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Immunosenescence in some but not all immune components in a free-living vertebrate, the tree swallow

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A wide diversity of free-living organisms show increases in mortality rates and/or decreases in reproductive success with advancing age. However, the physiological mechanisms underlying these demographic patterns of senescence are poorly understood. Immunosenescence, the age-related deterioration of immune function, is well documented in humans and laboratory models, and often leads to increased morbidity and mortality due to disease. However, we know very little about immunosenescence in free-living organisms. Here, we studied immunosenescence in a free-living population of tree swallows, *Tachycineta bicolor*, assessing three components of the immune system and using both *in vivo* and *in vitro* immunological tests. Immune function in tree swallow females showed a complex pattern with age; acquired T-cell mediated immunity declined with age, but neither acquired nor innate humoral immunity did. *In vitro* lymphocyte proliferation stimulated by T-cell mitogens decreased with age, suggesting that reduced T-cell function might be one mechanism underlying the immunosenescence pattern of *in vivo* cell-mediated response recently described for this same population. Our results provide the most thorough description of immunosenescence patterns and mechanisms in a free-living vertebrate population to date. Future research should focus on the ecological implications of immunosenescence and the potential causes of variation in patterns among species.

Keywords: immunocompetence; lymphocyte proliferation; natural antibodies; phytohemagglutinin test; ageing

1. INTRODUCTION

Until recently, it was assumed that individuals in the wild were highly unlikely to show signs of senescence (Holmes & Austad 1995; Kirkwood & Austad 2000); however, we now know that a wide diversity of free-living populations show age-related increases in mortality rates and/or decreases in reproductive success indicative of senescence (e.g. Promislow 1991; Holmes *et al.* 2001; Bonduriansky & Brassil 2002; Bryant & Reznick 2004; Morbey *et al.* 2005). Less clear are the physiological mechanisms underlying these demographic patterns (Reznick *et al.* 2004; Bronikowski & Promislow 2005). Physiological senescence is a multifaceted phenomenon involving irreversible deterioration of many organ systems in the body (Rose 1991). One main survival-related system affected by age is the immune system, consisting of complex defence mechanisms evolved in response to ubiquitous threats from parasites and diseases (Roitt *et al.* 1998). In humans, efficient function of the immune system is essential for survival, and immunosenescence, the deterioration of immune function with age, results in increased risk of infection, autoimmune disease and cancer (Miller 1996; Effros 2003) that lead to higher morbidity and mortality among the old (Pawelec *et al.* 2002).

The vertebrate immune system can be divided into two main arms: innate immunity (the first line of defence) and acquired immunity. Both arms can be further subdivided into humoral and cell-mediated components (Roitt *et al.* 1998). Most knowledge about immunosenescence comes from studies of humans and mammalian laboratory models, which suggest that the ageing immune system is characterized by altered activity of most of its components (reviewed by Miller 1996; Grubeck-Loebenstein & Wick 2002). Decline occurs in acquired cell-mediated immunity (reviewed by Aspinall 2003) and acquired humoral immunity (reviewed by Weksler & Szabo 2000), but the latter is thought to be caused by the former, given that important aspects of B-cell function depend on helper T-cell function (Pawelec *et al.* 2002). Regarding innate immunity, certain components may decline with age (e.g. function of phagocytes, Pawelec *et al.* 1998), although others may actually increase with age (e.g. inflammation, Franceschi *et al.* 2000).

Recent studies on ecoimmunology suggest that immunosenescence may be a common phenomenon in free-living animals, including both invertebrates (Adamo *et al.* 2001; Kurtz 2002) and vertebrates (Cichon *et al.* 2003; Lozano & Lank 2003; Saino *et al.* 2003; Haussmann *et al.* 2005; Ujvari & Madsen 2006). However, we still know very little about immunosenescence patterns, mechanisms and their implications in the wild, including whether

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immunosenescence is a causal factor in demographic senescence patterns (Saino *et al.* 2003). In particular, studies to date on free-living vertebrates (with the exception of humans) have measured only one component of the immune system in a given species (either acquired cell-mediated or humoral immunity) and information on innate immunity is lacking altogether. In addition, previous studies have used only *in vivo* assays of immune function that involve complex integrated responses by several immune effectors and therefore preclude elucidation of specific mechanisms underlying the observed immune patterns (Silliman & Wang 2006).

In this study, we examine immunosenescence patterns and mechanisms in a free-living population, using tree swallows (*Tachycineta bicolor*, Viellot 1808) as our model system. Tree swallows are secondary cavity-nesting aerial insectivores members of the family Hirundinidae (Robertson *et al.* 1992). We assessed three components of the immune system (acquired cell-mediated immunity, acquired humoral immunity and innate humoral immunity) and used *in vitro* assays, in addition to *in vivo* assays, to shed light on potential mechanisms underlying the immunosenescence patterns.

We focused on two basic questions: (i) how the different immune components behave with age and (ii) what mechanisms underlie the immunosenescence patterns. With respect to the latter, we know that tree swallows in our study population show immunosenescence in their *in vivo* acquired cell-mediated immune response assessed by the standard phytohemagglutinin (PHA) skin test (Haussmann *et al.* 2005). This test is the most widely used estimate of cell-mediated immune function in studies of wild birds and has been used as a general index of *in vivo* T-lymphocyte activation and proliferative potential (reviewed by Fairbrother *et al.* 2004). However, the response to PHA also involves innate components such as heterophils, macrophages and basophils (Goto *et al.* 1978; Martin *et al.* 2006), suggesting that the reduced swelling in older individuals could be due to reduced T-lymphocyte activation and proliferative potential, reduced recruitment of cells of the innate immune system, or both. We therefore used *in vitro* assays to assess whether the observed age-related decline of *in vivo* cell-mediated response is associated with reduced T-lymphocyte activation and proliferation. In addition, we measured *in vivo* acquired humoral immunity mediated by B-lymphocytes to determine whether this component also declines with age in tree swallows, and if so, whether the decline is associated with reduced *in vitro* B-lymphocyte activation and proliferation.

2. MATERIAL AND METHODS

(a) *Study population*

We conducted this study in a tree swallow nest-box population in Tompkins County, NY (42°29′ N, 76°27′ W), which has been studied since 1985 (Winkler & Allen 1996; Winkler *et al.* 2004) and provided an adequate number of known-age individuals. Several factors besides age can affect immune responses. Individuals with higher body mass and/or body condition tend to mount stronger responses (e.g. Saino *et al.* 1997; Navarro *et al.* 2003). In tree swallows, individuals that start breeding earlier show stronger immune responses than later breeders (Hasselquist *et al.* 2001; Ardia 2005). In addition, stress can influence immune responses through elevated corticosterone (CORT) levels (reviewed by Padgett & Glaser 2003). Therefore, to assess the unique contribution of age to variation in immune responses and enhance the detectability of immunosenescence patterns, we statistically controlled for these potentially confounding factors (see below).

(b) *Field sample collection*

During the 2005 breeding season, we monitored nests daily to determine clutch completion date. We captured 45 females at their nest-boxes between 05:00 and 13:00 h on 3 June 2005. At this time, females were in late incubation (last 4 days) or early nestling period (first 4 days). Females ranged from 1 to 10 years of age, as determined by their ringing history. We knew exact ages for 26 females, and for the remaining, age was a minimum estimate based on each female's plumage in the year she was first ringed. We collected blood (approx. 200 µl) by jugular venipuncture and immediately 100 µl were diluted 1 : 1 in AIM-V lymphocyte medium supplemented with 25 mM HEPES, 2 mM L-glutamine and 50 µg ml^{-1} gentamicin (all from Life Technologies, Rockville, MD) and stored on ice for use in the lymphocyte proliferation assay the following morning. The remainder of the blood (approx. 100 µl) was transferred into capillary tubes for use in the humoral immunity assays (both innate and acquired) and for measuring CORT concentration. We stored capillary tubes on ice until centrifugation, after which we isolated the plasma for storage at −20°C. Females were weighed to the nearest 0.1 g and head–bill length (a measure of structural body size) was measured to the nearest 0.1 mm to estimate a body condition index (Schulte-Hostedde *et al.* 2005). Finally, we injected females intraperitoneally with 100 µl of a 2% suspension of sheep red blood cells (SRBC, HemoStat Laboratories, Dixon, CA) in phosphate buffered saline (PBS) and then released them. Following Ardia *et al.* (2003), females were recaptured 8 days later and a second blood sample (approx. 60 µl) was obtained to determine anti-SRBC antibody production.

(c) *Immune function assays*

In vitro lymphocyte proliferation—we used a whole-blood mitogenic stimulation assay (Cunnick *et al.* 1994) that we optimized for use in tree swallows; parameters we report provided the highest proliferation responses for tree swallow blood. We had previously determined that response of blood cells kept on ice for 24 h did not differ from that of fresh cells (paired samples *t*-test: $t=0.516$, $p=0.616$, $n=12$). In the laboratory, blood samples were further diluted to 1 : 20 using supplemented AIM-V lymphocyte medium (same as above) and 50 µl were dispensed into 96-well microplates containing 50 µl of mitogen (stimulated wells) or 50 µl of medium (non-stimulated wells). We stimulated lymphocytes using standard mitogens used in poultry immunology (e.g. Hovi *et al.* 1978; Cunnick *et al.* 1994): two T-cell mitogens, phytohaemagglutinin (PHA, 60 µg ml^{-1}) and concanavalin A (ConA, 60 µg ml^{-1}) and one B-cell mitogen, lipopolysaccharide from *Salmonella typhimurium* (LPS, 100 µg ml^{-1}), all from Sigma (St Louis, MO). Blood cultures were incubated in a 7% CO_2, 41°C humidified atmosphere for a total of 48 (LPS plates) or 72 h (PHA and ConA plates). Plates were pulsed with tritiated [^{3}H] thymidine (0.5 µCi/well) for the last 24 h, then harvested onto glass-fibre filters using a cell harvester (Combi Cell Harvester, Skatron Instruments, Sterling, VA) and counted in a scintillation counter. Each sample was

tested in triplicate for each mitogen and the medium and the counts per minute (c.p.m.) for triplicates were averaged. The proliferative response was expressed as a stimulation index (SI) calculated by dividing the mean c.p.m. of mitogen-stimulated wells by the mean c.p.m. of non-stimulated wells (Cunnick *et al.* 1994).

In vivo acquired humoral immune response (SRBC test)—we quantified antibody production by B-lymphocytes in response to immunization with SRBC following Ardia *et al.* (2003). We heat inactivated plasma (56°C for 30 min) and placed 10 µl in the first well of a 96-well plate. Next, we serially diluted samples starting with 10 µl plasma in 10 µl PBS along the row. Then, 10 µl of a 2% SRBC suspension was added to each well and plates were incubated at 37°C for 90 min. Titres (SRBC Ab titres) are expressed as the $\log_2$ of the highest dilution factor of plasma that showed hemagglutination. We recorded half scores between two titres when the termination of hemagglutination was intermediate. All plasma samples were run in duplicate and average titres were used. Pre- and post-immunization samples, as well as negative and positive controls, were run in each plate. None of the pre-immunization samples showed hemagglutination.

Constitutive innate humoral immunity—we used a haemolysis–hemagglutination assay developed in birds (Matson *et al.* 2005) to assess the levels of natural antibodies (NAbs) in peripheral blood and complement-mediated cell lysis. Lysis of cells probably results from the interaction between NAbs and complement, while hemagglutination depends only on NAbs (Matson *et al.* 2005). We performed a base-2 serial dilution of plasma in PBS as described for the SRBC test, except that plasma was not heat-inactivated in order to preserve complement proteins. Next, we added 10 µl of a 2% suspension of rabbit red blood cells (RRBC, HemoStat Laboratories) to each well and incubated plates at 37°C for 90 min. The scoring of hemagglutination (NAb titre) and lysis titres was performed as described by Matson *et al.* (2005). Titres are expressed as the $\log_2$ of the highest dilution factor of plasma that showed each response. We recorded half scores between two titres when the termination of hemagglutination or lysis was intermediate. All plasma samples were run in duplicate and average titres were used. Negative and positive controls were run in each plate.

(d) *Corticosterone assay*

We quantified CORT level in plasma to control for its concentration at the time of blood sampling and determine its effect on immune responses. All samples were run in triplicate in a single radioimmunoassay (no. 07-120102, MP Biomedicals, Irvine, CA) that has been validated for use in passerines (Washburn *et al.* 2002). Intra-assay coefficient of variation was 9.7%.

(e) *Statistical analyses*

All variables were checked for normality and $\log_{10}$ transformed when necessary. Lysis titres were not normally distributed even after transformation, so we used non-parametric tests (Spearman's rank correlation) when analysing this variable. We used multiple regression analyses to determine the relationships between immune function and age while controlling for potential confounding factors (clutch completion date, body mass, body condition index and CORT concentration). Body condition index and body mass were highly correlated ($r=0.910$, $p<0.0001$, $n=45$), therefore we only present the results for models using body mass. We used backward elimination to determine the final regression models, using $\alpha=0.05$ and 0.1 as entry and removal probabilities, respectively. Sample sizes vary among analyses because we could not perform all assays in some samples due to limited blood volume. All statistical analyses were performed using SPSS v. 13.

3. RESULTS

Neither time of day nor breeding stage explained significant variation in immune responses (all $p>0.05$). CORT levels increased with handling time ($r=0.543$, $p=0.011$, $n=31$), but after controlling for handling time, CORT levels were not significantly correlated with age ($r_p=0.005$, $p=0.980$, $n=31$).

(a) *Acquired cell-mediated immunity*

In vitro lymphocyte proliferation in response to T-cell mitogens (PHA and ConA) decreased with age among adult females (figure 1*a*,*b*). The final regression model for PHA included age as the only significant explanatory variable ($r=-0.503$, $p=0.001$, $n=43$), whereas the final model for ConA included age ($r_p=-0.490$, $p=0.001$, $n=43$) and body mass ($r_p=-0.414$, $p=0.006$, $n=43$). Forcing CORT concentration and/or clutch completion date into the above models did not change the relationships.

(b) *Acquired humoral immunity*

In vitro lymphocyte proliferation in response to the B-cell mitogen (LPS) did not change with female age (figure 1*c*, $r=0.079$, $p=0.613$, $n=43$). The final regression model for LPS included CORT concentration as the only explanatory variable ($r=-0.443$, $p=0.013$, $n=31$), with higher CORT concentrations associated with lower proliferation. However, forcing body mass and/or clutch completion date into the model rendered that relationship non-significant. *In vivo* antibody response to SRBC did not change significantly with age (figure 2*a*). None of the variables explained significant variation among adult females in this immune response.

(c) *Innate immunity*

The two measures of innate humoral immunity (NAbs titre and lysis titre) did not change with age among female tree swallows (figure 2*b*,*c*, respectively). The final regression model for NAbs titre included only CORT concentration ($r=-0.401$, $p=0.031$, $n=29$), with higher concentrations associated with lower titres. Forcing body mass and/or clutch completion date into the model, however, rendered that relationship non-significant. No significant model was found for lysis titre.

4. DISCUSSION

In vitro proliferative response to T-cell mitogens (PHA/ConA) decreased with age in free-living adult female tree swallows ranging from 1 to 8 years of age, with the oldest individuals showing a response 84% weaker than the youngest. Indeed, age was the main predictor of variation in *in vitro* response to PHA/ConA among individuals, with the potential confounding variables (i.e. body mass, clutch completion date and CORT concentration) explaining little or no variation. An age-related decrease in T-lymphocyte proliferative ability in response to mitogens is a hallmark of immunosenescence in humans and

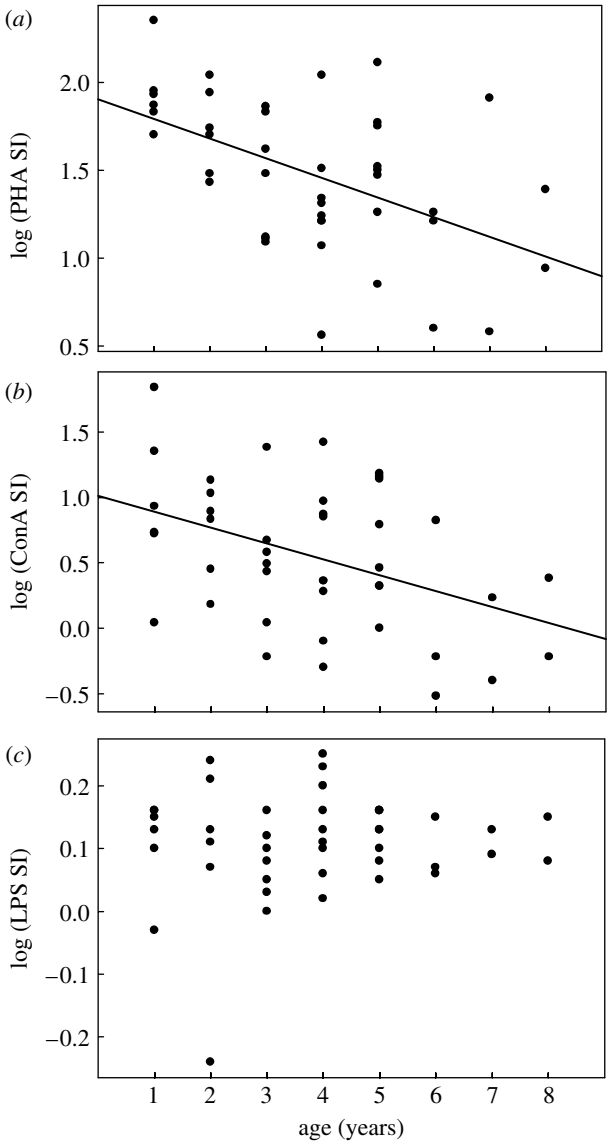

Figure 1. *In vitro* lymphocyte proliferation responses of adult female tree swallows as a function of age ($n=43$ in all cases). (*a*) Lymphocyte proliferation in response to PHA (PHA SI; $r=-0.503$, $p=0.001$, regression line: $\log y=-0.112 \log x+1.9$), (*b*) lymphocyte proliferation in response to ConA (ConA SI; $r=-0.422$, $p=0.005$, regression line: $\log y=-0.121 \log x+1.0$), (*c*) lymphocyte proliferation in response to LPS (LPS SI; $r=-0.079$, $p=0.613$).

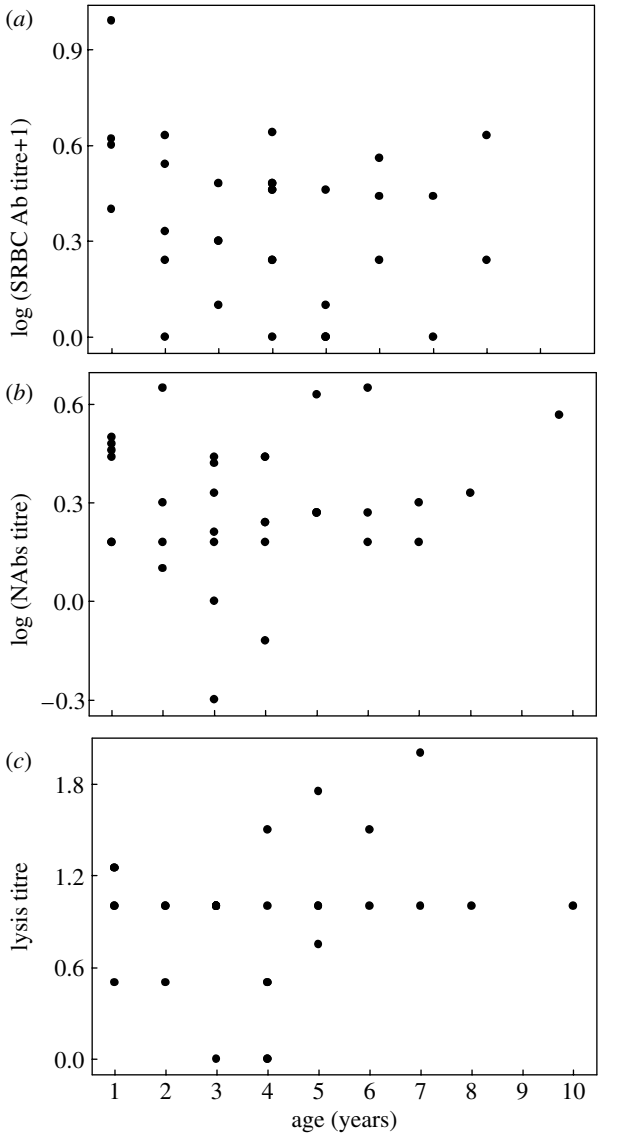

Figure 2. Humoral immune responses of adult female tree swallows as a function of age. (*a*) Acquired humoral response to SRBC challenge (SRBC Ab titre; $r=-0.273$, $p=0.120$, $n=33$). Note: 1 was added to the argument of the logarithm to show titres $=0$ corresponding to undiluted plasma, (*b*) levels of natural antibodies (NAbs titre; $r=0.111$, $p=0.531$, $n=34$), (*c*) complement-mediated cell lysis (Lysis titre; Spearman $r=0.065$, $p=0.724$, $n=32$).

mammalian laboratory models (Effros *et al.* 1994; Miller 1996; Franceschi *et al.* 2000), and together with other parameters of T-lymphocyte function, can predict survival among old individuals, suggesting an association between longevity and well-preserved immunity (reviewed by Pawelec *et al.* 2002). To our knowledge, this immunosenescence pattern had never been documented in a non-mammalian vertebrate or any free-living population with the exception of humans.

Haussmann *et al.* (2005) reported immunosenescence in this same tree swallow population based on the PHA skin test, with older individuals mounting weaker responses (i.e. smaller swellings) than younger ones. Data from this study support the hypothesis that reduced activation and/or proliferative potential of T-lymphocytes from older tree swallows is one mechanism underlying the pattern of immunosenescence in the PHA skin test response. The importance of T-cell function in the *in vivo* response to PHA challenge has been experimentally demonstrated in chickens thymectomized as neonates, which show a 50% reduction in swelling responses compared with controls (Goto *et al.* 1978). Since T-cells are not the only cells involved in the PHA skin response, future studies should assess the potential contribution of other immune components to the age-related reduction in swelling. In particular, studies of the histology of the skin swelling response to PHA (e.g. Martin *et al.* 2006) in young and old individuals could help determine the relative contribution of lymphocytes versus innate components such as heterophils, macrophages and basophils.

Ecoimmunology studies have shown that increased reproductive effort results in reduced immune function (see Schmid-Hempel 2003 for review). Therefore, an alternative explanation for the decline in immune function

with age is a shift in resource allocation from self-maintenance to reproductive effort as individuals get older (Cichon *et al.* 2003). This scenario predicts that during the non-reproductive period any age differences in immune function would vanish (Cichon *et al.* 2003), which would not be the case if immunosenescence were due to a progressive and unavoidable deterioration of the immune system. Studies with laboratory mammals and humans indicate that immunosenescence occurs even in the absence of reproduction; however, reproductive costs may indeed accelerate immunosenescence (Helle *et al.* 2004). Future studies in ecoimmunology should focus on understanding the interaction between immunosenescence and reproductive effort in free-living organisms.

Female tree swallows did not show immunosenescence in our measured aspects of acquired humoral immunity. This result differs from two recent studies in free-living passerine birds. Antibody responses against Newcastle disease virus vaccine (NDV) declined with age in female barn swallows (*Hirundo rustica*) ranging from 1 to 3 years old (Saino *et al.* 2003), and older female collared flycatchers (*Ficedula albicollis*; 5–6 years old) mounted weaker responses to SRBC than younger ones (less than 4 years old). We did not find evidence of immunosenescence in acquired humoral immunity in tree swallows, even when analysing a broader range of ages (i.e. 1–10 years old) and performing an *in vitro* assay of lymphocyte proliferation in addition to the *in vivo* antibody response to immunization.

An interesting aspect of our results on acquired humoral immunity is that we did not find an age-related reduction in the production of antibodies against SRBC by B-cells, which is dependent on T-helper cells (a subtype of T-cell), despite finding reduced response to T-cell mitogens with age. One possible explanation for this result is that an increase in B-cell responsiveness with age compensated for the decrease in T-cell responsiveness resulting in no net difference in antibody production across ages. However, this seems unlikely as we did not find support for increased B-cell responsiveness with age through our *in vitro* test, and we are not aware of any study demonstrating increased B-cell function with age. Another possible explanation is that the response to SRBC does not decline with age because rather than T-helper cells, it is T-cytotoxic cells that show reduced response with advancing age in the *in vitro* assay, as has been shown in humans (Grossmann *et al.* 1989).

Different patterns of immunosenescence among species are likely the consequence of differences in demography and selection, possibly reflecting the link between life history and physiology (Ricklefs & Wikelski 2002). Indeed, avian species with higher survival rates and longer lifespans show a slower senescent decline of *in vivo* PHA response than species with the opposite life-history traits (Haussmann *et al.* 2005). North American tree swallows appear to have higher annual survival rates (50%, Robertson *et al.* 1992) and maximum recorded lifespans (12 years, Robertson & Rendell 2001) than those reported for the European barn swallow (35–40%, 7 years, Saino *et al.* 2003) and collared flycatcher (35–40%, 8 years, Cichon *et al.* 2003) populations on which immunosenescence data were reported; therefore, we would predict slower senescence in tree swallows. In addition, differences in diversity and/or abundance of parasites in the environment can favour different levels of investment in immune function and/or different immune strategies (Zuk & Stoehr 2002; Schmid-Hempel 2003; Lindstrom *et al.* 2004), but this has not yet been studied in relation to comparative patterns of immunosenescence.

Finally, tree swallows did not show immunosenescence in either aspect of innate humoral immunity measured. Neither the level of circulating NAbs against RRBC nor complement-mediated cell lysis showed a decline with age. It remains to be determined whether cellular aspects of innate immunity, such as the function of phagocytes (Pawelec *et al.* 1998), show immunosenescence in this species.

In summary, we present the most thorough study of immunosenescence patterns in a free-living population to date. Immune function of tree swallows shows a complex age-related pattern with some aspects of immune function declining with age and others remaining unchanged. Our results in a free-living bird coincide with general patterns in humans and mammalian laboratory models that show that acquired immunity declines more pervasively with age than does innate immunity (Franceschi *et al.* 2000) and that T-cell function is more affected than B-cell function (Pawelec *et al.* 2002). Our study also provides evidence for a potential mechanism underlying immunosenescence of the *in vivo* response to PHA, the most widely used immune test in ecological immunology. Finally, our data also suggest that species might differ not only in the rate of senescence of a given immune component (Haussmann *et al.* 2005), but also in which components are more affected by age. An important next step for ecoimmunology is to understand the ecological and evolutionary implications of immunosenescence patterns in the wild.

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