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## Cell-mediated immunosenescence in birds

Received: 30 October 2004 / Accepted: 5 April 2005 / Published online: 16 July 2005  
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**Abstract** The phytohaemagglutinin (PHA) skin test response, used to assess cell-mediated immunity, is known to vary with many social and energetic factors, but the effects of age have received little attention. We found that the PHA response of immature birds was lower than those of the youngest breeding adults and were decreased in adults. Whenever possible, age should be included as a covariate when the PHA skin test is used to assess immunocompetence in ecological immunology. The rate of decline in PHA response differed between species and was inversely correlated with survival. The decrease in the PHA response averaged 57% over an average 80% of the maximum life span, but the absolute rate varied with species lifespan such that the short-lived species showed a greater loss per year than the long-lived species. This link between declining immune function and survival may reflect differences in resource partitioning between species, and suggests that selection may act on investment in immune function to influence maximum life span.

**Keywords** Ecological immunology · Life history · Aging · Phytohaemagglutinin skin test · PHA

Communicated by Mark Chappell

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### Introduction

The immune system is one of the most important defense and repair systems within vertebrates (Roitt et al. 1998). Immune function is complex with multiple components, but all classes of immune function are characterized by high energetic cost (Raberg et al. 1998; Lochmiller and Deerenberg 1999; Martin et al. 2003; Bonneaud et al. 2003). Resources required to mount an immune response must often be diverted from other systems. This plasticity underlies the emerging field of ecological immunology, which focuses on how immune responses of individuals are influenced by their environment and condition.

We used the phytohaemagglutinin (PHA) skin test to measure cell-mediated immunity across age in three species of birds that vary in survival. The PHA test was originally developed for use in poultry science (Goto et al. 1978; Cheng and Lamont 1988), but more recently has been used widely in avian field biology (for review see, Tella et al. 2002). A subcutaneous injection of PHA causes T cells to proliferate, which induces erythema, induration, cellular infiltration and dermatitis (Roitt et al. 1998). The resulting swelling can be measured and used as an index of an individual's cell-mediated immunity (Goto et al. 1978).

Despite conflicting results on whether the PHA skin test is harmless (Merino et al. 1999; Moreno et al. 2001b; but see, Lifjeld et al. 2002; Martin et al. 2003), the PHA skin test has been widely used to assess correlation between cell-mediated immunity and body condition (Alonso-Alvarez and Tella 2001; Lifjeld et al. 2002), energy expenditure (Martin et al. 2003), hormone profiles (Casto et al. 2001), brood size (Sorci et al. 1997; Saino et al. 1997; Moreno et al. 1999; Tella et al. 2002; Lifjeld et al. 2002), feeding rate (Saino et al. 1997; Moreno et al. 1999), sexual ornaments (Gonzalez et al. 1999; Velando et al. 2001; Saks et al. 2003) and survival (Gonzalez et al. 1999; Ardia et al. 2003). However, we know very little about how the PHA response varies with age.

Decreasing immune function with age, immunosenescence, has been documented in humans (Pawelec et al. 1999; Effros 2003) and domesticated animals (Blasco 2002; Jayashankar et al. 2003), but data on free-living animals are sparse (Cichon et al. 2003). We examined the PHA response across ages in three species of birds and determined how rate of immunosenescence correlates with survival and lifespan (Table 1).

## Methods

### Species

We determined the relationship between cell-mediated immunity and age in three species; the zebra finch (*Taeniopygia guttata*), tree swallow (*Tachycineta bicolor*), and Leach's storm-petrel (*Oceanodroma leucorhoa*). Zebra finches were from a captive colony maintained at Iowa State University since 1996 (Hausmann and Vleck 2002). The zebra finch is a 10- to 13-g passerine, which forms large, loosely associated breeding colonies and is noted for its opportunistic breeding strategy and high reproductive effort under good conditions (Farner and Serventy 1960). In our captive colony, zebra finches are housed in 2×1×2 m flight cages provided with perches and ad libitum finch seed mix and water. The room is under controlled temperature (~25°C), and light (14L:10D). One week prior to the experiment (June 2003), thirty nonbreeding males ranging from 5 months to 48 months of age (representing ~72% of zebra finch lifespan) were removed from the flight cages and placed into six smaller cages (0.5×0.5×0.5 m), containing five individuals each. Owing to conflicting experiments at the time of this study, we were unable to test cell-mediated immunity in female zebra finches.

The tree swallow study population near Ithaca, New York, has been studied since 1985 (Winkler and Allen 1996). Tree swallows are 18- to 22-g hole-nesting passerines, with a socially monogamous mating system, breeding in loose colonies. In the Ithaca population, nest boxes erected at 20 m intervals are checked throughout the breeding season to determine the identity of the individuals using the box and their breeding success. In May 2003, we measured PHA response of 14 male and 18 female tree swallows ranging from 1 year to -8 years

of age, and seven hatchlings (9 days old and sex unknown) (representing ~67% of tree swallow lifespan). At this time, adult swallows were either incubating completed clutches of eggs or brooding chicks.

The Leach's storm-petrel population on Kent Island, New Brunswick, has been studied since 1947 (Huntington et al. 1996). Leach's storm-petrels are 40- to 50-g procellariid seabirds, which breed in large colonies on islands in the northern Atlantic and Pacific Oceans. These birds nest in pairs in burrows, where they lay and incubate a single egg and then brood the chick. The Kent Island population is comprised of three main study areas in which burrows and the individuals that inhabit them are identified at the beginning of each breeding season. Because Leach's storm-petrels are highly philopatric (Huntington et al. 1996) and the study areas are monitored closely, any individual found in a study area that is not banded can be assumed to be a first-time breeding adult. We measured PHA response in both known-aged individuals (banded as nestlings) and individuals banded as breeding adults. Birds that were first captured as breeding adults were assumed to be 4-years-old at that time, the minimum age of first breeding in Leach's storm-petrels (Huntington et al. 1996). In July 2003, we sampled male and female Leach's storm-petrels ranging from 4 years to at least 34 years of age (representing ~86% of Leach's storm-petrel lifespan), when birds were incubating. Owing to an abnormally late breeding season in July 2003, we were not able to sample nestlings.

For these three species, we obtained information on annual adult survival from the literature. Annual adult survival is the proportion of banded adults returning in subsequent years (Table 1).

### Immune challenge

We tested the proliferative response of T lymphocytes to phytohaemagglutinin (PHA-P) using the simplified protocol of Smits et al. (1999). Birds were retrieved from the cage (zebra finch), nest box (tree swallow), or burrow (Leach's storm-petrel) and weighed with a Pesola spring balance. One person held the bird with its left wing extended and another person swabbed the mid-patagium (wing-web) with alcohol, and used a waterproof marker

**Table 1** Avian species studied, annual adult survival (AS), observed maximum lifespan in the wild (MLS), sample size (*n*), dose of PHA in PBS, repeatability of the PHA measurement

Species	AS <sup>a</sup>	MLS (year) <sup>a</sup>	<i>n</i>	Dose (µg)/injection volume (µl) <sup>c</sup>	Repeatability <sup>b</sup>
<i>Taeniopygia guttata</i> <sup>c</sup>	0.19	5	30	20/20	$r^2 = 0.99$ , $F = 982$ , $P < 0.0001$
<i>Tachycineta bicolor</i> <sup>d</sup>	0.50	12	39	40/20	$r^2 = 0.98$ , $F = 435$ , $P < 0.0001$
<i>Oceanodroma leucorhoa</i> <sup>e</sup>	0.94	36	41	50/25	$r^2 = 0.99$ , $F = 563$ , $P < 0.0001$

<sup>a</sup>Annual adult survival and observed maximum lifespan in the wild for each species, sources indicated after species name

<sup>b</sup>Similar injection dosage and volumes as previous studies (Smits et al. 1999)

<sup>c</sup>Zann (1996)

<sup>d</sup>Robertson et al. (1992)

<sup>e</sup>Huntington et al. (1996)

to mark the measurement and injection site. We injected PHA-P (Sigma L8754) dissolved in sterile PBS (Sigma P0261) at the marked site in a dose that was appropriate for the size of the species (Smits et al. 1999) (Table 1).

We measured the thickness of the patagium at the marked site immediately prior to injection and  $24 \pm 1.2$  h after injection. PHA response was determined by subtracting the preinjection measurement from the 24-hour measurement. Measurements were made using a digital micrometer precise to 0.001 mm (Mitutoyo, Tokyo, Japan) and modified with a detachable head that reduced the spindle's surface to  $2 \text{ mm}^2$ . All measurements were done by the same individual (M.F. Hausmann). Down feathers lying beneath the micrometer spindle or tissue pliability can make accurate measurements of patagium thickness difficult. To avoid these potential problems, the anvil of the micrometer was held firmly beneath the patagium, and as soon as the spindle came into contact with the tissue surface and caused the skin to begin twisting, the spindle was gently released until the skin orientation returned to its original conformation. Skin thickness both before and after injection was taken as the mean of four repeated measurements (see Table 1 for measurement repeatabilities (Lessells and Boag 1987)).

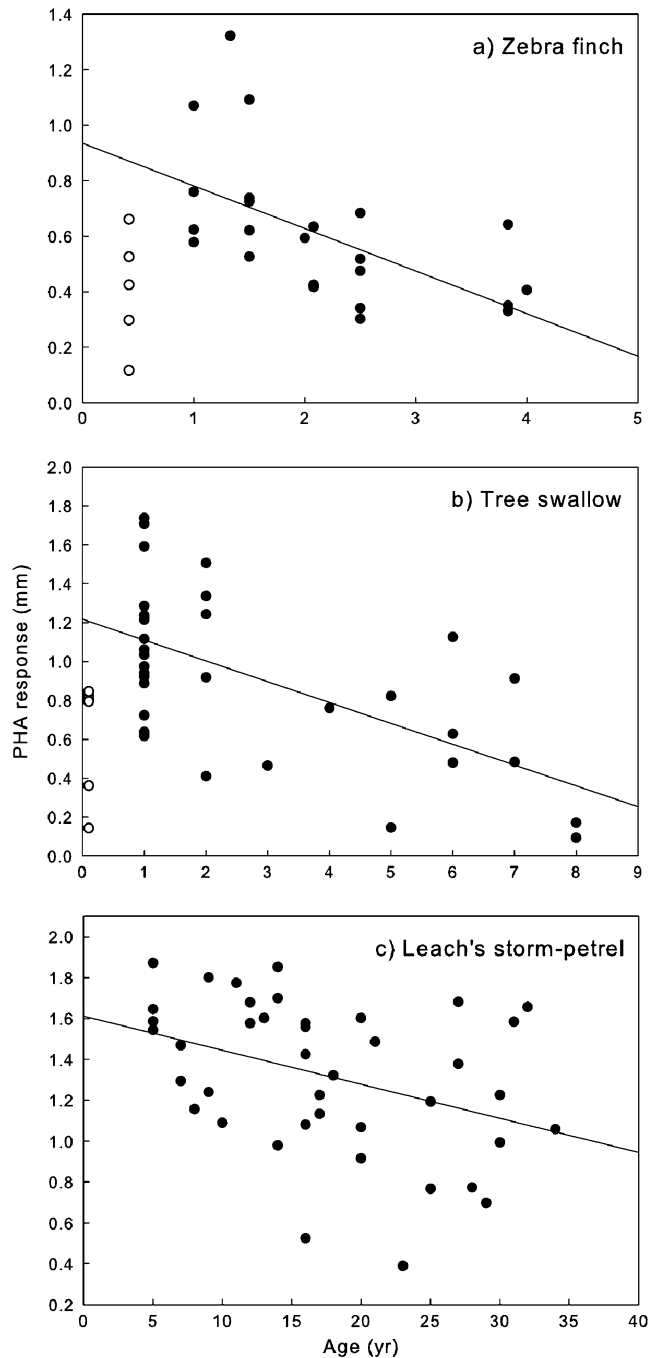
#### Data analysis

We used least-squares regression to examine the effects of age on PHA response for each species. To examine potential confounding effects of mass, sex and time of day of injection on PHA response, we included all variables in a multiple regression model for each species. Sex and time are fixed effects, and mass and age are random effects. Pearson correlations were used to determine relationships between the rate of immunosenescence and survival.

## Results

Phytohaemagglutinin response did not vary with mass, sex, or time of PHA injection in any of these species ( $P > 0.5$ ). PHA response decreased with age (Fig. 1) in adult zebra finches ( $F_{1,24} = 12.28$ ,  $P = 0.002$ ,  $r^2 = 0.35$ ), tree swallows ( $F_{1,30} = 15.25$ ,  $P = 0.001$ ,  $r^2 = 0.35$ ), and Leach's storm petrels ( $F_{1,40} = 6.65$ ,  $P = 0.014$ ,  $r^2 = 0.15$ ). The PHA response of the immature birds was lower than that of the youngest breeding adults in both finches and swallows (finches,  $t_{14} = -3.19$ ,  $P = 0.01$ ; swallows,  $t_{22} = -3.29$ ,  $P = 0.005$ ; Fig. 1). Consequently, we excluded the data from the immature birds when we calculated the rate of immunosenescence, defined as the proportional decrease in PHA response with age, which was calculated from the equation for the regression line.

The rate of immunosenescence differed between species ( $F_{2,94} = 34.79$ ,  $P = 0.0001$ ). The zebra finch had the greatest rate of immunosenescence (16% loss in PHA response per year), followed by the tree swallow (9%



**Fig. 1** Phytohaemagglutinin (PHA) response as a function of age in three species of birds. The lines are the least-squares regressions through the data in **a** zebra finches [slope =  $-0.153 \pm 0.04$  (SE)]; **b** tree swallows [slope =  $-0.107 \pm 0.03$  (SE)]; and **c** Leach's storm-petrels [slope =  $-0.016 \pm 0.01$  (SE)], for breeding adults (filled circle) and immature birds (open circle). Data from immature birds were not included in the regression analysis

loss in PHA response per year) and Leach's storm-petrel (1% loss in PHA response per year), respectively. Among species, there was a negative correlation between rate of immunosenescence and adult survival ( $P = 0.05$ ,  $r^2 = -0.99$ ).

## Discussion

Zebra finches, tree swallows and Leach's storm-petrels all exhibited a decline in PHA-specific, cell-mediated immunity with age. Immunosenescence in birds has been studied primarily in the species of agricultural importance (Barua and Yoshimura 1999; Parmentier et al. 2004), but recently two papers reported evidence of immunosenescence in the bird populations. In captive ruffs (*Philomachus pugnax*), cell-mediated immunity, as measured by the PHA response, differed between two age-classes, with birds between three years and 10 years of age having a higher PHA response than birds over ten years of age (Lozano and Lank 2003). In collared flycatchers (*Ficedula albicollis*), humoral immune response to sheep red blood cells also decreased over three age-classes (Cichon et al. 2003). To our knowledge, ours is the first study to investigate whether cell-mediated immunity decreases with age in one captive and two free-living bird populations using known-age individuals over most of their life span.

Mounting an immune response is costly, and although the source of the direct costs are not always clear, many studies have shown that mounting an immune response results in decreased food intake, increased energetic expenditure, and a negative nitrogen balance (Lochmiller and Deerenberg 1999; Bonneaud et al. 2003). Individuals from a number of species that mount an immune response to vaccination or sepsis can have a 10–60% increase in resting metabolic rate compared to control individuals (Lochmiller and Deerenberg 1999). We found no effect of body mass on PHA response within species, possibly because we did not have enough variation in body mass to distinguish an effect. Others have reported a positive relationship between body mass and PHA response (Saino et al. 1997; Sorci et al. 1997; Merino et al. 2000; Tella et al. 2000; Tella et al. 2002; Garamszegi et al. 2003; Navarro et al. 2003; but see, Moreno et al. 1999), suggesting that an energetically costly immune response is more likely to be mounted when energy stores are greater (Lochmiller and Deerenberg 1999; Bonneaud et al. 2003). Lijfeld et al. (2002) showed that tree swallows that mount a higher PHA response lose more body mass during the challenge than birds that mount a smaller response, and they suggested that birds of low body mass may be incapable of mounting a normal immune response.

Unlike the free-living tree swallows and Leach's storm-petrels, the zebra finches used in this study were from a captive population that had access to ad libitum food and were not exposed to many of the hazards found in a natural setting. Despite this, we still found immunosenescence in this population. Other captive populations of birds, such as the domestic chicken (*Gallus domesticus*) also show immunosenescence (Barua and Yoshimura 1999; Parmentier et al. 2004). Zebra finches and chickens have both been selected for high reproductive output, either naturally or artificially, and

a trade-off with long-term self-maintenance is expected. This suggests that even when early maturing species with high fecundity are raised in an environment with ad libitum resources, low disease incidence and no threat of predation, allocation of resources to immune defense is not sufficiently plastic to overcome genetic constraints associated with their life history, and immunosenescence persists.

Phytohaemagglutinin response differed between sexes in sexually size-dimorphic ruffs (Lozano and Lank 2003) and Magellanic penguins (*Spheniscus magellanicus*) (Moreno et al. 2001a), with larger males mounting a higher PHA response, but there was no sex difference in PHA response in nonsize dimorphic pied flycatchers (Moreno et al. 2001b) or tree swallows (Lifjeld et al. 2002). We found no sex difference in tree swallows or Leach's storm-petrels, neither of which show size dimorphism. The PHA response has also been shown to vary seasonally. The PHA response is stronger outside the breeding season in ruffs, possibly because birds at this time are released from energetically expensive reproduction (Lozano and Lank 2003). Sorci et al. (1997) found that PHA response negatively correlated with the day of year in magpie nestlings (*Pica pica*), which they suggested, may reflect reduced food availability and low nestling body mass late in the season. Ambient temperature and food abundance can also affect immune response. Parent tree swallows tested during cold weather and at low food abundance have a suppressed PHA response compared to birds treated during more favorable conditions (Lifjeld et al. 2002). PHA response can vary diurnally; in house sparrow (*Passer domesticus*), PHA response is consistently stronger at night than during the daytime (Navarro et al. 2003). In each species we did not test for seasonal variation in PHA response, because our tests were completed in less than a week. We confined PHA injections to between 0800 hours and 1,200 hours and did not find any effect of injection time on PHA response.

Nestling tree swallows and young zebra finches studied here had approximately half the PHA response of young adults, and this could reflect an immature immune system in these birds. Whether this reduced PHA response results from a trade-off to favor growth cannot be determined here; future studies should assess immunity as a function of growth rate in developing birds. Cell-mediated and humoral immunity are lower in neonatal, domestic turkeys compared to adults (Moore and Siopes 2002) and genetic selection for fast-growing poultry strains reduces the bird's immune potential (Van der zijpp 1983). Nestling magpies provided with methionine, an amino acid that enhances T-cell immune response, have lower growth rates, but larger PHA responses than control chicks, also suggesting that a trade-off between growth and immune function can occur in immature animals (Soler et al. 2003). Other studies have reported that immature birds have either lower PHA responses (Lozano and Lank 2003; Soler



et al. 1999; Tella et al. 2002) or higher PHA responses (Møller et al. 2001; Tella et al. 2002) than adults. Whether these differences are due to differences in measurement technique or due to population and species differences is not yet known.

Ours was a cross-sectional study and therefore we cannot say with certainty that the oldest individuals were a random sample of the population. If immunocompetence has survival value, then we predict that on average only the most immunocompetent individuals will live to the oldest ages. If this is the case, the correlation between immune response and age in the population as a whole may be even steeper. To our knowledge, the necessary longitudinal studies have been done only in humans, and do show that individual variation in rate of immunosenescence is associated with longevity, morbidity and mortality (Pawelec et al. 1997).

Organisms with different life-history strategies may vary in the rate at which immunosenescence progresses. Species with low extrinsic mortality and long lifespans should have high investment in maintenance to enhance longevity (Kirkwood 1990). Increased allocation to maintenance, however, usually translates into fewer resources allocated to reproductive effort. One recent study manipulated brood size in tree swallows to increase reproductive effort and demonstrated reduced immune function and survival (Ardia et al. 2003), evidence for a reproduction/maintenance trade-off.

In our study, we found that the rate of immunosenescence is negatively correlated with adult survival, such that long-lived bird species lose immune function at a slower rate than the short-lived bird species. We have studied immunosenescence in only three species to date, and while this avoids the limitations of a two-species comparative study (Garland and Adolph 1994), these results highlight the need for a phylogenetically controlled study with more species. If a larger study with many species demonstrates the same results, it would accord with optimal resource allocation theory and suggest that selection may act on investment in immune function to influence maximum life span (Bernstein and Bernstein 1991; Lithgow and Kirkwood 1996). Over evolutionary time, increased growth rate or breeding effort may translate into a quicker onset or higher rate of immunosenescence.

Immune function is a multifaceted system that cannot be completely assessed using only a single measure (Norris and Evans 2000; Blount et al. 2003). The PHA response provides information primarily about cell-mediated immunity (Roitt et al. 1998), but future ecological studies of immunosenescence should incorporate multiple measures of immune function in one individual. While investigation of immunosenescence in all three branches of the immune system would be informative, the PHA response is one of the most widely used measures of immunocompetence in field biology, and a decline in PHA response with age is noteworthy. Field biologists who wish to interpret PHA response relative

to sex, condition, breeding stage, etc., should account for the potentially confounding factors of age whenever possible.

**Acknowledgements** We thank Amber Cribbs, Alexis Blackmer and Nathaniel Wheelwright for assistance in the field, and Corey Freeman-Gallant for other assistance. This work was supported in part by a Cooper Ornithological Society, Joseph Grinnell Student Research Award for Basic Biological Avian Research to MFH, NIH Grant, RO3 AG022207 to Carol M. Vleck, and NSF grant, IBN-013437 to David W. Winkler. This manuscript represents contribution no. 165 from the Bowdoin Scientific Station. All procedures described were approved by the Iowa State University Committee on Animal Care (COAC, 5-04-5653-Q) and are in compliance with the United States of America's laws and policies governing the care and use of live vertebrate animals in research.

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