Does innate immune function decline with age in captive ruffs *Philomachus pugnax*?

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Abstract

Immunosenescence, the decline of immune function with age, results in increased risk of infection as an individual ages. The underlying reasons are still poorly understood. Here, we ask whether the rate of decline of an immune indicator is positively correlated with the cost of maintaining it, as predicted by optimal resource allocation theory. Using 30 female ruffs *Philomachus pugnax* ranging in age from 0.5-12 years we found no effect of age on five indicators of constitutive innate immunity, which is cheap to use and maintain. Body temperature increase following injection with lipopolysaccharide is an indicator of induced innate immunity, which is energetically expensive, and showed a curvilinear relationship with age, with a maximum in middle-aged birds. Our results suggest that changes in immune function with age may depend on the energetic cost of using an immune trait.

Introduction

Immunosenescence, the decline of immune function with age, is well-documented in humans, and leads to increased susceptibility to infection, resulting in higher

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mortality rates with age (Dorshkind et al., 2009). Immunosenescence has mainly been studied in humans and laboratory rodents, but also in some free-living vertebrates (see Palacios et al., 2011). The mechanisms underlying immunosenescence are, however, still poorly understood (Agarwal & Busse, 2010). There is some evidence that such an age-related decline is not uniform across all immune indicators (Palacios et al., 2007), possibly because with increasing age, an individual may allocate fewer resources to self-maintenance while investing more in reproduction (Cichoń, 1997). Optimal resource allocation thus dictates that an energetically more expensive trait should be down-regulated more rapidly with increasing age than a cheaper trait, and this hypothesis may also apply to immune indicators.

The vertebrate immune system can be divided into two arms: innate immune function is immediately effective against a broad range of pathogens, whereas acquired immune function is pathogen-specific and takes longer to develop (Shanley et al., 2009). Innate immune function can be further divided into constitutive (constantly maintained) and induced aspects, the latter being energetically much more expensive (Klasing, 2004). Here, we used captive ruffs *Philomachus pugnax*, a migratory sandpiper, to assess immunosenescence in constitutive and induced innate immunity. Immunosenescence for a cell-mediated trait has previously been found in this population of ruffs (Lozano & Lank, 2003).

To assess constitutive innate immunity, we measured the antimicrobial capacity of plasma using three different strains of bacteria, and complement-like activity, which provides early protection against infections via cell lysis (Ochsenbein & Zinkernagel, 2000). We also measured levels of haptoglobin, which is an acute phase protein that protects against harmful end products of the immune response (Delers et al., 1988). To assess induced innate immunity, we mimicked bacterial infection by injecting lipopolysaccharide (LPS) from the cell walls of gram negative bacteria. Within hours of exposure, an acute phase response characterized by sickness behaviour and fever is induced (Janeway et al., 2001). This challenge can be used to assess the symptoms of an acute phase response (e.g., increased body temperature) without causing the actual disease, since no live bacteria are present.

Material and methods

Bird husbandry

We obtained ruffs from a captive breeding flock originally derived from eggs collected near Oulu, Finland prior to 1990. Since 1994, the flock has been held under natural photoperiod in a communal outdoor pen at Simon Fraser University, Burnaby, Canada, with *ad libitum* water and food (Lozano & Lank, 2003). In the summer of 2009, 24 adult females ranging in age from 1-12 years were relocated to the Advanced Facility of Avian Research (AFAR) at the University of Western Ontario, London, Canada. They were housed in large indoor aviaries where each room had a pool with continuously running fresh water. Birds were free to move between rooms. Eggs from the same colony were shipped to AFAR and six juvenile females,

hand-raised in August and September 2010, were used. All animal protocols were approved by the University of Western Ontario Animal Use Sub-committee (protocols 2006-011-04, 2010-216) and birds were obtained under Canadian Wildlife Service permit CA0256.

Experimental design

The experiment was conducted during the non-breeding season. All individuals were blood sampled once on either December 8 or 10, 2010. This sample was used to assess constitutive innate immunity. A week later, we induced the acute phase response by injecting the birds with LPS: on December 15th, 8 individuals had their temperature measured and 5 of these were injected with LPS immediately afterwards. Three 0.5-year old individuals served as negative controls (results not presented because of low sample size) and did not receive LPS injections. The remaining 12 individuals were processed in the same way on December 16th. For both groups, temperature was measured again 20 h post LPS injection, and a second blood sample was taken. Birds were returned to their holding pens immediately after processing. LPS injection in all birds took place between 13:17 and 15:21 on the injection day.

Data collection

We measured body temperature to the nearest 0.1°C (cloacal insertion of high accuracy thermocouple, VWR 23609-232). We sterilized the area around the brachial vein with 70% ethanol and collected *ca.* 1 ml blood with a 26 ½ gauge needle into heparinized 1.5 ml microcentrifuge tubes. Plasma was obtained by centrifuging blood samples for 10 min at $2000 \times g$ and then stored at $-80°C$ until processing.

Constitutive innate immunity

We performed a microbicidal assay using the gram negative bacteria *Escherichia coli* [American Type Culture Collection (ATCC) #8739], *E. coli* (ATCC #51813) and the fungus *Candida albicans* (ATCC #10231). We optimized the assay described by Liebl & Martin (2009) for ruffs. Microbial concentrations were determined by measuring the absorbance of each sample at 300 nm using a spectrophotometer (Thermo Scientific NanoDrop 2000). Microbicidal activity was calculated as the proportion of microbes killed in samples relative to positive controls [1 − (absorbance of sample/absorbance of control)] (Liebl & Martin, 2009). The hemolysis-hemagglutination assay was used to assess complement-like activity by measuring the amount of hemoglobin released from the lysis of rabbit red blood cells (Matson et al., 2005). We quantified plasma haptoglobin levels following the 'manual method' in a commercial kit (no. TP801; Tri-Delta Diagnostics, Morris Plains, New Jersey, USA).

Induced innate immunity

Using a 23 gauge needle and a 1 ml syringe, we injected birds intra-peritoneally with a solution of 0.25 mg/ml LPS (Sigma L 7261, source strain *Salmonella typhimurium* ATCC #7823) in phosphate buffered saline at a dosage of 1 mg/kg (Koutsos & Klasing, 2001).

Statistical analyses

We used paired *t*-tests to compare body temperature pre- and post-LPS injection, and a Generalized Linear Model (GLM) with Type III sum of squares to test for an effect of age on body temperature and LPS-induced changes in body temperature and haptoglobin levels, using the statistical software SYSTAT (SYSTAT, 2009). Because of the curvilinear relationship of changes in temperature following LPS injection with age we included age and age^2 as factors. Baseline haptoglobin values were transformed using the natural logarithm to stabilize variance. Values for bactericidal killing, lysis (baseline and acute phase) and acute phase haptoglobin levels did not conform to normality, and we used the Spearman rank-order correlation coefficient *rho* to test for a correlation with age (Wessa, 2012). To eliminate baseline-variation among individuals, we present changes in lysis and haptoglobin in response to LPS by subtracting baseline from acute phase levels for each individual. Sample sizes vary among immune measures because of limited blood volume.

Results

Constitutive innate immunity

We did not find a correlation between the proportion of microorganisms killed and bird age in *Escherichia coli* ATCC #8739 ($N = 30$, $rho = -0.04$, $P = 0.82$), *E. coli* ATCC #51813 ($N = 30$, $rho = -0.10$, $P = 0.58$), nor in *C. albicans* ATCC #10231 ($N = 29$, $rho = -0.21$, $P = 0.29$). Likewise, age was not correlated with lysis ($N = 25$, $rho = -0.23$, $P = 0.26$). Haptoglobin levels were not correlated with age ($N = 21$, $F_{1,19} = 0.75$, $P = 0.40$) nor with age and age^2 ($N = 21$, $F_{2,18} = 0.36$, $P = 0.70$; table 1). The distribution of the values for bactericidal killing and lysis did not permit a GLM with a quadratic model, but visual inspection of the data distribution did not suggest a curvilinear relationship with age.

Induced innate immunity

Body temperature showed a significant increase ($t_{24} = -5.18$, $P < 0.001$) of on average 1.3°C (SD = 1.27) 20 h post LPS injection. Body temperature prior to LPS injection did not vary with age ($F_{1,28} = 1.45$, $P = 0.24$), but age did affect the change in body temperature following LPS injection: the difference in body temperature increased towards 6 years of age, after which it started to decrease (fig. 1). A model that included age and age^2 was supported by the data ($F_{2,20} =$

Table 1.

Immune indicators (mean; SD; N) by age group in captive non-breeding female ruffs.

Age (years)	Baseline					Acute phase		
	E. coli #8739	E. coli #51813	C. albicans	Lysis	Haptoglobin (mg/ml)	Δ temperature (°C)	Lysis	Haptoglobin (mg/ml)
0.5	0.59; 0.42; 6	0.25; 0.11; 6	0.38; 0.31; 6	5.00; n/a; 1	1.24; 0.50; 4	0.2; 0.20; 2	–	–0.95; 0.24; 2
1	0.50; 0.59; 2	0.04; 0.06; 2	0.46; 0.08; 2	5.75; 0.35; 2	0.50; 0.07; 2	0.5; 1.98; 2	–0.50; n/a; 1	–
2	0.71; 0.41; 8	0.19; 0.32; 8	0.33; 0.15; 7	5.44; 0.56; 8	0.58; 0.22; 7	1.4; 1.14; 8	0.50; 0.50; 3	0.48; n/a; 1
3	0.93; n/a; 1	0.75; n/a; 1	0.15; n/a; 1	6.50; n/a; 1	0.84; n/a; 1	–	–	–
6	0.53; 0.43; 6	0.07; 0.08; 6	0.28; 0.30; 6	5.12; 1.18; 6	0.69; 0.30; 4	2.7; 1.29; 5	1.00; 2.83; 2	–0.25; n/a; 1
7	0.71; n/a; 1	0.04; n/a; 1	0.20; n/a; 1	4.50; n/a; 1	–	0.5; n/a; 1	–	–
8	0.10; n/a; 1	0.08; n/a; 1	0.18; n/a; 1	6.00; n/a; 1	–	1.2; n/a; 1	–2.00; n/a; 1	–
11	0.97; 0.01; 2	0.22; 0.11; 2	0.22; 0.07; 2	4.25; 1.06; 2	0.46; n/a; 1	1.3; 0.42; 2	–	–
12	0.66; 0.48; 3	0.22; 0.07; 3	0.39; 0.32; 3	5.17; 0.76; 3	0.72; 0.06; 2	0.6; 0.85; 2	0.500; n/a; 1	1.03; n/a; 1

Values for the two strains of E. coli and for C. albicans are the proportion of colonies killed by the blood. Lysis levels show the step in the serial dilution of the plasma at which the lytic reaction stopped. Values for haptoglobin indicate plasma concentrations. Note that three of the 0.5-year old birds served as controls in the LPS trial and that in several cases it was not possible to obtain a sufficient quantity of blood the day after the first blood sample.
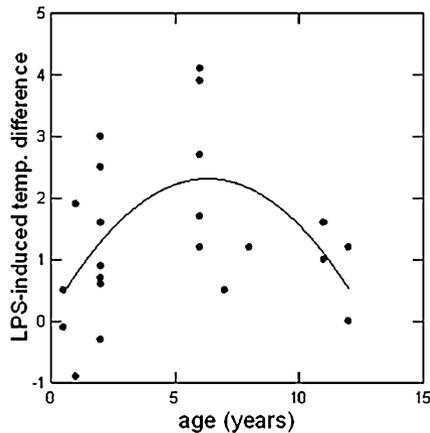
Figure 1. Body temperature (°C) was temporarily changed 20 h post lipopolysaccharide (LPS) injection and the magnitude of this change varied with individual age in ruffs: it increased towards 6 years of age, after which it decreased again.

3.74, $P = 0.042$). During the acute phase, there was no correlation between age and lysis levels ($N = 8$, $rho = 0.03$, $P = 0.94$) or age and haptoglobin levels ($N = 4$, $rho = 0.80$, $P = 0.33$; table 1). Again, visual examination of the data distribution did not suggest a curvilinear relationship with age.

Discussion

We used non-breeding captive female ruffs ranging in age from 0.5 to 12 years to assess whether the energetically more costly induced innate immunity declines more rapidly with age than the less costly constitutive innate immunity. We found no relationship between age and five measures of constitutive and two measures of induced innate immune function. While our small sample sizes (table 1) may have limited the ability to detect patterns in the data with small effect size, it is worth noting that in free-living tree swallows *Tachycineta bicolor*, indicators of innate immunity, including lysis, also did not change with age (Palacios et al., 2007). We did, however, detect a curvilinear relationship in the change in body temperature during the acute phase response with age. Mounting an acute phase response, with the concomitant increase in body temperature, is energetically much more expensive than the constitutive innate immune function (Klasing, 2004).

The strength of the acute phase response peaked at 6 years of age, which raises the possibility that the decline with more advanced age is due to immunosenescence. While this does not offer an explanation for the weak response in younger birds, it is interesting to note that these age-related changes in body temperature in response to LPS (fig. 1) followed the same curvilinear relationship as those of the cell-mediated immunity, which is part of the acquired immune response, in male ruffs with age (Lozano & Lank, 2003). A study on bactericidal activity in wild stonechats *Saxicola torquata* ranging in age from 1 to 7 years showed that

in older birds, the bactericidal ability against *E. coli* (ATCC #8739) was higher than in younger birds (Tieleman et al., 2010). The authors concede that they cannot distinguish whether the birds' capacity to fight these microbes improves with age or whether the birds that have poorer bactericidal ability die younger. Our finding of no change in bactericidal ability with age in captive birds supports the second interpretation.

Studies on immunosenescence conducted on animals in a controlled setting are important complements to studies on wild populations because in the wild weak individuals may be eliminated due to poor survival skewing immune parameters in those that survive. In the wild, very few ruffs live to be 12 years of age, but in captivity, 10% of females in this study population survived to age 12 ($N = 304$, DBL, unpublished data). Therefore, it is unlikely that weak individuals were eliminated due to poor survival in our experiment, partly because in a controlled setting, the cost of immunosenescence is independent of environmental factors (Palacios et al., 2011), something that cannot be controlled in the wild. Nevertheless, mortality still occurs in captivity and we cannot exclude the possibility of selective mortality correlated to immune characteristics.

Different aspects of the immune system incur different use and maintenance costs (Klasing, 2004), and it may be that the different aspects of the immune system are subject to energetic trade-offs (Lee, 2006). Control of the expensive acute phase response maybe surrendered towards the end of an individual's life, as has been suggested in humans (Giunta et al., 2008), or the inflammatory defenses may be enhanced while the acquired defenses are waning (Martin et al., 2006). Seen from this perspective, differences in the effect of age on different aspects of immune function may themselves be adaptive. Understanding the factors underlying immunosenescence is relevant to human medical as well as ecological and evolutionary disciplines as it contributes towards being able to explain intra- and interspecific variation in mortality rates.

Acknowledgements

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