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No Evidence for Constitutive Innate Immune Senescence in a Longitudinal Study of a Wild Bird

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ABSTRACT

Aging is associated with declines in physiological performance; declining immune defenses particularly could have consequences for age-related fitness and survival. In aging vertebrates, adaptive (memory-based) immune responses typically become impaired, innate (nonspecific) responses undergo lesser declines, and inflammation increases. Longitudinal studies of immune functions in wild animals are rare, yet they are needed to understand immunosenescence under evolutionarily relevant conditions. Using longitudinal data from a tropical passerine (*Malurus coronatus*) population, we investigate how population trends emerge from within-individual changes and between-individual heterogeneity (e.g., selective disappearance) in immune status. We quantified constitutive immune indexes (haptoglobin [inflammation associated], natural antibodies, complement [lytic] activity, and heterophil-lymphocyte ratio; $n = 505–631$) in individuals sampled one to seven times over 5 yr. Unexpectedly, longitudinal analyses showed no age-related change within individuals in any immune index, despite sufficient power to detect within-individual change. Between individuals, we found age-related declines in natural antibodies and increases in heterophil-lymphocyte ratios. However, selective disappearance could not adequately explain between-individual age effects, and longitudinal models could not explain our data better than cross-sectional analyses. The lack of clear within-individual immunosenescence is itself notable. Persistent levels of haptoglobin, complement activity, and natural antibodies into old age suggests that these immune components are maintained, potentially with adaptive significance.

Keywords: aging, ecoimmunology, gerontology, immunosenescence, inflammaging.

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Introduction

Like humans, animals exhibit senescence—an overall decline in individual performance and fitness with advancing age across a diverse range of life history strategies (Jones et al. 2008, 2014; Baudisch 2011). In the wild, there is strong evidence that senescence commonly manifests as actuarial senescence (a decline in survival probability with age) or reproductive senescence (a decline in reproductive output with age; Brunet-Rossinni and Austad 2005; Nussey et al. 2013; Gaillard et al. 2017; Lemaître and Gaillard 2017). Age-related declines in physiological traits and processes underpin decreasing survival and fecundity (Ricklefs 2008). However, such traits are relatively understudied in the wild in the context of aging. Senescent decline in immune function—immunosenescence (Pawelec 2018; Peters et al. 2019)—is one such trait likely to be particularly consequential for survival and reproduction in wild organisms (Sadd and Schmid-Hempel 2008; Cheynel et al. 2017; Froy et al. 2019), notably when infection might exacerbate the threat of predation or reduce competitiveness for critical resources (Møller and Erritzøe 2000; Verhulst et al. 2014).

In humans, the aging immune system renders the elderly more susceptible to contracting and succumbing to infectious disease, especially novel infections (Pawelec et al. 2010; Pera et al. 2015). A few consistent patterns are emerging from wild vertebrate studies suggesting that there may be congruent age-related changes in immunity in humans and wild vertebrates (Froy et al. 2019; Peters et al. 2019). In vertebrates, innate immune components that rapidly deal with novel infections and adaptive immune components that deal with repeated infections through specific (acquired, memory-based) immune responses form two major branches of the immune system (Litman et al. 2010; Riera Romo et al. 2016). Although some senescence of innate immunity has been observed in vertebrates (Müller et al. 2013), there is much stronger and consistent evidence for senescence of adaptive immunity, primarily through thymic involution and depletion of naive T cells (Shanley et al. 2009; Müller et al. 2013)—the cells responsible for generating new memory-based immune repertoire (Dowling and Hodgkin 2009). The

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balance between innate and adaptive immunity could therefore change with advancing age (McDade et al. 2016) and result in a "remodeling" of the immune system (Müller et al. 2013; Fulop et al. 2018). Despite detailed knowledge of human aging, humans are unusually and exceptionally long-lived after reproduction (Ellis et al. 2018). Consequently, a better understanding of senescence and, more broadly, age-related changes of immune components in different species is needed to refine our evolutionary perspectives on how physiological aging processes may drive diverse life histories and actuarial and reproductive senescence.

Although innate immune components initially appeared less prone to age-related decline, age-dependent dysregulation of innate immunity is now considered an important aspect of immunosenescence (Shaw et al. 2013). Closely linked to this immune dysregulation is the increase in chronic low-grade inflammatory activity, or "inflammaging," that characterizes elderly humans (Bruunsgaard et al. 2001; Franceschi et al. 2006) and possibly other older animals (Peters et al. 2019). At the physiological level, various markers of immune function and inflammation may undergo different age-related changes that contribute to immunosenescence within individuals, which ultimately reduces fitness with advancing age (Pawelec 2018). Crucially, these age-related changes within individuals are distinct from changes that are observed between individuals of different age classes in a population, which can arise from individual heterogeneity in trait values associated with disappearance from the population (e.g., mortality risk; Vaupel et al. 1979). Longitudinal studies are therefore necessary to distinguish independent age-related trends both within and between individuals that comprise cross-sectional population demographic patterns (van de Pol and Verhulst 2006; Nussey et al. 2008; van de Pol and Wright 2009). However, the difficulties of repeatedly capturing and measuring wild individuals have meant relatively few studies have longitudinally assessed age-related change in immune function (Graham et al. 2010; Schneeberger et al. 2014; Beirne et al. 2016; Vermeulen et al. 2017; Froy et al. 2019; reviewed by Peters et al. 2019). These studies have helped demonstrate that similar to humans, wild vertebrates might undergo immunosenescence, which interacts with extrinsic sources of mortality to affect survival. Still, the multifaceted nature of immunosenescence means that more longitudinal studies examining various immune components are required to affirm findings of previous cross-sectional studies (Peters et al. 2019) and decipher the evolutionary role of immunosenescence in a natural ecological context (Nussey et al. 2008; Maizels and Nussey 2013; Kowald and Kirkwood 2015).

To examine longitudinal patterns of immune function, we repeatedly captured individuals from a long-term individual-based study of a wild tropical passerine, the purple-crowned fairy-wren (*Malurus coronatus*). We addressed two main questions: (1) what is the evidence for age-related changes in a variety of immune markers and (2) do age-related trends emerge from longitudinal within-individual change and/or between-individual processes (such as selective disappearance from individual heterogeneity)? To do this, we quantified commonly measured (Peters et al. 2019) indexes of constitutive immune function that are maintained regardless of infection status (Schmid-Hempel and Ebert 2003) from >500 captures of >280 individuals over 5 yr: natural antibodies (NAbs) and lytic complement activity (CA; both innate), haptoglobin-like protein (Hp; an inflammation-associated marker), and heterophil-lymphocyte (HL) ratio (ratio of the primary cell types of innate [heterophil] and adaptive [lymphocyte] cell-mediated immunity). Using longitudinal analyses to distinguish within-individual senescence from population trends, we tested whether the innate immune indexes exhibited a decline with age, as predicted if they showed age-dependent dysregulation (Shaw et al. 2013). As an inflammation-associated marker, baseline Hp can thus be predicted to increase with age if there is inflammaging (Peters et al. 2019). Last, because senescence in adaptive immunity is expected to be more pronounced than for innate immune functions (Shanley et al. 2009; Peters et al. 2019), we predicted that HL ratio would increase with age.

Methods

Study Population

Purple-crowned fairy-wrens are riparian habitat specialists in tropical savannahs of northern Australia. This cooperatively breeding species defends year-round stable territories (Kingma et al. 2011), with social groups composed of a dominant breeding pair, usually with subordinate adults, and any offspring (Hall and Peters 2008; Kingma et al. 2010). Our study population (~250 individuals at any time) inhabits 15 km of Annie Creek and Adcock River at the Australian Wildlife Conservancy's (AWC) Mornington Wildlife Sanctuary (126.1°E, −17.5°N). Since 2005, all individuals have been uniquely color banded to monitor social group composition, territory boundaries, individual movements, dispersal within the population, and survival. Birds are declared dead after resighting all other group members ≥3 times. During this study, the estimated census detection rate of individuals per sampling season in the core population was 98%; 28 birds were initially presumed dead but subsequently rediscovered (equivalent to ~2 birds per total population size per biannual census). Survival estimate reliability is strengthened further as this species disperses only along waterways, and ~95 km of waterways with suitable habitat in the wider catchment (up to 60 km away) are surveyed annually using a 90% successful detection survey technique, discovering dispersed emigrants from the core population (Hidalgo Aranzamendi et al. 2016).

Aging, Capture, and Sampling

All individuals that comprised the final data set either were (*a*) banded as nestlings ($n = 62$) or were (*b*) aged as fledglings ($n = 119$) or (*c*) aged as subadults ($n = 113$) by estimating a hatch date based on behavioral, morphological, and plumage traits displayed at first capture, with known acquisition ages (for age descriptors and uncertainty calculation, see sec. 3 of the supplemental PDF, available online). Estimated hatch dates had small uncertainty for birds first captured as fledglings (mean uncertainty = 2 wk) but were slightly larger for birds captured as subadults (mean uncertainty = 1.9 mo). These hatch dates were used to calculate the ages of individuals at capture, ranging from 3 mo to nearly 11.6 yr in our data set (mean = 1.9 yr, median = 1.0 yr;

for final sample age structures, see fig. S1; figs. S1–S5 are available online). Additionally, for fully mature immigrants displaying no immature plumage traits ($n = 64$), we (*d*) assigned an age that was equivalent to the age at which emigrants were last seen in the core population before dispersal into the wider catchment (median = 8.3 mo; table S1; tables S1–S10 are available online). The oldest bird ever recorded in the population was 14.4 yr old, and the mean life span for individuals that reach independence is 2.35 yr (based on 644 individuals over 16 yr of study). Although longer-lived animals typically exhibit stronger senescence (Turbill and Ruf 2010), we still expect purple-crowned fairy-wrens to exhibit senescence during their life span because shorter-lived congeners, superb fairy-wrens (*Malurus cyaneus*), show clear survival senescence (Cooper et al. 2021)—potentially underpinned by immune function.

Birds reach independence at ~90 d, so samples from birds younger than 91 d at capture ($n = 201$) were excluded from analyses because early age-related changes in immune indexes were expected to relate to maturation (Killpack et al. 2013) rather than to senescence. We excluded several samples from birds banded as adults at the inception of the long-term study because their ages could not be estimated ($n = 10$–12, dependent on the immune index). Excluded samples are not included in the sample total for the final data set.

From April 2012 to June 2017, a total of 773 samples from 358 individuals contributed to the final data set. Because not all immune indexes could be quantified from every sample, sample sizes for each index differ and are subsets from this total (see further final sample details in "Immune Indexes" and "Statistical Analysis"). Captures were made during two sampling periods each year from mid-April to mid-June and from mid-October to late November, before and after the main breeding peak (Hidalgo Aranzamendi et al. 2019; Hp: $n = 11$ field sampling periods, 2012–2017; NAbs and CA: $n = 7$, 2012–2013 and 2016–2017; HL ratio: $n = 9$, 2012–2014 and 2016–2017). Birds were captured in mist nets (≤15 min between net checks), extracted, and kept in holding bags until blood sampling, which was started as quickly as possible (from extraction: median = 23 min, SD = 19.6 min, range = 1 to [exceptionally] 125 min) to minimize handling stress known to influence some immunological indexes (Davis 2005; Zylberberg 2015). Following brachial venipuncture, up to 100 μL of blood was collected in heparinized capillary tubes that were sealed, stored on ice, and centrifuged at 16,060 *g* for 5 min later that day. Plasma was frozen at −20°C and transferred to −80°C within 8 wk. Samples remained frozen for several months after capture (median = 4, SD = 4.9) before thawing to assay NAbs and CA. Plasma was refrozen and thawed before assaying Hp, which these indexes are robust to (Hegemann et al. 2017), and plasma storage time did not influence our results. At capture, a blood smear was created using the wedge-pull method (Campbell 2015*a*), air-dried, and fixed in methanol the same day for at least 15 min.

Immune Indexes

We quantified four innate immune indexes that form integral components of immune surveillance and initial defense against infection. First, NAbs identify nonspecific foreign antigens and opsonize them for phagocytosis (Matson et al. 2005; Holodick et al. 2017), as well as linking the innate and adaptive immune systems (Panda and Ding 2015). In addition, NAbs can initiate the complement system via the classical pathway (Panda and Ding 2015). Second, CA then lyses and breaks down foreign bodies toward elimination of infection through the protein cascade of the complement system (Trouw and Daha 2011). Third, Hp-like heme-binding scavengers mitigate damage incurred from reactive oxidative heme groups released by cells that are damaged by infection or inflammation (Quaye 2008). These scavengers are major positive acute-phase proteins that are tightly linked to inflammatory responses, with baseline levels to some extent predictive of immune responsiveness (Matson et al. 2012). Last, the HL ratio is composed of heterophils that exhibit bactericidal and phagocytotic ability important for cellular innate immunity and lymphocytes that secrete antibodies crucial to the adaptive immune response (Minias 2019). Additionally, the HL ratio is an indicator of chronic stress (Davis and Maney 2018), but it can also be predictive of immune responsiveness (Krams et al. 2012). The specific assay methods are described fully by Roast et al. (2019).

NAbs and CA were both quantified using the hemolysis-hemagglutination assay (Matson et al. 2005) with minor modifications as in Roast et al. (2019). Sample size for these two indexes differs because some samples were limited by plasma volume and lysis titers and could not be measured ($n = 12$). Serial dilutions were run across two adjacent 96-well plates to accommodate high agglutination values for $n = 120$ assay runs. On each plate, interplate chicken plasma standards were scored for both agglutination (mean = 10.1, $n = 247$ standards) and lysis (mean = 3.55, $n = 265$ standards) titers, resulting in coefficient of variation (CV) = 0.13 and CV = 0.11, respectively. While titers for CA are comparable to those observed in other species (mean = 2.3, SD = 1.8), titers for NAbs are unusually high in purple-crowned fairy-wrens (mean = 14.8, SD = 1.9). While the reason for this is unknown, interestingly, this species has persistently low incidence of avian malaria despite high community prevalence (Eastwood et al. 2019), and NAbs have been putatively linked to malarial resistance (Atkinson and Paxton 2013).

Baseline Hp was assayed using a commercial kit (Phase Range TP801, Tridelta Development) and microplate reader, with a modified protocol from Matson et al. (2012; see details in Roast et al. 2019). Prescans at 630 nm before addition of kit reagents were used to correct for plasma turbidity. All samples were run in duplicate; standards run in triplicate were used to assess interplate variation (CV = 0.24, $n = 25$ plates). Of all samples initially assayed, the majority (89%) formed a normal distribution (mean = 0.63 mg/mL, SD = 0.27; fig. S4) falling below the 1.25 mg/mL optical saturation threshold of the assay. For 82 samples, Hp levels fell above this threshold, presumably representing individuals undergoing an acute-phase response (a multiplefold induced increase, not comparable to baseline concentrations; Quaye 2008; Matson et al. 2012). These instances of optical saturation were unrelated to any variable included in this study or subsequent survival and were excluded from Hp analyses (fig. S5). Plasma redness was quantified by measuring absorbance at 450 nm before the

addition of kit reagents and within-subject centered for inclusion as a covariate in statistical analyses (450_{dev}; Matson et al. 2012).

Blood smears stained with May-Grünwald then Giemsa stains were examined at ×1,000 magnification, and heterophils, lymphocytes, basophils, eosinophils, and monocytes were counted for the first 100 leukocytes observed. The majority of all cells identified (87%) were heterophils or lymphocytes, with other cell types observed infrequently among individuals or in low numbers. As proportions, heterophils and lymphocytes were highly negatively correlated ($r = -0.78$, $P < 0.001$) and therefore analyzed as a single composite metric, the HL ratio (mean = 0.22, SD = 0.19). Scoring was undertaken by four individual scorers, following Campbell (2015*b*). Scorer ID was included in all statistical models to account for variation between individuals; all further details are in Roast et al. (2019).

Statistical Analysis

Statistical analyses were performed using R software version 3.4.0 (R Development Core Team 2017). All equations referenced are provided in the supplemental PDF. Hp and NAbs immune indexes were normally distributed, while CA titer scores were natural log transformed, and HL ratios were square root transformed (all observed raw values were >0 and <1) to normalize distributions. Outlying observations beyond ±2.5 SDs of the mean were excluded (Hp: $n = 2$; NAbs and HL ratio: $n = 11$; CA: $n = 0$). Final sample sizes were as follows: $n = 631$ from 329 individuals for Hp, $n = 506$ from 294 individuals for NAbs, $n = 505$ from 293 individuals for CA, and $n = 521$ from 289 individuals for HL ratio. These sample sizes include both single and multiple repeated measures of individuals. Repeated measures formed 75% of the final sample with up to seven measures per individual for Hp and 70% with up to five measures for all other indexes, while single measures constitute the remaining 25%–30% of the sample (for repeated-measures sample structure, see fig. S2).

We initially constructed four generalized additive mixed models (GAMMs) using the gamm4 package (Wood and Scheipl 2017) to investigate potential nonlinear changes in immune indexes with advancing age, without prior assumption of the shape of any nonlinear relationship. Each GAMM contained an immune index response variable and age at capture fitted as a smooth function. Additional fixed effects and random intercepts were applied to each model that controlled for relevant sources of methodological quantification error, biological variation not of primary interest, and nonindependent sampling structure, providing a null model (for specific model structures, see table S2). The fixed effects included sex, time bled (the time of day relative to sunrise; range = 35–730 min, median = 117 min), time wait (the holding time between net extraction and sampling), 450_{dev} to control for plasma redness in colorimetric assays (Hp), and plate standard to control for assay interplate variation (NAbs, CA). The random intercepts included plate ID (Hp), scorer ID (HL ratio), individual ID, and field season (a multilevel factor of each field sampling period). These GAMMs indicated no support for nonlinearity, with the estimated degrees of freedom (edf) equal to 1 for age smooth functions in each immune index GAMM (edf values close to 1 show linear terms; table S3). Consequently, only linear mixed models (LMMs) were used further for direct comparison with a longitudinal analysis method requiring linear models.

To disentangle within-individual change and between-individual effects, we used a longitudinal within-subject centering method (eq. [2]; van de Pol and Wright 2009). Advantageously, this method accommodates the single measures and individuals still extant in our data set. Between-individual (β_B) and within-individual (β_W) effects were estimated by replacing age at capture in each model with mean (μ) age of each individual across all captures and Δ age at capture (age at capture minus mean age); the β_B effect shows how immune function changes between individuals in the population as a function of individual mean age, while the β_W effect shows the overall age-related change that occurs within individuals captured repeatedly at different ages (e.g., senescence). Single measures were included with $\Delta = 0$ and $\mu =$ the age at capture. These two age parameters were added to LMMs with relevant null model structures (table S2).

Some within-subject centered models showed support for between-individual effects but not within-individual change, which could indicate selective disappearance. In our study system, we know that many individuals survived long after their final repeated measurement but were not recaptured (88%–90% of individuals for each immune index). To provide a more direct estimate of selective disappearance (β_S), a complementary model was fitted with age and "estimated life span" (ω_i) included as a covariate in respective null models for each response variable (ω_i; eq. [1]; van de Pol and Verhulst 2006). At the time of analysis, ~75% of sampled individuals had died with known life spans, and the extant ~25% required estimation. Expected life spans for the remaining ~25% were estimated according to age-dependent mean life expectancy based on data from all previously fledged individuals in the study population of known age ($n = 876$). Including these individuals with estimated life spans will introduce some error, but it avoids bias resulting from removing the remaining potentially longer-lived individuals while assessing selective disappearance. These models provided no additional evidence of clear selective disappearance (β_S) and are therefore not reported further (for models including this estimated life span parameter, see table S4). To estimate selective appearance (possible from nonrandom sampling), minimum sampling age can also be included in models (α_i; van de Pol and Verhulst 2006); however, because each individual's first observation has exactly the same value for age and minimum sampling age, these variables are highly correlated (Spearman's $\rho =$ 0.70–0.75 for each index), precluding the estimation of selective appearance.

For longitudinal within-subject models where 95% confidence intervals for any standardized age effect did not include zero, the difference between the β_B and β_W parameter slopes was formally tested (eq. [3]; van de Pol and Wright 2009). The β_B and β_W parameter slopes did not differ significantly from one another for these immune indexes (table S5), suggesting a single age effect might be more parsimonious. To assess whether a cross-sectional population-level model (eq. [1]; van de Pol and Wright 2009) better described age-related changes in immune

indexes, age at capture as an explanatory variable was added to null models for each immune index. Longitudinal within-subject centered models were compared with cross-sectional models and null models using Akaike information criterion (AIC), and model weights were calculated for each immune index respectively (table S6). We present these models and comparison in our results.

In exploratory analyses with cross-sectional models, we also included cohort as a random factor, which explained minimal (<0.2%) variance and was therefore removed from analyses. Social status (dominant vs. subordinate) was significant in CA models as a main effect only ($t = -2.2$, $P = 0.027$), but differences in status did not change age effect estimates in any model (i.e., there was no interaction with age), and given the correlation with age (as subordinates only progress to become older dominants), status was excluded from further models (for status models, see table S7). Importantly, being an immigrant (16%–17% individuals, $n = 80$–101) did not affect any immune index, so immigrant samples were retained (for models assessing immigrant inclusion, see table S8).

Results

Longitudinal Within-Subject Centering Analyses

In longitudinal models, where age effects were split into within- and between-individual components, no clear senescence or age-related change within individuals was detected (table 1; coefficients for all models and terms are reported in table S9). Within-individual repeatability is low for all indexes (Hp: $R_{adj} = 0.18$; NAbs: 0; CA: 0.09; HL ratio: 0.16). Only NAbs and HL ratio exhibited any effect of age in respective longitudinal models (table 1; figs. 1, 2), where clear between-individual age (β_B) effects were found rather than within-individual change (β_W). NAbs decreased linearly with age between individuals, showing that individuals with a higher average age of sampling had lower level of NAbs (table 1; figs. 1, 2*c*). Only a weak and nonsignificant decline was observed within individuals (table 1; figs. 1, 2*b*). HL ratio increased linearly with age between individuals, showing that individuals with a higher average age of sampling had higher HL ratios (table 1; figs. 1, 2*f*). Within individuals there was no significant age-related change (table 1; figs. 1, 2*e*).

For Hp and CA, no clear age-related changes were found in longitudinal models. Neither index showed β_W or β_B effect sizes that were distinct from zero (table 1). However, although nonsignificant, the slightly positive within-individual age effects on CA had highly asymmetric 95% confidence intervals almost entirely in positive space (table 1; fig. 1), more suggestive of a possible maintenance or even slight enhancement with age but not immunosenescence.

The between-individual effects in both NAbs and HL ratio could indicate selective disappearance of individuals with high and low values of these indexes, respectively, and therefore some heterogeneity in mortality risk. To further estimate selective disappearance (β_S), an additional model for each immune index was fitted with age and estimated life span. For all immune indexes the estimated life span parameter was not significant in these models (table S4). Thus, we cannot conclude that the observed between-individual age-related changes in NAbs (decline) and HL ratio (increase) are due to selective disappearance of individuals. Additionally, we formally tested whether the slopes of within- and between-individual effects differed significantly from one another in each longitudinal model for NAbs and HL ratio. Despite the evidence for stronger between-individual effect sizes than for within-individual effects in each immune index, the two slopes were not significantly different from one another for either index (table S5).

Cross-Sectional Analyses

Cross-sectionally, only NAbs and HL ratio show any age-related change, with clear age effects bound by 95% confidence intervals not containing zero, while CA and Hp showed no evidence for

Table 1: Standardized age effects from longitudinal and cross-sectional age models

	Longitudinal				Cross-sectional		
Response variable	ΔAIC	μ/Δ	β (std)	CI (std)	ΔAIC	β (std)	CI (std)
Hp	3.7	μ	.010	−.058 to .078	2.0	.000	−.068 to .067
		Δ	−.017	−.082 to .048			
NAbs	−11.1	μ	−.134	−.203 to −.065[a]	−12.4	−.141	−.213 to −.069[a]
		Δ	−.040	−.122 to .042			
CA	1.7	μ	.034	−.056 to .124	.3	.061	−.032 to .153
		Δ	.067	−.031 to .164			
HL ratio	−3.7	μ	.110	.023 to .197[a]	−5.7	.128	.038 to .217[a]
		Δ	.065	−.027 to .155			

Note. For within-subject-centered longitudinal models both within-individual (Δ) and between-individual (μ) age effects are shown. Standardized (std) β estimates were calculated per 2 SDs using the arm package standardize function (Gelman and Su 2016). The difference in Akaike information criterion (ΔAIC) is shown between each model and the null model (i.e., models with ΔAIC values <0 are a better fit than the null model). CI = confidence interval; Hp = haptoglobin-like protein; NAbs = natural antibodies; CA = complement analysis; HL = heterophil-lymphocyte.

[a]Does not contain zero (calculated using the lmerTest package confint function; Kuznetsova et al. 2017), which is interpreted as showing a clear effect.

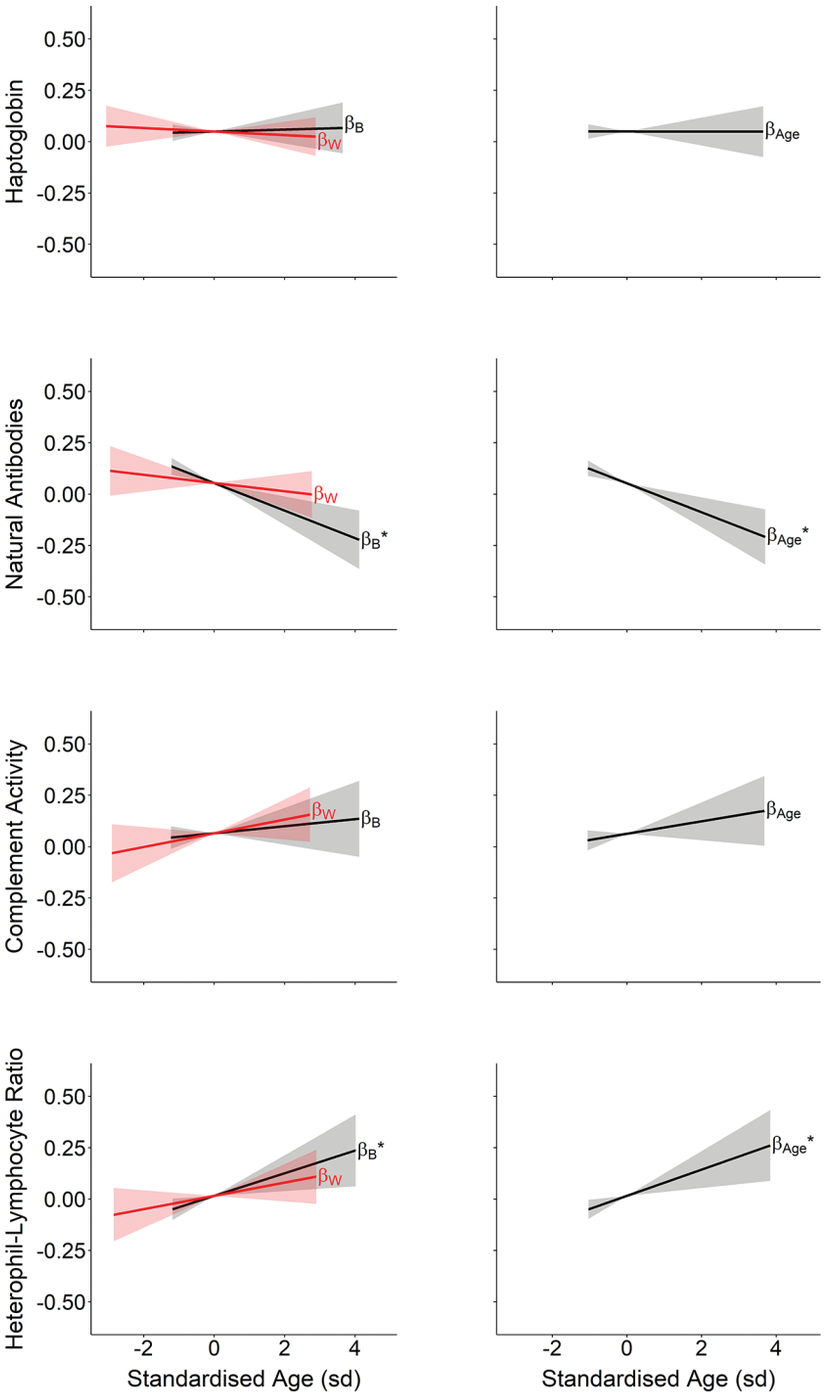

Figure 1. Overview of standardized age effects from longitudinal and cross-sectional models. Predicted values of haptoglobin, natural antibodies, complement activity, and heterophil-lymphocyte ratio showing the separate between-individual (β_B) and within-individual (β_W) age effects from within-subject-centered longitudinal models (*left*) and the overall age effect (β_{Age}) from cross-sectional models (*right*). Fitted values were calculated from β estimates of respective standardized models (table 1), while all other fixed effects were set to mean values. Ribbons show 95% confidence intervals of age effect β estimates only. Effects labeled with asterisks show confidence intervals not containing zero.

age-related changes (fig. 1). Older individuals had decreased levels of circulating NAbs (table 1), while the HL ratio increased with advancing age (table 1). These effects are congruent with, and apparently driven by, between-individual effects detected in longitudinal models. Comparison of AIC values between cross-sectional and longitudinal models show that longitudinal models explain these data no better than the simpler and more parsimonious cross-sectional models (tables 1, S6).

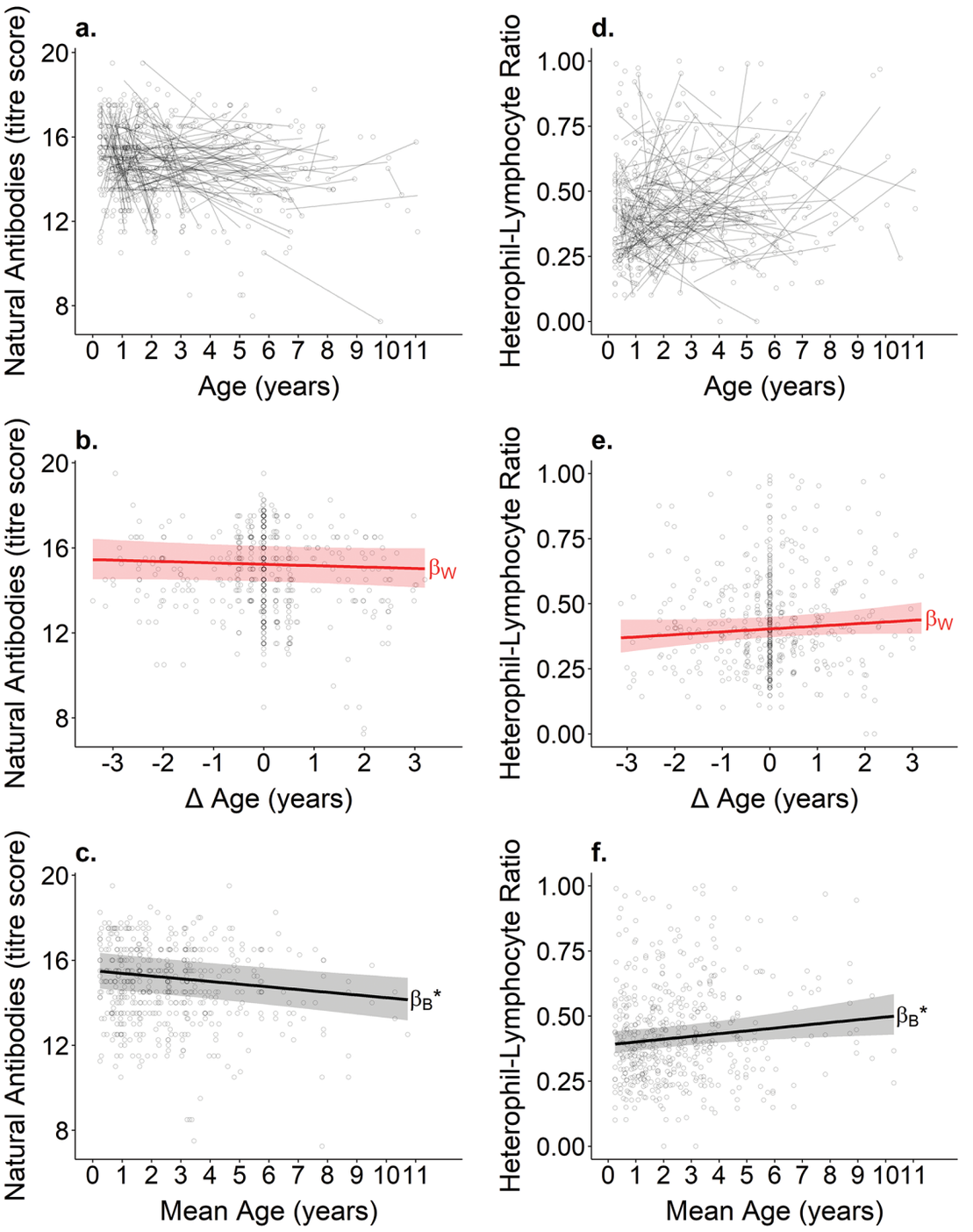

Figure 2. Effects of age on natural antibodies (*a–c*) and heterophil-lymphocyte ratio (square root transformed; *d–f*): there is stronger evidence for between-individual effects (β_B) than age-related changes within individuals (β_W). Plots *a* and *d* show raw data and reaction norms for each individual with multiple repeated measures (gray lines), obtained from a simplified linear regression of immune index ~ age, while individuals with single measures have no lines. The length of these lines along the *x*-axis indicates the time window between first and last repeated measures. Plots *b* and *e* show age-related changes within individuals (β_W), while plots *c* and *f* show between-individuals effects (β_B), with all fitted lines derived from nonstandardized longitudinal models (table S9). Confidence ribbons incorporate uncertainty of all modeled fixed effects. β estimates with 95% confidence intervals that do not contain zero are indicated with asterisks.

Discussion

This study aimed to assess age-related changes in several immune indexes in a wild tropical passerine, the purple-crowned fairy-wren, and to distinguish within- and between-individual processes. Overall, we found no convincing evidence for within-individual immunosenescence in any immune index. Within-subject centering showed that differences in NAbs and HL ratio that were observed cross-sectionally across age classes could not be clearly attributed to within-individual age-related changes and instead appear driven primarily by differences between individuals in the population. These between-individual effects could not be attributed to selective disappearance (mortality) of individuals. Finally, we found no age-related trends for Hp and CA within or between individuals.

No Decline in Immune Function: Complement Activity and Haptoglobin

Although we predicted senescence in CA, this function was clearly maintained into older age, with a small positive effect size both within and between individuals (though not statistically distinct from zero; table 1; fig. 1). With advancing age, there is the theoretical expectation that the decline in self-maintenance accelerates as the age-adjusted risk of mortality increases (Kirkwood and Rose 1991). Consequently, at a cellular level, organisms can accumulate senescent or apoptotic cells (Vicencio et al. 2008). One less well-known—but important—function of the complement system is to remove apoptotic cells from the body (Ricklin et al. 2010). In humans, the age-related increase in apoptotic cells stimulates an otherwise chronically attenuated CA response (fig. 1*c* in Ricklin et al. 2010). This can explain levels of CA being maintained (as appears overall common; Peters et al. 2019) or possibly increasing with age rather than declining, as maintaining these humoral components might mitigate or delay the consequences of not adequately clearing dead cells (Nagata 2010).

The cumulative effects of damage and cellular debris acquired with age are also thought to result in a chronic systemic increase in the inflammatory system (i.e., inflammaging), a process closely linked to immunosenescence and age-related illness (Bruunsgaard et al. 2001; Franceschi et al. 2006, 2017; Pawelec 2018). We thus predicted that Hp would undergo an age-related increase as a part of inflammaging (Nussey et al. 2012; Cheynel et al. 2017; Vermeulen et al. 2017). Although Hp can increase multiplefold from baseline levels during an inflammatory response at any age, its baseline levels are primarily controlled by interleukin-6 (IL-6), an inflammatory cytokine found to increase with age in humans (Franceschi et al. 2006; Quaye 2008). Considering that Hp production is stimulated by inflammatory cytokines to dampen the effects of inflammation (Quaye 2008), our observed absence of age-related increase in Hp suggests either that IL-6 does not increase or does not stimulate Hp or possibly that there is no inflammaging overall in aging purple-crowned fairy-wrens. This apparent lack of inflammaging contrasts with the general inflammaging seen in other wild animals (Peters et al. 2019), though some evidence indicates that reduced inflammaging may be beneficial for longevity (Shanley et al. 2009) and potentially adaptive for a long-lived fairy-wren.

Natural Antibodies and Heterophil-Lymphocyte Ratio: Between-Individual Processes

NAbs showed a clear decline with age in the population overall, in line with our predictions and some published avian studies (Møller and Haussy 2007; Vermeulen et al. 2017; although a general decline is not found across species; Peters et al. 2019). From the within-subject centered model, however, the lack of support for within-individual senescence suggests that NAbs levels might not change with age, as is also reported in cross-sectional avian studies elsewhere (Palacios et al. 2007; Lecomte et al. 2010). As NAbs function for frontline immune defense and surveillance against novel infections but are presumably relatively low cost to produce (Klasing 2004), maintaining NAbs levels would remain beneficial for survival (Holodick et al. 2017). Alternatively, because certain classes of NAbs are self-reactive (autoantibodies), NAbs can become increasingly important with age as part of a debris clearance system (Grönwall et al. 2012; Nagele et al. 2013; Panda and Ding 2015). In the context of aging, natural autoantibodies produced in response to increasingly dysregulated cellular and molecular components (Dansereau et al. 2019) could result in the maintenance of NAbs into old age.

We also predicted that HL ratio might increase with age as a result of faster senescence of cellular adaptive immunity relative to cellular innate immunity, particularly through reduced lymphopoiesis in older individuals (Shanley et al. 2009). Congruent with our prediction, the population showed a clear increase in HL ratio with age, which could indicate immune remodeling and a greater dependence on innate immune defenses (Fulop et al. 2018). However, the longitudinal analysis showed no clear evidence of distinct within-individual age-related change that would more strongly support immune remodeling through differential rates of senescence among innate and adaptive components of cellular immunity.

The between-individual effects found for both NAbs and HL ratio could result from several possible processes. First, there could be selective disappearance of individuals through trait-dependent heterogeneity in mortality risk. By testing explicitly for selective disappearance by including estimated life span as a covariate (table S4), we found no support for heterogeneity in mortality risk. Second, disappearance from the sample due to trait-dependent dispersal could theoretically explain these between-individual effects. A particular strength of our study system, however, is known dispersal outcomes (Hidalgo Aranzamendi et al. 2016), which allow us to identify that directly after immune sampling only six individuals dispersed and were not sampled further. As these individuals had NAbs and HL ratio values throughout the sampled range, trait-dependent dispersal does not explain these between-individual effects. Conversely, trait-dependent appearance in the sample would appear as a statistically equivalent effect, yet we found no differences in immune values of immigrants arriving into the study population (table S1). Last, any heterogeneity in mortality typically assumes that disappearance from the sample signifies death, yet sampled birds were often not recaptured and lived beyond their final measurement. It therefore remains possible that selective recapture explains our results. For example, individual personality (boldness, explorative tendency) has been linked to

immunity (Guenther et al. 2018), including in a closely related fairy-wren (Jacques-Hamilton et al. 2017), such that more exploratory, bolder individuals might be (re)captured more easily (Michelangeli et al. 2016) and systematically bias the sampled immune traits. Such effects of selective (re)trapping could be widespread in natural studies of age-related change in any traits that require capture to measure them, such as physiological or morphological parameters (as opposed to many observable forms of reproductive investment and survival), and such effects might even extend into human studies (e.g., retention of study subjects).

Longitudinal Analyses: Limitations

Within-subject centered models can be open to alternative interpretations regarding the presence and role of immunosenescence if results are not clear-cut, limiting the conclusions that can be drawn. For example, when within- and between-individual trends align in the same direction, it becomes challenging to tease them apart. In such a case, between-individual effects can also be a consequence of within-individual effects (van de Pol and Wright 2009). Stronger between-individual than within-individual effects similar to those that we observed, however, can be confidently interpreted as distinct effects only when effect slopes are significantly different from one another (van de Pol and Wright 2009). It also remains possible that our data structure biased the estimation of between-individual effects (Westneat et al. 2020). We applied a power analysis and established that our large sample size, multiple repeated measures (fig. S2), and representative sampling across all older age classes (fig. S1) provide sufficient power to detect within-individual change and slightly more power to detect between-individual processes (for power analysis, see sec. 4 of the supplemental PDF; fig. S3). The average sampling window within each individual was ~2 yr (range = 0.5–4.5 yr), which might representatively sample our study population (mean life span = 2.35 yr) but might not fully capture within-individual change across the entire lifetimes of the longest-lived individuals. To our knowledge the sampling window relative to the species' life span has not explicitly been considered previously, and it may be an important consideration in the design of future longitudinal studies.

Conclusion

Our study utilized a long-term data set to provide an assessment of immunosenescence based on several immune markers, with one of the largest samples examined to date. There is a clear absence of within-individual immunosenescence in Hp, lytic CA, and NAbs and within-individual age-related change in HL ratio, supported further by our within-study power analysis. Immunosenescence could be selected against if innate immunity remains critically important throughout life. The overall persistence of innate immunity into old age might compensate for a reduced ability of the adaptive immune system to develop specific antibodies to novel antigens with fewer circulating naive T cells. However, innate immune components also have different self-maintenance roles (e.g., cellular debris and apoptotic cell clearance), which become more important later in life as other physiological systems become dysregulated. This alternative functionality has been well documented in humans (Quaye 2008; Ricklin et al. 2010; Holodick et al. 2017) but is rarely considered in ecological studies. Such dual function could show a functional shift with age, with a defensive role becoming less relevant in older age. It is therefore essential that alternative functions, and the possibility of immune remodeling, are explicitly considered and integrated when interpreting age-related trends in immune parameters. This will be a challenge for wild ecoimmunology, even for such widely measured parameters.

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