterioration with age (e.g. Ottinger et al. 1983; Dhondt 1989; Catchpole et al. 2000; Wilson et al. 2007; Alonso-Alvarez et al. 2010; Rivera- Gutierrez et al. 2012).

In this study, we investigated how baseline innate immune parameters of free-living great tits (*Parus major*) changed with age. We compared four innate immune parameters among birds belonging to different age classes, using a cross-sectional approach that makes use of the most extensive data set. For those individuals that have been measured repeatedly we also performed a longitudinal analysis in order to distinguish within-individual changes in performance from processes such as selective disappearance of specific, often low-quality individuals due to mortality, which is likely to bias the phenotypes of the remaining population. We hypothesize that age-related changes in innate immunity will be visible at the individual level, reflecting progressive deterioration of baseline

immunity. However, as we hypothesize that younger animals still have a developing immune system (Klasing and Leshchinsky 1999), the general pattern will likely be bell-shaped.

2. Materials and methods

2.1 Study sites and data sampling

The study was performed in three great tit populations near Antwerp, Belgium (Wilrijk: N51 9 56.3, E4 22 36.7; N51 9 47.2, E4 24 10.2; Mortsel: N51 10 28.2, E4 27 37.3). All study sites have the same habitat type which can be specified as deciduous park area. Each population had between 35 and 127 great tit nest boxes, with the number of nest boxes depending on the size of each plot. Every year, all nestlings and all immigrants (new individuals without a metal ring) are ringed with an uniquely coded metal ring. Birds were sexed either molecularly or based on plumage traits (Griffiths et al. 1998, Svensson 1984). Since 2012 birds are also provided with a passive integrated transponder tag (PIT tag) inserted in a colour ring, which makes identification with minimal disturbance possible (e.g. to identify birds in the nest box without opening it and subsequently only opening the nest boxes of target birds to collect a blood sample). For all birds used in this study, the exact year of hatching was known. For first year birds this was determined based on their plumage characteristics (Svensson 1984), which enabled us to distinguish between juveniles (hatched that same year) or adults (>1 year). Birds caught as adult for the first time were not further used in this study. Age of a bird is indicated in months. During the winter of 2012 (November to February) we conducted several night controls (great tits roost in nest boxes during winter at night which makes capturing/recapturing highly successful) in all study areas. Birds of which we knew the exact age were blood sampled, taking ± 150 µL of blood from the brachial vein using a Microvette CB 300 lithium-heparin tube (Sarstedt). Further, we also measured their body mass (precision 0.1 g) and tarsus length (precision 0.01mm). During the winter of 2013 and 2014 we repeated the night controls and the blood sampling of target birds. Further, we tried to retrieve as many of the birds we sampled

131 in the previous winters as possible in order to investigate the within individual effects of ageing on
132 innate immunity, but each year we also sampled additional birds of known age.

133 In total, we collected blood of birds belonging to six different age classes (N_{total} = 147; hatched in
134 2008 N= 3, 2009 N= 13, 2010 N = 16, 2011 N= 36, 2012 N= 49, 2013 N= 30), of which 100 birds were
135 sampled only once during their life, 35 birds were sampled in two and 12 birds in three - subsequent
136 years/winters (not always consecutive).

137 To minimize the potential effects of stress on baseline immune parameters, all blood samples were
138 collected immediately after taking the bird out of the nest box (Matson et al. 2006; Millet et al. 2007;
139 Buehler et al. 2008). All blood samples were stored under cool conditions during transport and were
140 centrifuged at 7000 rpm for 10 minutes. Plasma samples were transferred to a new Eppendorf tube
141 and stored at -80°C until the start of the immunological assays.

142 We subsequently measured natural antibodies (NAbs, HA scores) and complement activity (HL
143 scores) which are two interrelated non-cellular components of innate immunity. These NAbs are able
144 to broadly recognize and bind antigens which can than result in activation of the complement
145 cascade and end with the lysis of foreign cells (Boes 2000; Ochsenbein and Zinkernagel 2000; Matson
146 et al. 2005; Murphy et al. 2012). Further, we determined the concentrations of the acute phase
147 protein (APP) haptoglobin (Hp, mg/mL). APPs have several antimicrobial functions, such as
148 opsonizing bacteria and activating the complement cascade (Murphy et al. 2012) and are typically
149 synthesized by hepatocytes in response to cytokines released by macrophages when bacteria are
150 present (Owen-Ashley and Wingfield 2007; Cray et al. 2009; Coon et al. 2011; Murphy et al. 2012).
151 Further, their concentrations are known to rise significantly in response to an acute infection, trauma
152 or inflammation (Murata et al. 2004; Quaye 2008; Cray et al. 2009; Matson et al. 2012). Finally, we
153 also measured nitric oxide (NO) concentrations (mmol/L) in the plasma. NO is a multifunctional
154 signalling molecule which acts as a vasodilator, neurotransmitter and a modulator of inflammatory
155 processes. It is biosynthesized by the oxidation of L-arginine by the enzyme nitric oxide synthase

(NOS), which has three isoforms: a neuronal isoform (nNOS) which produces NO in nervous tissue, an endothelial isoform (eNOS) which produces NO in blood vessels and the heart and an inducible isoform (iNOS) which increases when induced by for example inflammatory cytokines, endotoxins or microorganisms. Assessing nitric oxide can provide useful information on individual variation in work load, physiological condition and health state (Bourgeon et al. 2007; Sild and Hõrak 2009).

The above mentioned techniques are field-friendly since they do not require resampling (a single blood sample is sufficient) and furthermore they also require only small blood volumes, which enabled us to assess multiple parameters of innate immunity, thereby accounting for the complexity of the immune system (Norris and Evans 2000; Tieleman et al. 2005; Martin et al. 2006; Demas and Nelson 2012).

2.2 Immunological assays

2.2.1 Haemolysis-haemagglutination assay

We used the haemolysis-haemagglutination assay as developed by Matson et al. (2005) to assess the levels of natural antibodies and complement activity, with some minor alterations: all plates were treated with a blocking solution containing milk powder and Dulbecco's phosphate buffered saline (PBS) and were thereafter washed three times with a PBS-TWEEN 20 solution. Further, we used a serial dilution (1:2) based on 15 µL of plasma and 15 µL of PBS (Vermeulen et al. 2015). In general, the assay is based on the interaction of great tit plasma and rabbit red blood cells which results in agglutination and natural antibody mediated complement activation. Agglutination scores (HA) represent the interaction between natural antibodies in the plasma and antigens present in the rabbit blood, while lysis scores (HL) reflect the interaction of the complement system and natural antibodies. Agglutination and lysis titers were blindly scored from digitized images as the negative $\log_2$ of the last plasma dilution at which agglutination or lysis occurred. Half scores were assigned to

wells which showed intermediate agglutination or lysis. All samples were scored twice on different days, by the same person.

2.2.2 Haptoglobin assay

We quantified plasma haptoglobin concentrations (Hp in mg/mL) using the manufacturer's instructions provided with the commercially available colorimetric assay (PHASE Haptoglobin assay, Tridelta Development Ltd). Additionally, we performed a pre-scan at 630 nm which allowed us to correct for potential differences in plasma cloudiness and plasma color (Matson et al. 2012). Absorbance of all plates was recorded at 630 nm using a Molecular Devices VersaMax Tunable Microplate Reader.

2.2.3 Nitric oxide assay

Concentrations of nitric oxide (NOx mmol/L) were quantified using a spectrophotometric assay based on the reduction of nitrate to nitrite by copper-coated cadmium granules (Sild and Hõrak 2009). This assay consists of three main steps: deproteinization, nitrate reduction, and a Griess reaction. In each plate, a standard curve and an among-plate standard were run in duplicate and absorbance was recorded at 542 nm using a Molecular Devices VersaMax Tunable Microplate Reader.

2.3 Statistical analysis

As our dataset contained measurements of the immune parameters at different ages, including repeated measures of the same individual, this dependency of the observations was taken into account in all our analyses. To achieve this, mixed models were constructed, which included bird ID and the interaction between bird ID and age as random effects. In this way, for each bird with repeated measurements, a random intercept and slope were estimated. The correlation between the random intercept and slope was set to equal zero to avoid numerical problems during the estimation of the parameters of interest. By using a mixed model like ours, we did not only make sure that tests of the fixed effect were corrected for the dependency of repeated measurements, but we also aimed

to separate processes such as selective disappearance of specific (poor quality) individuals from the population, from the within-individual effects of ageing (McCleery et al. 2008; Nussey et al. 2008; Bouwhuis et al. 2009; Hajishengallis 2010). We constructed four separate linear mixed models, one for each immune parameter, containing the immune parameters (HA, HL, Hp or NOx) as dependent variable, age (in months), age², age³, age^4, condition (defined as the residual from the linear regression of body mass on tarsus length; Geens et al. 2009, Ots et al. 1998), year sampled (either 2012, 2013, 2014 or 2015), sex and the interaction between sex and age as fixed effects. We hypothesized that immunity would change in a bell-shaped way with age, which should be reflected in a significant age² effect. However, we anticipated that the curvature between our selected immune parameters and age may be more complex. Hence we also tested for age³ and age^4 in all our models. To illustrate the interpretation of our final models, plots were generated in which the association between the immune parameters and age were plotted on the basis of the fixed effects parameter estimates. To highlight variation among individuals, we added individual changes in the associations between the immune parameters and age on the basis of the individual-specific estimates of random intercepts and slopes (the so-called BLUP's), in case the estimated variances were larger than 0 and only for those birds which contained repeated measurements. In this way, the raw data as well as the model predictions are provided in single plots. The fixed effects part of our model can be viewed as a cross-sectional study, correcting for repeated measurements, while the random effects part models the longitudinal aspect of our data, namely the changes of immune parameters with age within individuals. Even though the two interpretations come from one statistical model, they shed a different light on the variation in immune parameters with age.

Model estimation and testing was achieved using the lmer function imbedded in the package lmerTest in R (Kuznetsova et al. 2013). We started from a model containing all independent variables of interest and their interactions. Subsequently, we used a backward elimination procedure for model reduction. Decisions to keep parameters in the model, were based on a significance level of 5%. Tests of the fixed effects were based on F-tests with denominator degrees of freedom using

Satterthwaite approximations. Model assumptions were checked using histograms, Q-Q plots and Shapiro-Wilk normality tests. We excluded three outliers for Hp and one outlier for NOx from further analysis, since they deviated from the mean with more than three times the standard deviation plus the average. Afterwards, in all cases, the distribution of the residual values approximated normality (Shapiro-Wilks W>0.95). All statistical analyses were performed in R 3.1.2 (R development core team 2014-10-31 release; www.r-project.org).

3. Results

3.1 Effects of age on innate immune parameters: cross-sectional approach

Tests for associations between age and each of the four innate immune parameters showed contrasting patterns for the different measure (Table 1). For **HA**, there was no significant effect of age^3 or age^4. We found a significant effect of age^2, condition (β = -0.27) and sampling year, with birds sampled in 2012 having higher HA values than birds sampled in other years. No significant sex effect or sex*age interaction was found(Table 1). On the basis of the estimated association between HA and age, birds of approximately 25-30 months of age had the highest HA scores (NAbs) and HA scores (NAbs) subsequently declined again beyond that age (Figure 1). For **HL**, there was a significant effect of age^2, age and sex, with males having higher HL values than females (β = 0.91). All other factors were not statistically significant (Table 1). Here, young birds apparently had relatively high HL scores (complement mediated cell lysis), but around the age of 12 months, HL scores started to decline (Figure 2). For **Hp**, we only found a significant effect of age, while all other factors were not significant (Table 1). Young birds had relatively low Hp concentrations whereas these concentrations increased linearly with age (Figure 3). For **NOx**, there were no significant effects (Table 1, Figure 4)

3.2 Effects of age on innate immune parameters: longitudinal approach

On the basis of the repeated measurements of the immune parameters in birds sampled at least twice, the random effects part of our statistical model allowed to investigate to what extent changes in immune parameters with age varied among individuals. Due to the limited number of repeated observations, only variation in intercepts and linear slopes could be modelled. For **HA** and **HL**, we found variation in intercepts but not in slopes amongst the individual profiles, whereas for **Hp** the slopes, but not the intercepts appeared to vary among individuals. For **NOx**, no between-individual variances were detected (Table 1). Because it is difficult to interpret the longitudinal effects on the basis of the estimated variances alone, we added this aspect to the graphs (Figure 1, 2, and 3). For **HA**, the between-individual variation in intercepts appeared relatively low, which is not unexpected given the relatively high residual variation (Figure 1, Table 1). For **HL**, the variation in intercepts appeared relatively high compared to the residual variation (Table 1), which can also be seen by the wider variation in individual profiles for the birds with repeated measurements. All lines were parallel because there was no variation in slopes (Table 1, Figure 2). For **Hp**, slopes did vary across individuals, unlike the intercepts, such that Hp values did not differ strongly among individuals at young ages, but variation increased with age as individual profiles varied in slopes (Table 1, Figure 3). For **NOx**, no between-individual variation in intercepts or slopes was detected (Table 1, Figure 4).

4. Discussion

In this study we explored how age influences baseline innate immune parameters of free-living great tits. We found that three out of the four selected innate immune parameters were significantly affected by age, but the patterns of changes in innate immunity with age differed substantially between all innate immune parameters.

4.1 Effects of age on innate immune parameters: cross-sectional approach

We found evidence that three out of the four selected innate immune parameters were indeed affected by age (Figures 1- 4). NAb levels (HA scores) showed the expected bell-shaped pattern, with levels initially increasing through early life, highest levels in mid-aged birds that plateau, followed by decreased levels in older birds (e.g. Møller and Haussy 2007, Figure 1). This pattern is accordant with the idea that early in life, the immune system is still developing until adult levels are reached (Klasing and Leshchinsky 1999), to decline thereafter when individuals grow older (Cichon et al. 2003; Saino et al. 2003; Lavoie et al. 2007; Møller and Haussy 2007; Noreen et al. 2011). Further, innate defenses reach adult levels earlier than adaptive defenses (Palacios et al. 2009) as they are especially important in young animals which have only poorly developed adaptive immune responses (Klasing and Leshchinsky 1999; Grindstaff et al. 2006). Individuals gaining more immunological experience (memory cells of adaptive immunity) as they get older, may then again depend less strong on NAbs due to the increased availability of other (adaptive) immune traits. The observed bell-shaped pattern resembles previously described age-related changes in other (fitness related) traits [bird song: Rivera-Gutierrez et al. (2012), breeding performance: Dhondt (1989)].

However, such a typical pattern of senescence has not been observed consistently across species. This may relate to differences in life history, as species with longer life spans or slower pace-of-life are thought to invest more in their immune function, slowing down the rate of immunosenescence (Lee 2006; Demas and Nelson 2012). In the case of NAbs, it might be that NAbs play a more important role in long lived animals due to the fact that they have an important role in the elimination of autoantigens, which increase in the elderly (Elkon and Casali 2008; Holodick and Rothstein 2015). This could also explain why they are less important in short-lived species such as the great tit.

It has also been argued that the acquired immune system would show a steeper decline with age than the innate immune system (Noreen et al. 2011). Thus immunosenescence may not act in concert, but rather in complex interplay between the different arms of the immune system.

Unfortunately, we could not measure any other arm of the immune system besides the innate immunity, due to limitations in the sample volume. But based on the four measures of innate immunity investigated here, we show that there is already substantial inconsistency in the effects of ageing within the same arm of the immune system.

In addition to the patterns as described above we found for the complement activity (HL scores), that the highest levels of complement proteins occurred in young birds (≤ 10 months), while these levels slightly but continuously dropped to lower levels in older birds (Figure 2). This is in contrast with other studies investigating this immune trait in birds (Møller and Haussy 2007; Palacios et al. 2007; Nebel et al. 2013). Why agglutination (NAbs), but not lysis (complement) declines with age, is a highly interesting question. Immune components that increase rapidly at early age (e.g. NAbs) could show senescence, while not as rapidly increasing or maturing immune parameters (e.g. complement) may not (see also Møller and Haussy 2007). Another reason could be that great tits show only a small decrease in complement activity in their relatively short life (due to an increased availability of other immune traits), because they rather rely on this immune component due to the relevance of it in several immune responses (e.g. innate and adaptive) (Abbas and Lichtman 2009).

For the acute phase protein haptoglobin (Hp), we found on average increasing concentrations with age (Figure 3). Ageing results in chronic low-grade systemic inflammation and an increase in circulating levels of pro-inflammatory cytokines (Woods et al. 2012), which stimulate the production of acute phase proteins such as haptoglobin (Gruys et al. 2006). Such chronic low-grade inflammation associated with age could be responsible for the increase in Hp concentrations observed here. Yet evidence is limited and comes mainly from medical studies (Fowkes et al. 2006; Spagnuolo et al. 2014). We found no effect of age on nitric oxide concentrations (NOx, Figure 4). The reason why we did not find age-related changes on nitric oxide concentrations remains unclear. It again contrasts a number of studies that show a decline in the release of NO with age in mice and humans (Kissin et al.

1997; Toprakci et al. 2000), amongst others caused by a lack of the endogenous substrate L-arginine with increasing age (Reckelhoff et al. 1994).

4.2 Effects of age on innate immune parameters: longitudinal approach

Differences in traits according to age as obtained from cross-sectional, population-level analyses, do not necessarily reflect ageing processes at the individual level. Selective disappearance of individuals with a specific innate immune system, for example, may also give rise to a relationship between age and immunity at the population level. Identifying within-individual changes in innate immunity with age requires therefore longitudinal approaches as achieved via repeated sampling of individuals. Changes of immune parameters with age within individuals were here derived from the random effects part of our mixed models.

Age-related changes in both NAbs (HA scores, Figure 1) and complement mediated cell lysis (HL scores, Figure 2) are most probably reflecting within-individual changes in immunity, despite the general difference in the pattern of change, as we observed no significant variation in slopes. Both measures however showed significant variation in intercepts, indicating that there are consistent intrinsic differences in innate immunity among individuals. These are likely due to genetic and/or (early) environmental effects (Lifjeld et al. 2002; Cichon et al. 2006; Garvin et al. 2006; Pitala et al. 2007; Hegemann et al. 2012; Brodin et al. 2015). Distinguishing the causes of among-individual variation requires further studies, as we are not able to differentiate between common environment effects, more specifically parental and genetic effects. In the case of Hp however, we found variation in slopes but not in intercepts (Figure 3). This indicates that individual variation in Hp concentrations is smaller in young adults, while it increases with age. We previously explained that a chronic low-grade inflammation associated with age could be responsible for the increase of Hp concentrations. Another possible explanation could be that the high level of variability in Hp concentrations may be due to high a stochasticity in infections. Hp is known to rise significantly in response to an acute

infection, trauma or inflammation. Our limited number of sampling events might have led to the observed variation in slopes, because birds having an infection or inflammation response shortly before sampling becomes probably more likely later in life, which could explain the overall positive effects. Nitric oxide did not vary with age (Figure 4), which was also the case at the within-individual level, suggesting a lack of an age-effect on nitric oxide concentrations. Although this may be related to the central role nitric oxide fulfils during immune responses as well as during various other physiological processes (Bogdan et al. 2000). The fact NO declines with age as shown in a number of previous studies (Reckelhoff et al. 1994; Kissin et al. 1997; Toprakci et al. 2000) has to be interpreted carefully at least with regard to immune function. Indeed, the fact that nitric oxide synthesis respectively secretion was reduced with older age was in fact been interpreted in the context of endothelial (dys)function rather than immunity (Toprakci et al. 2000).

5. Conclusions

Baseline innate immunity of free-living great tits varied with age overall as well as within-individuals. Yet, the innate immune traits studied, differed in how they were affected. Only natural antibodies showed the expected bell-shaped pattern that corresponds with a still developing immune system early in life until adult levels are reached, followed by a decrease in concentrations when individuals age. Similar patterns have been observed for other physiological traits deteriorating with increasing age. Also complement activity declined to a slightly lower level in older birds, but already from early age onwards. A reduced innate protection against pathogens and infectious diseases may, however, to some extent be compensated by other (acquired) components of the immune system. Enhanced variation in haptoglobin levels in older individuals likely reflects stochasticity in infections as haptoglobin reacts quickly to trauma or infection. Finally, the signaling molecule nitric oxide seemed to be unaffected by age. Thus, our findings suggest that the different innate immune components studied may serve different functions throughout an individual's lifespan, at least in our study

species. But the adaptive significance of the observed patterns still needs to be unraveled to fully understand innate immunity variation with age.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This study was approved by the ethical committee of the University of Antwerp (ID number 201131) and it was performed in accordance with Belgian and Flemish laws.

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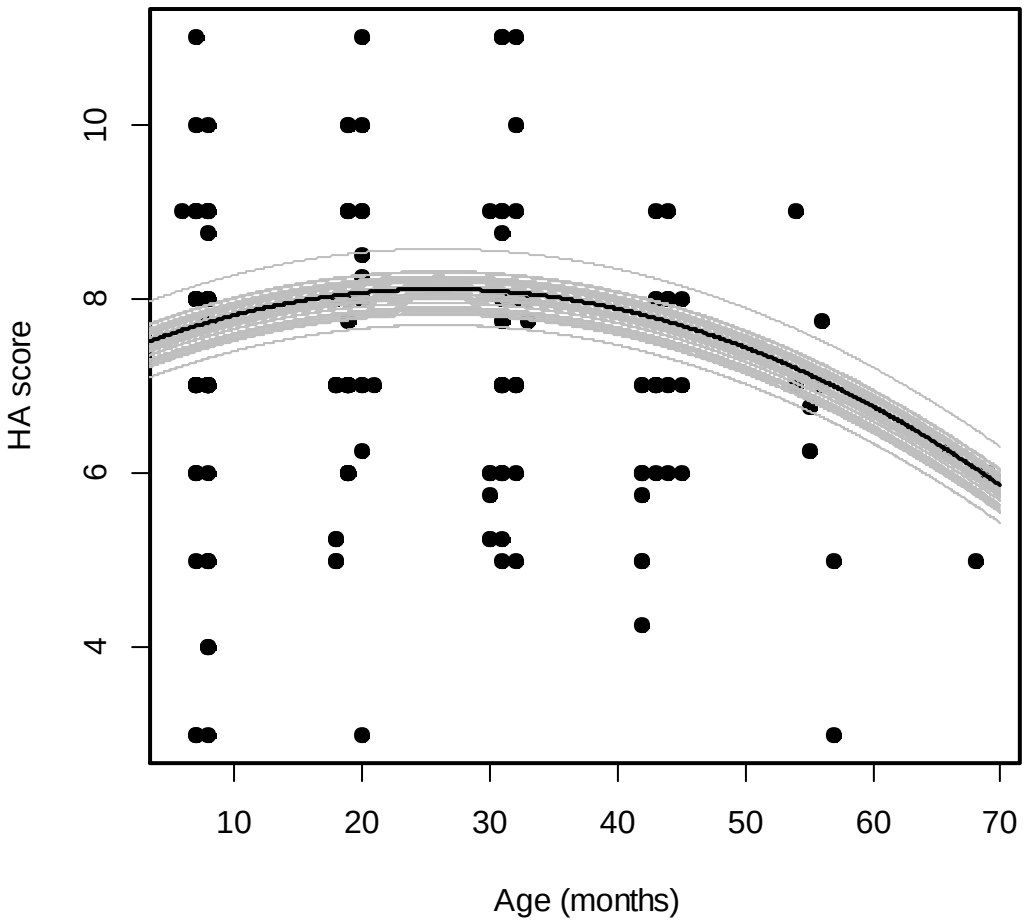
582 Table 1: Significance tests of the fixed effects in the mixed models testing the associations between
583 the immune parameters and age. Significance of other covariates (condition, year sampled, sex and
584 the sex*age interaction) are also provided. Significant F-tests are indicated as *: p<0.05; **: p<0.01;
585 ***: p<0.001 and highlighted in bold. Estimates of the variance components of the random intercept
586 (individual) and slope (individual:age interaction) and the residual variation are also provided.

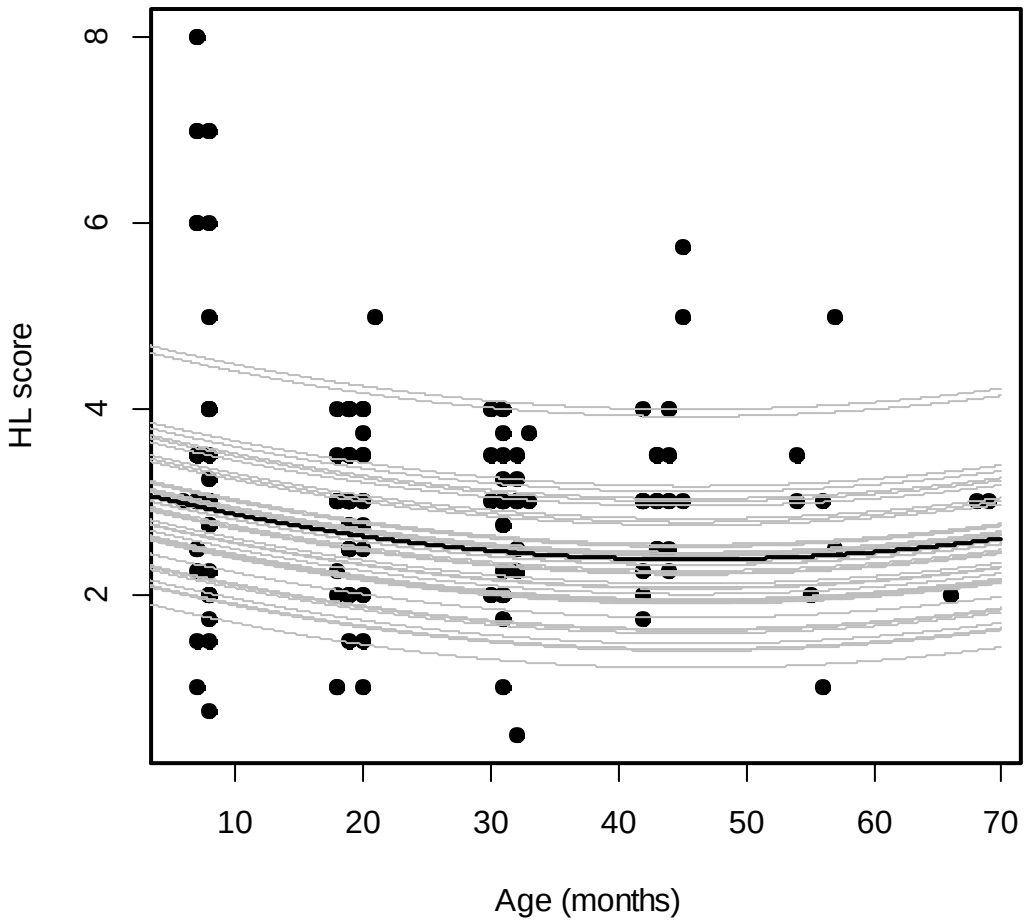
587

Effect	**Agglutination**	**Lysis**	**Haptoglobin**	**Nitric oxide**
Age	**$F_{1,198}$=5.59***	**$F_{1,109}$=8.91****	**$F_{1,86}$=5.41***	$F_{1,186}$=0.52
Age^2	**$F_{1,196}$=7.55****	**$F_{1,90}$=4.66***	$F_{1,113}$=0.15	$F_{1,185}$=2.01
Age^3	$F_{1,157}$=1.02	$F_{1,56}$=2.95	$F_{1,173}$=0.15	$F_{1,184}$=0.06
Age^4	$F_{1,155}$=0.04	$F_{1,34}$=0.28	$F_{1,157}$=0.14	$F_{1,183}$=0.01
Condition	**$F_{1,159}$=6.33***	$F_{1,140}$=1.51	$F_{1,169}$=3.46	$F_{1,175}$=0.61
Year sampled	$F_{3,179}$=5.59	$F_{3,79}$=1.90	$F_{3,147}$=2.19	$F_{3,175}$=0.16
Sex	$F_{1,109}$=0.24	**$F_{1,110}$=26.3*****	$F_{1,167}$=0.84	$F_{1,175}$=2.70
Sex:age	$F_{1,176}$=0.08	$F_{1,109}$=0.35	$F_{1,95}$=0.70	$F_{1,175}$=0.06
$\sigma^2_{intercept}$	0.21	0.91	0.00	0.00
σ^2_{slope}	0.00	0.00	4.87e^{-07}	0.00
$\sigma^2_{residual}$	1.96	0.44	9.55e^{-04}	1.86e^{-04}



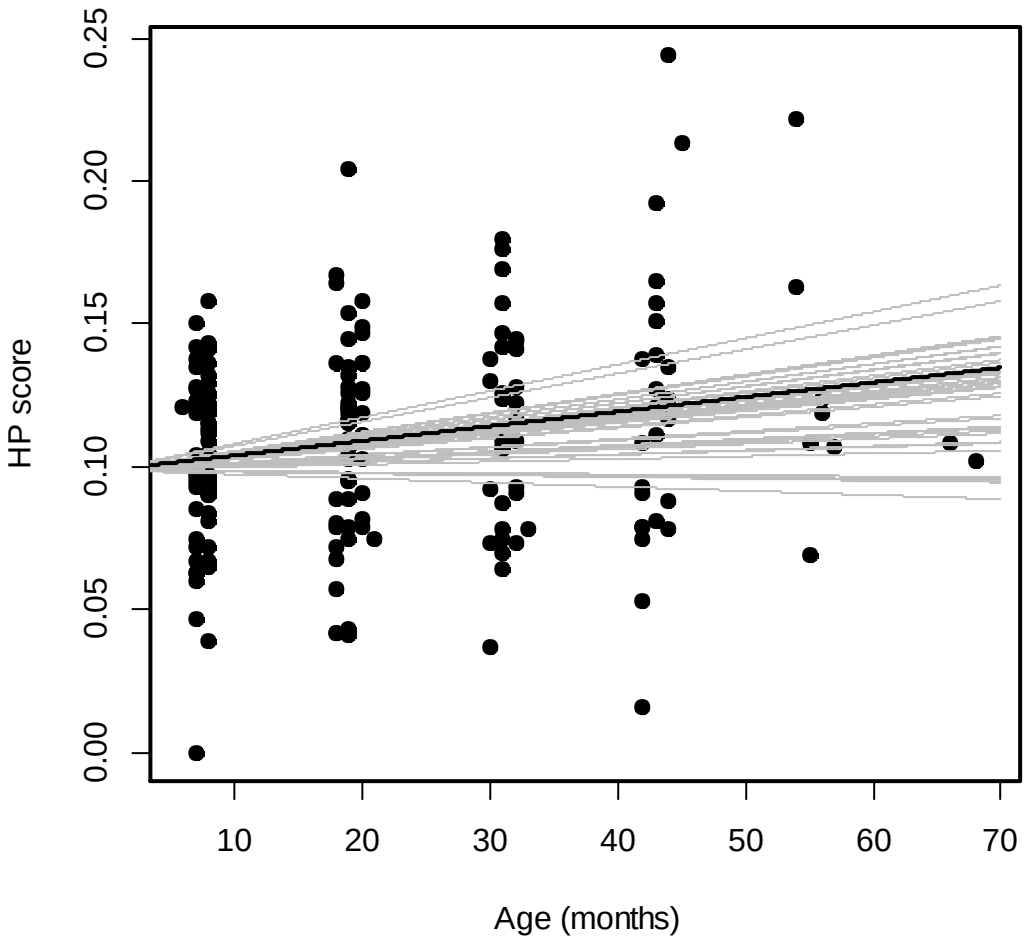
588

589 **Figure 1:** Relationship between agglutination (HA) scores and age (months). The black line represents the cross-
590 sectional pattern, whereas grey lines represent the individual profiles (longitudinal approach). Results show the
591 expected bell-shaped pattern with birds of approximately 25-30 month of age having higher HA scores
592 compared to younger and older birds. Furthermore, the individual profiles do not show a large amount of
593 variation around the cross-sectional line, indicating that the variation in intercepts (Table 1) is relatively small
594 compared to the residual variation.



595

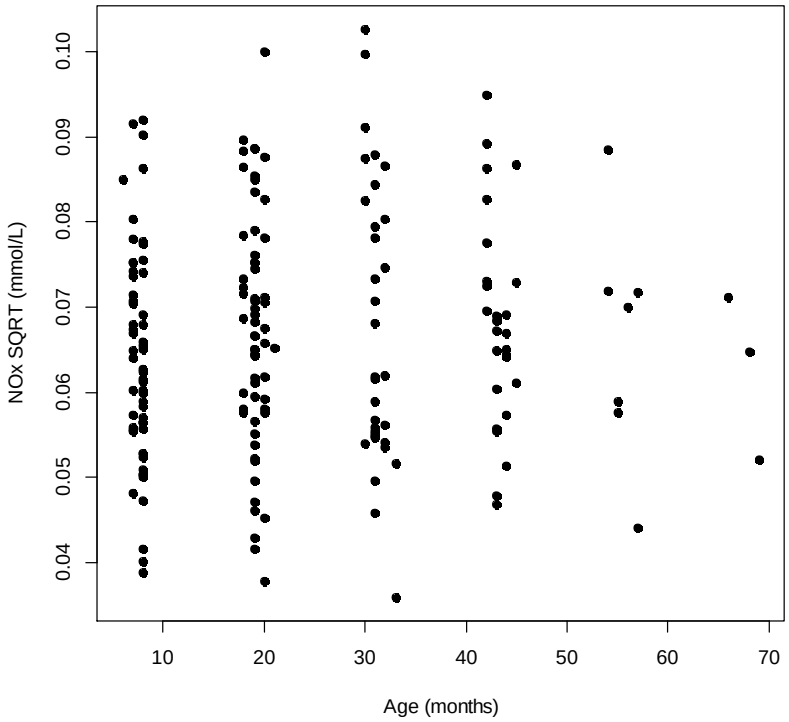
596 **Figure 2:** Relationship between lysis (HL) scores and age (months). The black line represents the cross-sectional
597 pattern observed, whereas grey lines represent the individual profiles (longitudinal approach). Here results
598 show that young birds have the highest levels of complement proteins, with levels slightly dropping to lower
599 levels in older birds. Further, individual profiles are positioned further apart, implying more intrinsic variation
600 between individuals relative to the residual variation (Table 1).



601

602 **Figure 3:** Relationship between haptoglobin concentrations (Hp (mg/mL)) and age (months). The black line
603 represents the overall (=cross-sectional) pattern observed, whereas grey lines represent the individual profiles
604 (longitudinal approach). Results show an increase in haptoglobin concentrations with age. Individual profiles
605 start at very similar levels at young ages as a result of the absence of variation in intercepts (Table 1). Due to
606 the variation in slopes, changes in Hp with age occur at different magnitudes for different individuals, such that
607 individual Hp values vary considerably at older ages.

608



609

610 **Figure 4:** Relationship between square root transformed nitric oxide concentrations (NOx (mmol/L)) and age
611 (months). No effect of age on nitric oxide concentrations was found (cross-sectional pattern), and profiles did
612 not vary across individuals (longitudinal pattern).

613

614