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Consequences of immune system aging in nature: a study of immunosenescence costs in free-living Tree Swallows

Maria G. Palacios,[1,5] David W. Winkler,[2] Kirk C. Klasing,[3] Dennis Hasselquist,[4] and Carol M. Vleck[1]

[1]*Department of Ecology, Evolution, and Organismal Biology, Iowa State University, Ames, Iowa 50011 USA*
[2]*Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, New York 14853 USA*
[3]*Department of Animal Science, University of California, Davis, California 95616 USA*
[4]*Department of Biology, Lund University, Ecology Building, 223 62 Lund, Sweden*

Abstract. Immunosenescence, the aging of the immune system, is well documented in humans and laboratory models and is known to increase infection risk, morbidity, and mortality among the old. Immunosenescence patterns have recently been unveiled in various free-living populations, but their consequences in the wild have not been explored. We investigated the consequences of immunosenescence in free-living Tree Swallows *Tachycineta bicolor* through a field experiment simulating a bacterial infection (challenge with lipopolysaccharide, LPS) in females of different ages during the nestling rearing period. We assessed behavioral and physiological responses of females, as well as growth and quality of their offspring, to determine the costs associated with the simulated infection. Results of the experiment differed between the two years of study. In the first year, old females challenged with LPS lost more body mass and reduced their nest visitation rates more, and their offspring tended to grow slower compared to similarly challenged younger females. In contrast, in the second year, old females did not appear to suffer larger costs than younger ones. Interestingly, immunosenescence was only detected during the first year of the study, suggesting that it is the dysregulated immune function characteristic of immunosenescent individuals rather than age per se that can lead to higher costs of immune defense in old individuals. These results provide the first evidence of costs of immunosenescence in free-living animals and support the hypothesis that old, immunosenescent individuals pay higher costs than younger ones when faced with a challenge to their immune system. Our results also suggest that these costs are mediated by an exaggerated sickness behavior, as seen in laboratory models, and can be modulated by ecological factors such as weather conditions and food availability.

Key words: acute phase response; bacterial killing; disease; ecological immunology; immunocompetence; lipopolysaccharide; lysozyme; senescence; sickness behavior.

Introduction

Aging, or senescence, is a pervasive phenomenon in the natural world. Individuals in a wide variety of free-living animal populations, including both vertebrate and invertebrate taxa, show reductions in survival and/or reproductive success with advancing age (e.g., Promislow 1991, Holmes et al. 2001, Bonduriansky and Brassil 2002, Bryant and Reznick 2004, Morbey et al. 2005). Several physiological mechanisms likely interact to cause this irreversible age-related deterioration in individual performance (Vleck et al. 2007), but for the most part the main mechanisms remain undetermined in wild animal populations. Immunosenescence, i.e., the deterioration or dysregulation of immune function with advancing age (Miller 1996, Grubeck-Loebenstein and Wick 2002, Shanley et al. 2009), is an obvious candidate. The immune system involves critical survival-related mechanisms that have evolved to overcome the negative impacts of parasites and disease; therefore, deterioration in its function is expected to have important fitness costs. Indeed, in humans, immunosenescence results in increased risk of infectious diseases and cancer (Miller 1996, Effros 2003) that leads to higher morbidity and mortality among the elderly (Pawelec et al. 2002).

Although current knowledge of immunosenescence comes almost exclusively from studies in humans and laboratory or domesticated animals (mostly mammals), several recent studies in free-living vertebrates have documented patterns of immunosenescence in the wild (Table 1), suggesting that the aging of the immune system is a widespread phenomenon in natural populations and warrants further study. These studies highlight several points: (1) The study of immunosenescence in the wild has so far been dominated by studies in birds—and to a lesser degree, reptiles—contrary to the pattern for laboratory and domesticated animals. Thus, a broader taxonomic approach including fish, amphibians, and mammals would be important for determining the extent and generality of immunosenescence in wild vertebrates.

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[5] E-mail: mgp@iastate.edu

TABLE 1. Studies assessing senescence of immune defense components in wild animals.

Immune component, technique used, and species	Evidence of senescence?	Reference
Acquired cell-mediated immune component (T-cell function)		
Tested by in vivo skin swelling in response to PHA		
Ruff (*Philomachus pugnax*)†	yes	1
Storm Petrel (*Oceanodroma leucorhoa*)	yes	2
Zebra Finch (*Taeniopygia guttata*)†	yes	2
Tree Swallow (*Tachycineta bicolor*)	yes	2
Tested by in vitro T-cell proliferation in response to PHA and ConA		
Tree Swallow	yes	3
Acquired humoral immune component (B-cell function)		
Tested by in vivo antibody production in response to antigenic challenge (NDV, SRBC, KLH, SRBC, respectively)		
Barn Swallow (*Hirundo rustica*)	yes	4
Collared Flycatcher (*Ficedula albicollis*)	yes	5
Water python (*Liasis fuscus*)‡	yes	6
Tree Swallow	no	3
Tested by in vitro B-cell proliferation in response to LPS		
Tree Swallow	no	3
Tested by total IgG (= IgY) level in plasma		
Common Tern (*Sterna hirundo*)	no	7
Innate humoral immune component		
Tested by natural antibody level in plasma		
Barn Swallow	yes	8
Tree Swallow	no	3
Garter snake (*Thamnophis elegans*)	possible	9
Tested by complement-mediated lysis		
Barn Swallow	no	8
Tree Swallow	no	3
Garter snake	possible	9

Notes: Measurements are from free-living individuals unless specified otherwise. Abbreviations: PHA, phytohemagglutinin; ConA, concanavalin A; LPS, lipopolysaccharide; NDV, Newcastle disease virus; SRBC, sheep red blood cells; KLH, keyhole limpet hemocyanin. References: 1, Lozano and Lank (2003); 2, Haussmann et al. (2005); 3, Palacios et al. (2007); 4, Saino et al. (2003); 5, Cichon et al. (2003); 6, Ujvari and Madsen (2006); 7, Apanius and Nisbet (2003); 8, Moller and Haussy (2007); 9, Sparkman and Palacios (2009).

† Captive colony.

‡ Animals brought into captivity before measurement.

(2) Most studies to date have focused on a single immune defense component, either acquired cell-mediated, acquired humoral, or innate humoral immunity. More integrative studies that incorporate various aspects of immune defense (e.g., Palacios et al. 2007) would help us understand the patterns of immune system aging, including whether decline in one component might be somewhat compensated by increase in a different one. (3) Declines in each of the major immune defense components have been documented in at least one wild population to date, while an increase with age has never been reported. (4) Immune function involving T-lymphocytes seems to be the most pervasively affected by age in free-living vertebrates, which is consistent with patterns of immunosenescence in humans and laboratory models (Pawelec et al. 2002), while acquired and innate humoral components show signs of senescence in some but not all free-living species studied to date. (5) Based on the study in Tree Swallows (Palacios et al. 2007), it appears that not all immune defense components are equally affected by age in a given species. While some components seem to decline, others seem to be well preserved into old age. More studies would be important to determine the generality of this pattern in wild animals. (6) Even from the few studies performed to date, it is evident that patterns of immunosenescence vary across species, even closely related ones (e.g., compare Tree Swallows with Barn Swallows in Table 1). Thus, further description of immunosenescence patterns in diverse taxa will be important for documenting the extent and elucidating the causes of these disparate patterns. (7) Finally, despite the pervasiveness of immune system aging documented so far, nothing is known at present about the consequences of immunosenescence for animals in nature. Free-living animals are subjected to harsh weather, food scarcity, and other environmental stresses that can impact immune function (e.g., Lifjeld et al. 2002). These factors could potentially interact and exacerbate any age-related effects on immune function, so that the costs of immunosenescence in the wild can be expected to be high and likely dependent on environmental conditions. Understanding the nature of these costs is not only important for increasing our knowledge about immunosenescence and aging in vertebrates, but can also have important implications for wild populations, particularly in the face of increased incidence of emerging wildlife diseases such as West Nile virus disease, bat white-nose

syndrome, and amphibian chytridiomycosis, to name a few.

To start to elucidate the consequences of immunosenescence in wild animals it is important to consider how the immune system responds to infection and how a dysregulation of these responses with advancing age might affect the chances of successful reproduction and/or survival of individuals. Upon infection by a pathogen, the immune system of the host becomes activated, resulting in local inflammation and a suite of highly organized behavioral, hormonal, and immunological changes collectively known as the acute phase response (Dantzer 2001). The acute phase response is characterized by fever, secretion of antimicrobial proteins (e.g., lysozyme produced by macrophages and acute phase proteins by the liver), elevation of stress hormone levels, and highly stereotyped sickness behaviors (Hart 1988, Dantzer 2001). These sickness behaviors include loss of appetite and thirst that lead to loss of body weight, increased somnolence and lethargy, reduced locomotor activity, and depressed social behaviors (Hart 1988, Owen-Ashley and Wingfield 2007). Despite the benefits of the acute phase response in improving the chance of successfully coping with an infection, this response does not come without costs (Hart 1988, Adelman and Martin 2009). Fever and hepatic production of acute phase proteins are metabolically costly processes that might use up energy reserves, especially if individuals are also reducing their food intake due to sickness behavior (Hart 1988, Lochmiller and Deerenberg 2000). In addition to these energetic costs, which could potentially reduce the chances of individuals survival during energetically demanding periods (e.g., migration, harsh weather), sickness behavior can also interfere with reproductive behaviors, as has been demonstrated both in captive and in free-living animals (reviewed by Adelman and Martin 2009).

Recent studies in laboratory rodents suggest that the costs of immune defense and sickness behavior are age dependent, with costs being disproportionately large for old compared to young individuals. For example, healthy old mice challenged with lipopolysaccharide (LPS, a non-replicating component of gram-negative bacterial cell walls commonly used to elicit sickness behavior without causing actual disease) suffer greater reductions in food consumption leading to larger body mass loss and more pronounced depression of locomotor and social behaviors compared to younger mice (Godbout et al. 2005, Huang et al. 2008). This exaggerated and prolonged sickness behavior in old individuals is thought to be linked to their immunosenescent phenotype, specifically, dysregulated inflammation (Godbout et al. 2005, Gaykema et al. 2007). Although the fitness consequences of the age-related differences in response to infection are not understood at present, these laboratory studies suggest that immunosenescence can have important impacts on sickness behavior, even in mild environmental conditions such as ad libitum access to food, thermoneutrality, minimal activity, and freedom from predators. Thus, they provide a solid basis for beginning to understand the implications of immunosenescence for animals in the wild.

In the present study, we investigate the consequences of immunosenescence in free-living animals, with particular focus on the effects of age on the acute phase response to infection and sickness behavior, using Tree Swallows as our model system (see Plate 1). Tree Swallows are small (~20 g) passerine birds in the family Hirundinidae and are widespread in North America (Robertson et al. 1992). Although most individuals live only two to three years in the wild, the maximum reported lifespan for the species is 12 years (Robertson and Rendell 2001). Tree Swallows constitute an ideal system to study the costs of immunosenescence because we have already demonstrated that individuals in our study population show immunosenescence in the in vivo skin swelling (Haussmann et al. 2005) and the in vitro lymphocyte proliferation (Palacios et al. 2007) responses to phytohemagglutinin (PHA, a common mitogen that stimulates T-lymphocytes), with immune function decreasing steadily with age in adults ranging from 1 to 8 years of age.

We performed a field experiment simulating a bacterial infection (challenge with LPS) in adult females during their nesting season to test the hypothesis that older, immunosenescent individuals pay higher fitness costs of immune defense than younger individuals. We predicted that when challenged with LPS, older, immunosenescent adult females may show any or all of the following responses compared to younger ones: (1) exaggerated sickness behavior, reflected by a larger loss of body mass and decrease in locomotor and parental behaviors; (2) greater reduction in offspring quality, in terms of reduced growth, immune function, and/or larger increase in the stress hormone corticosterone, if females cannot maintain feeding rates to offspring (and their male partners cannot compensate by increasing their contribution); and (3) a greater reduction in fitness in terms of current reproductive success and/or survival until the following breeding season. In addition, we measured corticosterone level in females, which is known to increase during an acute phase response and can be an important modulator of sickness behavior (Goujon et al. 1995, Wingfield 2003), and we investigated the effects of age on the immunological response to LPS challenge by measuring changes in several aspects of immune function that are known to be important for fighting bacterial infections: bacterial killing capacity of plasma, plasma activities of the antimicrobial proteins lysozyme, natural antibodies and complement, and specific antibody response against LPS. Finally, we measured the in vivo skin swelling response to PHA to corroborate the immunosenescence pattern previously documented in our study population (Haussmann et al. 2005).

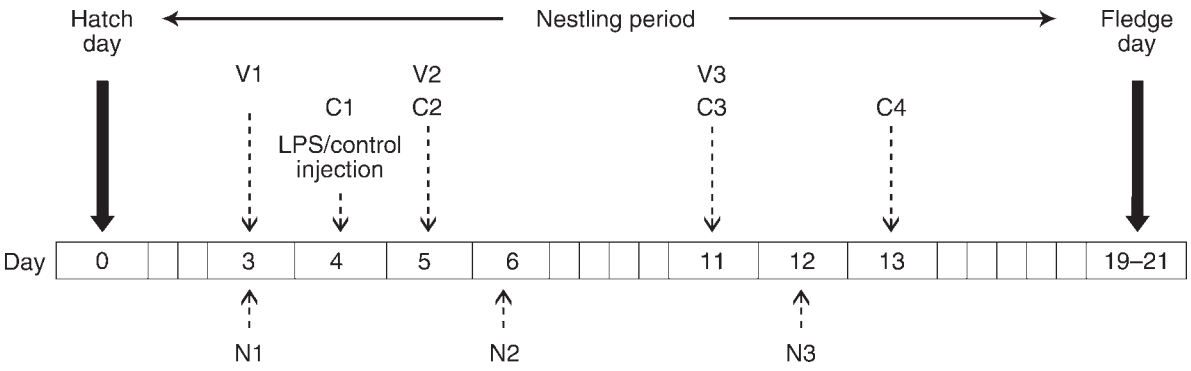

FIG. 1. Diagram of the experimental timeline during the nestling period in Tree Swallows. Key to abbreviations: V, video recording of nest visitation by parents; C, capture and sampling of females; N, measurement of nestlings. See *Methods* for details.

METHODS

Field experiment

We conducted the field experiment during the breeding seasons (May–June) of 2007 and 2008 in a Tree Swallow nest-box population in Tompkins County, New York, USA (42°29′ N, 76°27′ W) that has been the subject of long-term study since 1985 (Winkler and Allen 1996, Winkler et al. 2004). We used a factorial experimental design with one factor being adult female age (young, 1 year; mid-age, 2 and 3 years; old, 4 years and older), following the age categories used by Robertson and Rendell (2001). Only individuals that could be unambiguously classified into these age categories based on their banding history were included in the experiment. The other main factor was experimental treatment (LPS injection vs. control injection). Nests of young, mid-age, and old females were matched by hatching date and brood size (three to seven nestlings) and then randomly assigned within maternal age categories to the experimental treatment. Sample sizes for young, mid-age, and old females differed between 2007 and 2008 because of availability of birds of a given age group and needs of other researchers working in the same population. Birds sampled in 2007 were not reused in 2008. We collected data for each female before (pre-treatment) and twice after the experimental injections (post-treatment) to determine the behavioral and physiological changes caused by LPS challenge. All measurements in this study were made in accordance with standard animal care protocols approved by the Institutional Animal Care and Use Committees at Iowa State University and Cornell University (protocol #2001-0051) and permits from the U.S. Fish and Wildlife Service and New York State Department of Environmental Conservation.

Experimental timeline

The experiment was started for each female on day 3 of the nestling period (hatch day = day 0, Fig. 1). We videotaped nests to determine initial nest visitation rates by parents and we measured all nestlings for initial growth parameters. The next day (day 4), we trapped females in their nest boxes (capture 1), collected a sterile blood sample (~120 μL) within 3–5 minutes of capture, and prepared a thin blood smear. Next we weighed females to the nearest 0.01 g with a digital scale, measured the head–bill length (structural size) to the nearest 0.1 mm using calipers, and then performed the experimental injections (see next section). After the injections, females were released near their nest boxes to continue caring for their young. The following morning, approximately 24 h after the injections, nests were videotaped again, and immediately after, females were captured for a second time (capture 2), bled (~100 μL), weighed, and then released. We measured nestlings for a second time when they were 6 days old (i.e., ~ 48 h after their mothers received the injections). One week after the injections, nests were videotaped for a third time and females were captured (capture 3) to obtain a third set of body masses, blood samples, and blood smears to determine whether the effects of the experimental treatments persisted after the sickness behavior (which lasts ~48 h, see *Experimental injections*) had subsided. At this time, we also challenged females with PHA to corroborate the immunosenescence pattern, necessitating a fourth capture after the PHA challenge to measure the swelling response. This fourth capture was performed 48 h after the PHA injection given that previous work in our study population indicated that the swelling measured after 48 h was no different from that measured after 24 h (Ardia 2005). Nestlings were measured a third time when they were 12 days old, and at this time we also collected a blood sample from one randomly chosen nestling from each nest to assess bacterial killing capacity and stress hormone level (the latter only in 2008). All blood samples were maintained on ice in the field, centrifuged within 3 hours of collection, and plasma was stored at −80°C until analyzed.

Experimental injections

LPS birds received a single subcutaneous injection of 50 μL of an LPS water–oil emulsion in the dorsal apterium located immediately anterior to the wing. The

LPS water–oil emulsion consisted of LPS from *Escherichia coli* (serotype 055:B5; Sigma, St. Louis, Missouri, USA) dissolved in 0.9% sterile saline and emulsified in a 1:1 ratio with Freund's incomplete adjuvant. Use of adjuvant prolongs the expression of sickness symptoms to ~48 h, so that responses can be measured 24 h after the injections (e.g., Owen-Ashley and Wingfield 2006). Control birds received a similar injection but containing the vehicle (water–oil emulsion) only. We determined the experimental dose of LPS for Tree Swallows in a dose response pilot study that included LPS doses of 0.1, 0.5, and 1 mg/kg of body mass. The intermediate dose (0.5 mg of LPS per kg of body mass) was selected for the experiment because it led to significant body mass loss without causing nest abandonment (M. G. Palacios, *unpublished data*).

Response variables

Body mass and body condition.—We calculated body condition of females as the residuals of the regression of body mass against head–bill length. This index of size-corrected body mass was strongly positively correlated to body mass of females in all three captures in both years (all $r > 0.97$, all $P < 0.0001$) and gave virtually indistinguishable results to those of body mass when entered in statistical models. We therefore present only the results using body mass.

Nest visitation rates.—Nest visitation rates were determined by video recording parental activity at the nest for 60 minutes in the morning (between 07:00 and 12:00; Winkler 1991, Ardia 2005) and later viewing the tapes to quantify the number of visits per hour. During the nestling period, most parental visits to the nest are to provision food to nestlings; therefore, nest visitation rate provides a measure of parental care behavior in addition to being an index of locomotor activity. We report both female nest visitation rates (number of trips to the nest made by females in one hour) and total nest visitation rates (number of trips to the nest made by both parents in an hour). In 2007, females were not marked before the onset of the experiment and we were therefore unable to distinguish females from their mates in the pre-treatment recording (although we could in both post-treatment measures). In 2008 we captured all females 10–18 days before the onset of the experiment and marked them with wite-out (BIC USA, Inc., Shelton, Connecticut, USA) on the tail to clearly distinguish them from their mates in all video recordings. Because measures of pre-treatment nest visitation rate by females were only available in 2008, and they did not explain significant variation in post-treatment nest visitation rates (data not shown), we did not include initial nest visitation rate as a covariate in the statistical models.

Corticosterone levels.—We quantified corticosterone level in plasma from females (in both years) and nestlings (only in 2008) using a radioimmunoassay kit that has been validated for use in passerines (Washburn et al. 2002) and which we have previously used in Tree Swallows (Palacios et al. 2007). Because some blood samples (~20%) could not be collected within 3 minutes of capture, we included the time between capture and bleeding (i.e., handling time, range = 1–15 minutes, median = 3, $n = 222$) as a covariate in the statistical analyses of corticosterone levels.

Immunological parameters.—We determined immunological parameters in Tree Swallows using ecoimmunology assays developed for use in avian species and requiring small volumes of plasma (~10–20 μL). We assessed the capacity of plasma to kill *E. coli* following a previously described protocol (Matson et al. 2006). In this assay, the percentage of *E. coli* killed by plasma is calculated by comparing the number of bacterial colonies growing in agar plates after bacteria have been exposed to plasma to those exposed to a saline solution (controls). We quantified the levels of lysozyme in plasma by exposing *Micrococcus lysodeikticus* to plasma and comparing the resultant bacterial lysis to that from a standard curve generated using known concentrations of chicken lysozyme (Millet et al. 2007). The level of natural antibodies and the ability of complement to lyse erythrocytes were determined using a hemagglutination-hemolysis assay (Matson et al. 2005), which we have previously used in Tree Swallows (Palacios et al. 2007, 2009). In this assay, the level of natural antibodies in plasma is estimated as the ability of plasma to agglutinate rabbit red blood cells (RRBC) while complement activity is estimated as the ability of plasma to lyse those cells. We also determined the plasma levels of specific antibodies against LPS (i.e., anti-LPS antibodies) using an enzyme-linked immunosorbent assay (ELISA) following the protocol by Grindstaff et al. (2006) but using a plasma dilution of 1:200 (i.e., the optimal dilution for our Tree Swallow samples). This assay uses a rabbit-anti-Red-Winged-Blackbird-immunoglobulin serum that binds both IgM and IgG and that has been previously demonstrated to detect antibodies in Tree Swallows (Hasselquist et al. 2001). Antibody titers are reported as the slope of the substrate conversion over time (in $10^{-3} \times$ optical densities per minute), with a higher slope indicating a higher concentration of anti-LPS specific antibodies in a sample. Finally, the PHA skin test was performed following a protocol previously used in Tree Swallows (Ardia 2005). We report the skin swelling response to PHA as the difference between the average of three post- and three pre-injection wing web thickness measures (Palacios et al. 2009).

Nestling growth.—Nestlings were measured to assess growth when they were 3, 6, and 12 days old. By day 12, Tree Swallow nestlings have already reached adult body mass (McCarty and Winkler 1999). We weighed each nestling to the nearest 0.01 g using a digital scale and measured their head–bill length and flat wing length to the nearest 0.1 mm using calipers and a wing rule. The means for all nestlings in a given nest were used for further analyses. We calculated the growth between days 3 and 6 for each variable (early growth) and between

days 3 and 12 (total growth) as final measure minus initial measure. Because of the high correlation among all three nestling measures (change in mass, head–bill length, and wing length), we used a principal component analysis to extract the first principal component (PC1) for the three measures of early and total growth and used these new growth variables for statistical analyses. Early growth PC1 and total growth PC1 explained 78.9% and 79.4% of the variation in nestling growth, respectively.

Fitness-related parameters.—We monitored the nests of all females during the nestling period to determine their fledging success (an index of reproductive success), defined as the proportion of hatchlings that survived to fledge. We estimated survival of females to the next breeding season using return rates of females to the breeding grounds, which is an index to survival in this highly philopatric species (Winkler et al. 2004).

Weather and insect data.—Tree Swallows and their nestlings are highly dependent on the availability of flying insects (their main food source during the breeding season), which in turn is highly dependent on weather. We therefore evaluated these factors as covariates in the models to test their potential effects on female and nestling responses to the experiment. Availability of flying insects was estimated from samples collected daily during the breeding season by an aerial insect sampler located at our study site (McCarty and Winkler 1999). The number of insects in each sample was counted and used as an indicator of flying insect availability. Weather data (hourly and daily) were obtained from the Network for Environment and Weather Applications (NEWA) webpage for the weather station located at the Ithaca airport (Ithaca, Tompkins County, New York, USA), which is within 5 km of the study area. Five daily weather variables—maximum, average, and minimum temperature (°C), total rain (mm), and average wind speed (km/h) for each study year for the month of June—were used as input variables in a principal components analysis. PC1 represented temperature (with positive values indicating higher minimum, average, and maximum temperatures) and explained 59% of the variation, while PC2 represented wind and rain (with positive values indicating higher wind speed and total rain) and explained 21% of the variation. For models of female variables (except nest visitation rates; see *Data analyses*), we included the average daily weather and insect availability experienced by the female on the day she was measured and on the previous day. For models of nestling variables, we included the average daily weather and insect availability experienced by the nestlings on the day they were measured and on the two previous days. For models of nest visitation rates, PCAs were performed on hourly weather data (temperature, relative humidity, and wind speed) and PC1 explained 55% of the variation (positive values indicate higher temperature and wind speed, while negative values indicate higher relative humidity), whereas PC2 explained 30% of the variation (positive values indicate higher temperature, while negative values indicate higher wind speeds). We used the weather experienced by females at the time and day their nests were being videotaped and the insect availability for the day of video recording, given that hourly insect data were not available.

Data analyses

To test for the effects of experimental treatments on female and nestling responses we used general linear models (GLM) that included the main factors of our experimental design: treatment (LPS, control), female age (young, mid-age, old), treatment × female age interaction (to test for differential responses to treatment by females of different ages), and pre-treatment measurements (to control for initial values on female responses). Because the experiment was performed in two separate years, we also tested for year (2007, 2008) effects in our models. Body mass (or condition) can affect the intensity of an acute phase response in small birds (Owen-Ashley and Wingfield 2006); we therefore tested for the effects of female body mass on their behavioral and physiological responses. Several additional variables could in principle affect female and nestling responses to the experimental treatment under field conditions and were also evaluated as covariates in the models: date (when the experiment was started for each female), time of day (during which nests were videotaped, variables measured, or blood samples collected), brood size (number of nestlings in the nest), weather (first and second principal components), and insect availability. Handling time was evaluated as an additional covariate in the models for corticosterone levels. Because of the large number of potential predictor variables relatively to the sample size available for the models, we used Akaike's information criterion (Akaike 1973) corrected for small sample bias (AIC_c) to select the best model for statistical inference. We followed the methodology of Burnham and Anderson (2002) to evaluate whether the addition of any of the covariates (entered one at a time) improved the fit over that of the basic model, i.e., the model including only the main factors of our experimental design. This approach is based on the principle of parsimony and allowed selection of the models that explained the most information with the fewest parameters (Burnham and Anderson 2002). Except where indicated, the AICc approach did not result in the addition of covariates to the basic models. Residual plots were visually inspected for signs of non-normality, and variables were ln-transformed when necessary. Female survival and fledging success were analyzed using logistic regression models with binomial distribution and logit link functions. Sample sizes differ among analyses within a given year because not all data were available for each female. All statistical analyses were performed using SAS version 9.2 (SAS Institute 2008).

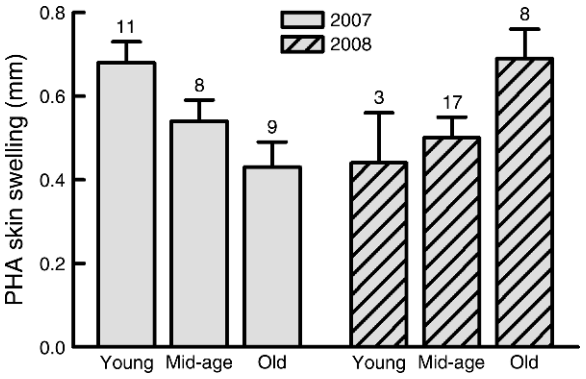

FIG. 2. Comparison of age-related variation in phytohemagglutinin (PHA) skin swelling response of female Tree Swallows between study years. The immunosenescence pattern is only observed in 2007. Graphs show means + SE, with sample sizes depicted above the error bars.

RESULTS

None of the variables measured in females (mass, head–bill length, immune parameters, stress-related parameters, clutch size, brood size) or their offspring (mass, head–bill length, wing length) differed significantly between control and LPS groups before the administration of the treatment injections in either year of the study (ANCOVA models for initial [pre-treatment] measurements, effect of treatment, all $P > 0.05$). Females sampled in 2007 showed the immunosenescent pattern in PHA response previously described in this population (age $F_{2,22} = 4.98$, $P = 0.017$; treatment $F_{1,22} = 0.07$, $P = 0.788$; treatment × age $F_{2,22} = 1.90$, $P = 0.174$). Surprisingly, however, the females sampled in 2008 did not show the immunosenescence pattern (age $F_{2,23} = 2.3$, $P = 0.123$; treatment $F_{1,23} = 1.44$, $P = 0.242$; treatment × age $F_{1,23} = 0.22$, $P = 0.646$). In fact, the PHA response declined with female age in 2007 as expected, whereas it actually tended to increase with age in 2008 (Fig. 2). This year effect provided a unique opportunity to test our hypothesis in years in which we would expect contrasting experimental outcomes given the presence (2007) or absence (2008) of immunosenescence in our study populations. Given this critical difference in the age-related immune pattern of females between years, and because statistical models showed a significant year effect (or interaction) for most response variables (Appendix A), we present separately the analyses and results of the experiment for each year of the study.

2007: Year showing immunosenescence

The effect of LPS challenge on body mass of females depended on female age (treatment × age $F_{2,34} = 3.71$, $P = 0.035$). As predicted, old females injected with LPS showed the lowest body mass 24 hours after the injection compared to their controls, while body mass of young females did not vary with treatment, and that of mid-age birds showed an intermediate response (Fig. 3A). Similarly, the effect of LPS challenge on nest visitation rate by females 24 h after the injection also depended on female age (treatment × age $F_{2,31} = 5.9$, $P = 0.007$). While young LPS females tended to visit the nest more frequently than controls, and mid-age LPS and control females did not differ in their behavior, old LPS females made significantly fewer trips to the nest compared to their controls (Fig. 3B). This same pattern was observed for total nest visitation rate (treatment × age $F_{2,32} = 6.56$, $P = 0.004$), i.e., both by male and female parents, indicating that males did not sufficiently compensate for their partners' responses.

Corticosterone level 24 h after the injections did not differ significantly between LPS and control females of any age group (age $F_{2,34} = 1.98$, $P = 0.15$; treatment $F_{1,34} = 0.71$, $P = 0.41$; treatment × age $F_{2,34} = 0.18$, $P = 0.84$), as was the case for natural antibodies against RRBC (age $F_{2,34} = 1.98$, $P = 0.15$; treatment $F_{1,34} = 0.71$, $P = 0.41$; treatment × age $F_{2,34} = 0.18$, $P = 0.84$) and complement-mediated lysis titers (age $F_{2,28} = 0.78$, $P = 0.47$; treatment $F_{1,28} = 2.18$, $P = 0.15$; treatment × age $F_{2,28} = 0.93$, $P = 0.41$). On the other hand, LPS challenge resulted in increased bacterial killing capacity (treatment $F_{1,31} = 6.28$, $P = 0.012$, Fig. 4A) and levels of lysozyme in plasma (treatment $F_{1,32} = 4.54$, $P = 0.041$, Fig. 4B) of females compared to their controls, but this effect was independent of female age (treatment × age: $P > 0.45$ in both cases).

The levels of specific antibodies against LPS, measured one week after the injections (capture 3), showed a significant interaction between treatment and female age (treatment × age $F_{2,30} = 5.76$, $P = 0.008$). While both young and mid-age LPS females showed increased levels of anti-LPS antibodies compared to their controls, old females did not show a response to the challenge (Fig. 4C). None of the remaining experimental effects described above were detectable one week after the injections, except for that of bacterial killing capacity, which remained elevated in LPS compared to control females irrespective of their age (Appendix B).

LPS challenge to females also affected their offspring. Offspring of LPS females showed a tendency to grow slower than those of control females between days 3 and 6 of the nestling stage (i.e., early growth, treatment $F_{1,25} = 3.67$, $P = 0.07$) once the effects of insects ($F_{1,25} = 25.03$, $P < 0.0001$) and weather (PC1, $F_{1,25} = 6.60$, $P < 0.017$) on early growth were taken into account. Although the treatment × age interaction was not significant in this model (treatment × age $F_{2,25} = 0.55$, $P = 0.58$), the effects on early growth appeared most pronounced for old females (Fig. 5A). These differences in growth were not detected when analyzing nestling growth between days 3 and 12 (i.e., total growth, treatment $F_{1,25} = 0.82$, $P = 0.37$; treatment × age $F_{2,25} = 0.52$, $P = 0.60$). However, offspring of LPS females tended to have lower bacterial killing capacity at 12 days of age compared to those of control females irrespective of female age (treatment $F_{1,28} = 4.19$, $P = 0.05$; treatment × age $F_{2,28} = 0.13$, $P =$

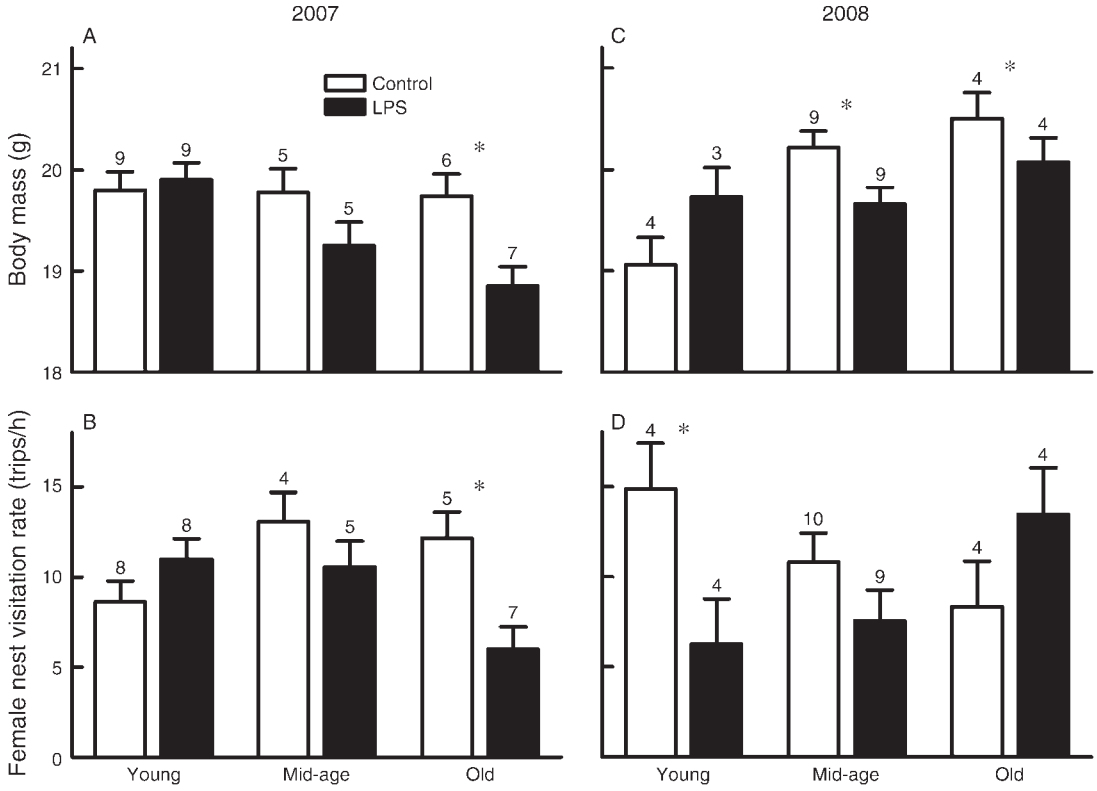

FIG. 3. Comparison of sickness behavior parameters of female Tree Swallows of different ages 24 hours after the experimental injections. Panels A and B show 2007 data, while panels C and D show 2008 data. (A, C) Female body mass; (B, D) female nest visitation rate. Data are least-square means and SE, with sample sizes depicted above the error bars. Asterisks indicate statistically significant differences ($P < 0.05$) between control and lipopolysaccharide (LPS) treatments within each age group.

0.88; Fig. 5B). No significant effects of LPS challenge were detected on female reproductive success or on female survival to the next breeding season (all effects $P > 0.12$, Table 2).

2008: Year not showing immunosenescence

The effect of LPS challenge on body mass of females also depended on female age in 2008 (treatment × age $F_{2,26} = 4.02$, $P = 0.03$), but the pattern differed from that seen in 2007. While body mass of young females tended to increase with LPS treatment, both mid-age and old females treated with LPS showed similar responses in 2008 (Fig. 3C). Interestingly, the effect of LPS challenge on nest visitation rate was actually reversed for young and old females from that observed the previous year (treatment × age $F_{2,29} = 3.77$, $P = 0.04$, Fig. 3D): in 2008, old LPS females tended to make more, and young LPS females made fewer visits to the nest compared to their controls. Total nest visitation rate did not vary significantly with treatment ($F_{1,29} = 0.13$, $P = 0.73$), age ($F_{2,29} = 1.11$, $P = 0.34$), or their interaction ($F_{1,29} = 1.73$, $P = 0.20$). Further exploration of these data showed that in 2008 six females were probably raising their broods alone (i.e., males did not feed nestlings during any of the three 60-minute video recordings), something that was not seen in 2007. Three of these females were young (two control, one LPS), two mid-age (one control, one LPS), and one old (LPS). We assessed the impact of these "single mothers" on the observed patterns by removing them from the analyses (Appendix C). While the difference in female nest visitation rate between old LPS females and their controls remained relatively unchanged, the difference between young LPS females and their controls was considerably reduced by the removal of "single mothers" (difference before removal = 8.6 trips/h, after removal = 3.3 trips/h), making the interaction term between treatment and age only marginally significant (Appendix C). In the case of total nest visitation rate, removal of the "single mothers" resulted in a marginally significant treatment × age interaction (Appendix C), with nests of old LPS females tending to have higher total nest visitation rate than all other groups (data not shown).

Contrary to the lack of pattern in 2007, corticosterone level of females showed a significant treatment × age interaction in 2008 ($F_{2,26} = 3.43$, $P = 0.05$). While LPS treatment tended to elevate corticosterone level in young (mean ± SE ln[corticosterone level] [measured in ng/mL], control = 1.16 ± 0.39, $n = 4$; treatment = 2.18 ± 0.45, $n = 3$) and mid-age (control = 0.72 ± 0.24, $n = 10$; treatment = 1.51 ± 0.27, $n = 8$) females, the opposite trend was observed in old females (control = 2.26 ±

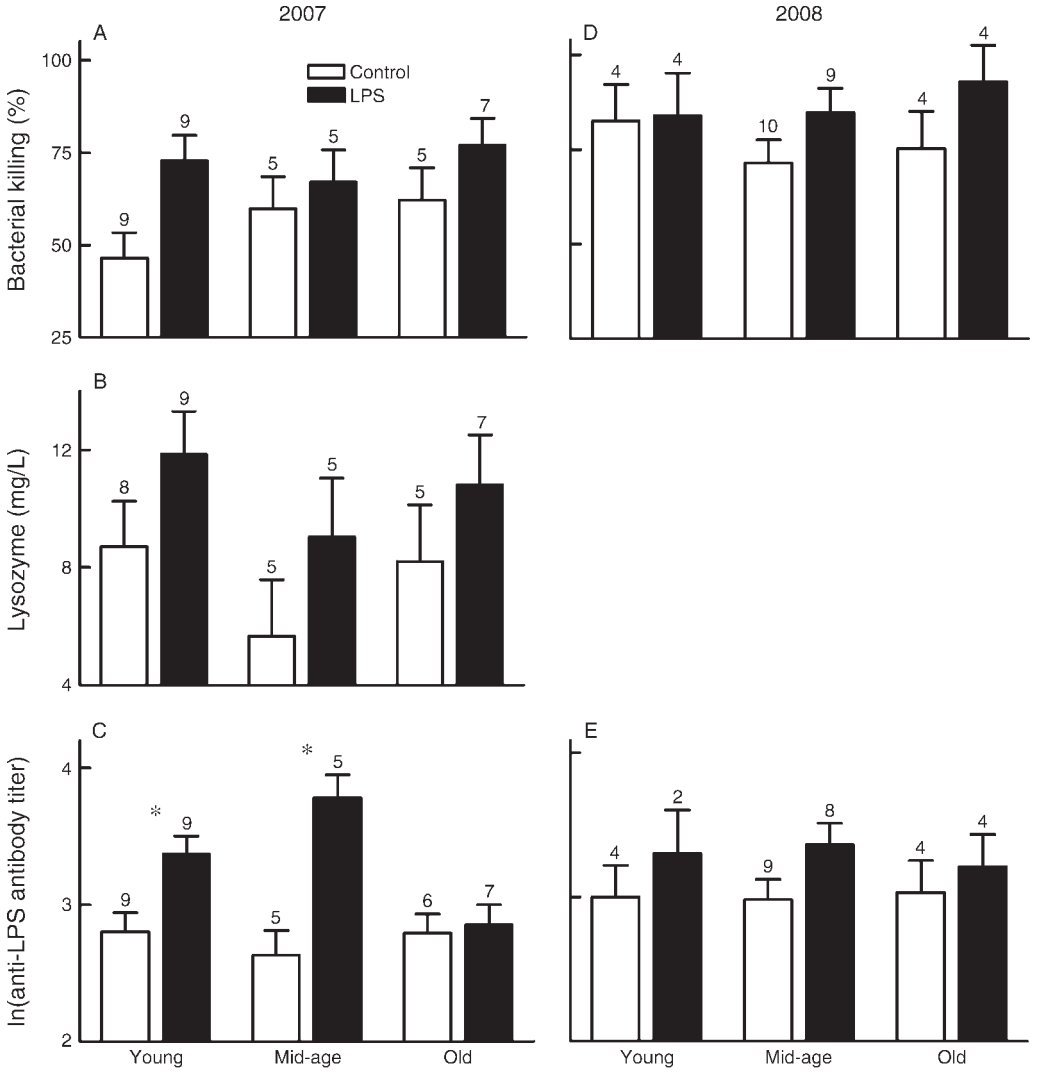

FIG. 4. Comparison of immune parameters of female Tree Swallows of different ages in response to the experimental injections. (A, D) Bacterial killing capacity of plasma (24 h post-injections); (B) lysozyme levels in plasma (24 h post-injections, measured only in 2007); (C, E) specific antibody response against LPS (one week post-injections). Antibody titers were measured as the slope of the substrate conversion over time (in $10^{-3} \times$ optical densities per minute), with a higher slope indicating a higher concentration of anti-LPS specific antibodies in a sample. Graph components are as in Fig. 3.

* $P < 0.05$.

0.39, $n = 4$; treatment = 1.57 ± 0.39, $n = 4$). This pattern, as well as those for all other female and nestling responses (except for nest visitation rates described above), was not changed by removal of the "single mothers" from the models (Appendix C). On the other hand, similarly to 2007, LPS challenge had no effect on the levels of natural antibodies against RRBC (age $F_{2,27} = 0.19$, $P = 0.83$; treatment $F_{1,27} = 0.10$, $P = 0.76$; treatment × age $F_{2,27} = 0.83$, $P = 0.45$) and complement-mediated lysis (age $F_{2,27} = 0.17$, $P = 0.85$; treatment $F_{1,34} = 0.73$, $P = 0.40$; treatment × age $F_{2,34} = 1.35$, $P = 0.28$) and while LPS females in 2007 had higher bacterial killing capacity than controls regardless of their age, this trend did not reach significance in 2008 (treatment $F_{1,34} = 2.12$, $P = 0.16$, Fig. 4D).

A difference with respect to the 2007 results was also observed in the levels of specific antibodies against LPS. In 2008, LPS injection did not elevate significantly the levels of specific antibodies over those of controls irrespective of female age (age $F_{2,24} = 0.04$, $P = 0.96$; treatment $F_{1,24} = 2.60$, $P = 0.12$; treatment × age $F_{2,24} = 0.14$, $P = 0.87$, Fig. 4E). One week after the injections, all experimental effects described here were no longer detectable (Appendix B).

Effects on offspring growth also differed between years (Fig. 5C). In 2008, offspring of LPS females tended to grow slower than those of control females between days 3 and 6 of the nestling stage irrespective of female age (treatment $F_{1,29} = 2.09$, $P = 0.16$; treatment × age $F_{2,29} = 0.14$, $P = 0.87$) once the effects of date on

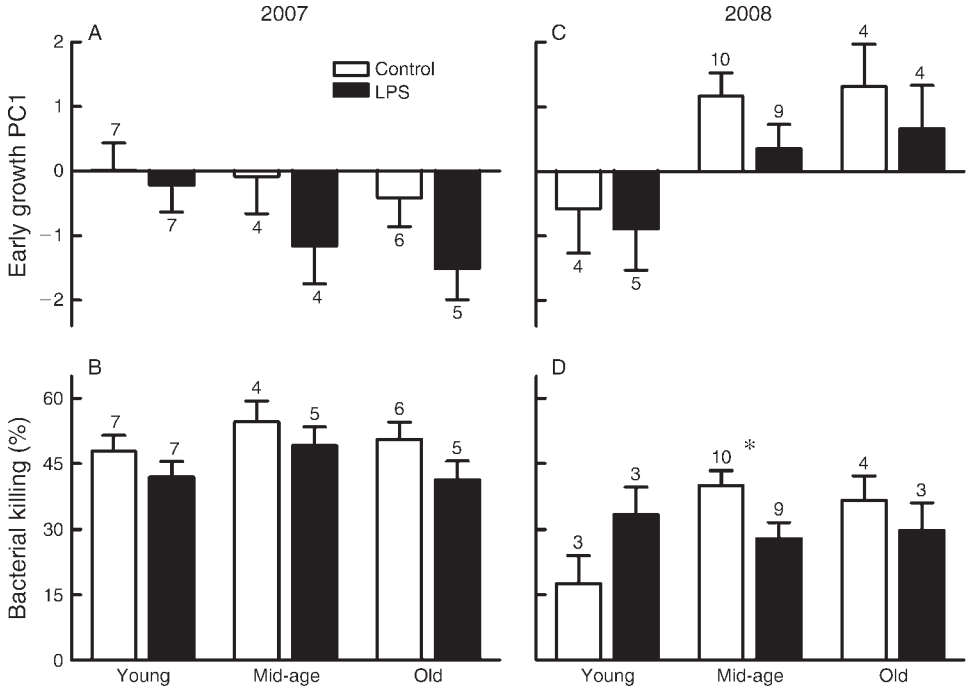

FIG. 5. Comparison of nestling parameters after their mothers received the experimental injections. (A, C) Early growth (growth between days 3 and 6 of the nestling period); (B, D) bacterial killing capacity of plasma on day 12 of the nestling period. Graph components are as in Fig. 3.

* $P < 0.05$.

growth were taken into account ($F_{1,29} = 4.17$, $P = 0.05$), but the trend was weaker than in 2007. In addition, offspring of young females in 2008 tended to grow slower than those of mid-age and old females (age $F_{2,29} = 2.81$, $P = 0.08$) irrespective of treatment. These effects on growth were stronger when considering total growth (i.e., between days 3 and 12) of the nestlings (age $F_{2,25} = 13.21$, $P < 0.001$; treatment $F_{1,25} = 4.45$, $P = 0.05$; treatment × age $F_{2,25} = 0.35$, $P = 0.41$). Bacterial killing capacity of nestlings showed a significant treatment × age interaction ($F_{2,26} = 3.70$, $P = 0.04$), with offspring of both mid-age and old LPS females showing lower levels than controls (as seen in 2007), but those of young LPS females having the opposite trend (Fig. 5D). Finally, corticosterone levels of offspring, which were available only in 2008, did not vary with maternal treatment ($F_{1,27} = 1.64$, $P = 0.21$), but tended to be lower for offspring of young females than for offspring of mid-age and old females ($F_{2,27} = 3.04$, $P = 0.07$, data not shown).

Fledging success in 2008 was not affected by LPS challenge (treatment $\chi^2 = 1.78$, $P = 0.18$, treatment × age $F_{2,25} = 1.94$, $P = 0.38$), as seen in 2007, but it was significantly lower for young females than for mid-age and old females (age $\chi^2 = 21.28$, $P < 0.001$, Table 2). Female survival to the next breeding season did not vary with treatment, age, or their interaction as seen in 2007 (all $P > 0.22$), but tended to be higher overall in 2008

TABLE 2. Values of fitness-related variables for each experimental treatment by female age combination in Tree Swallows in both study years.

	Sample size			Fledging success			Female survival		
Treatment	Young	Mid-age	Old	Young	Mid-age	Old	Young	Mid-age	Old
2007									
Control	9	5	7	0.60 ± 0.13	0.60 ± 0.24	0.69 ± 0.13	0.33	0.20	0.43
LPS	9	5	7	0.78 ± 0.11	0.93 ± 0.05	0.60 ± 0.16	0.44	0.40	0.43
2008									
Control	4	10	4	0.55 ± 0.26	0.88 ± 0.05	1.00 ± 0.00	0.75	0.60	0.75
LPS	5	9	4	0.10 ± 0.10	0.81 ± 0.13	0.92 ± 0.08	0.40	0.56	0.50

Notes: Depicted data are the sample sizes, which are common to both variables, and means ± SE (where applicable). Fledging success is (number of fledglings)/(number of hatchlings); female survival is (number of returning females the following breeding season)/(number total females).

than in 2007 (2007 mean ± SD, 0.37 ± 0.09; 2008, 0.59 ± 0.14; $\chi^2 = 2.8$, $P = 0.09$, Table 2).

Discussion

The costs of immunosenescence are well documented in humans and laboratory models, but are poorly understood in wild animals that must find food, thermoregulate, avoid predators, and reproduce in unpredictable environments. The results of our experimental study in 2007 provide the first evidence of the nature of such costs in a free-living animal. Old, immunosenescent Tree Swallows facing a simulated bacterial infection lost more body mass and reduced their nest visitation rate more than similarly challenged mid-age and young swallows, supporting our prediction that old, immunosenescent individuals would show exaggerated sickness behaviors, as observed in laboratory mice (Godbout et al. 2005, Huang et al. 2008). As a result, offspring of those old females tended to grow more slowly than those of similarly challenged mid-age and young females. Importantly, these higher costs to old females were not detected in 2008, when females in this year's study population did not show the age-related decline in cellular immune response to PHA, suggesting that it is indeed the immunosenescent phenotype that contributes to the higher costs among old individuals, and not old age *per se*. In the following sections, we first discuss our results for 2007, when immunosenescence was present, and then our results for 2008, when females did not show the immunosenescence pattern, presenting possible explanations for the discrepancy between years.

Consequences of immunosenescence in Tree Swallows

Immunosenescence and sickness behavior.—Loss of body mass, reduced locomotor activity, and depressed social and reproductive behaviors are hallmarks of the acute phase response to infection of vertebrates (Hart 1988, Dantzer 2001, Owen-Ashley and Wingfield 2007, Adelman and Martin 2009). Here we show that free-living Tree Swallows lose body mass and reduce their feeding rates to nestlings in response to a simulated bacterial infection (LPS challenge). Most importantly, however, we show that the magnitude of these sickness responses can be age dependent, as has been demonstrated in old laboratory mice (Godbout et al. 2005, Huang et al. 2008). The larger loss of body mass of old Tree Swallows compared to younger individuals is consistent with a stronger and/or more prolonged anorexia, adipsia, and lethargy (sickness behaviors) characteristic of immunosenescent individuals. In addition, the higher metabolic rate and impaired nutrient utilization characteristic of the acute-phase response (Dantzer 2001) might also have contributed to the patterns of body mass loss observed in Tree Swallows. Similarly, the larger reduction in nest visitation rate by old, immunosenescent Tree Swallows compared to younger birds is consistent with a greater suppression of locomotor activity and/or social behavior, similar to that demonstrated in old, immunosenescent laboratory mice (Godbout et al. 2005, Huang et al. 2008).

Several studies have shown that the behavioral aspects of the acute phase response to pathogens are plastic and their expression can be modulated depending on ecological context (e.g., Aubert et al. 1997, Bilbo et al. 2002, Owen-Ashley and Wingfield 2006, Weil et al. 2006). For example, female mice challenged with LPS fail to build a nest for their pups when housed at mild ambient temperatures, but their nest-building behavior is unaffected when they are housed at low temperatures that threaten offspring survival (Aubert et al. 1997). Although the ability of individuals to modulate sickness behaviors depending on the ecological context is likely adaptive (reviewed by Adelman and Martin 2009), this ability has limits. For instance, the severity of an infection can influence the ability of mice to modulate sickness behaviors, as suggested by studies using a range of doses of LPS (e.g., Weil et al. 2006). We suggest that age might also be an important factor limiting the modulation of sickness behaviors, with older, immunosenescent individuals being less able to modulate behavioral aspects of sickness than younger ones. This hypothesis warrants further testing.

Immunosenescence and immune responses to infection.—Another important component of the acute phase response to pathogens is the elevation of antimicrobial defenses that enhances the probability of overcoming the infection (Dantzer 2001). Increases in acute phase proteins, lysozyme levels, and bactericidal capacity in response to LPS challenge have all been documented in mammals and chickens (reviewed by Owen-Ashley and Wingfield 2007), but to our knowledge, our study is the first to show these antimicrobial responses in free-living animals. Tree Swallows challenged with LPS showed enhanced bacterial killing capacity and elevated levels of lysozyme in plasma. The effect of LPS on these innate immune components was, however, independent of female age, consistent with previous findings indicating that Tree Swallows in this population do not show age-related changes in innate immunity (Palacios et al. 2007). The latter result implies that older, immunosenescent Tree Swallows mounted similar innate immune responses to LPS challenge compared to those of mid-age and young individuals, but that they did so at the cost of larger body mass loss and greater depression of parental behavior; indicating these immune responses were, therefore, more costly to older, immunosenescent females than to young and mid-age ones.

On the other hand, while young and mid-age females mounted specific antibody responses to LPS, old females did not show such a response. This result is intriguing given that our previous study had found no evidence of immunosenescence in B-lymphocyte function in Tree Swallows (Palacios et al. 2007). Perhaps the higher cost of the acute phase response paid by these old, immunosenescent females left them without sufficient resources (e.g., energy, amino acids) to mount an

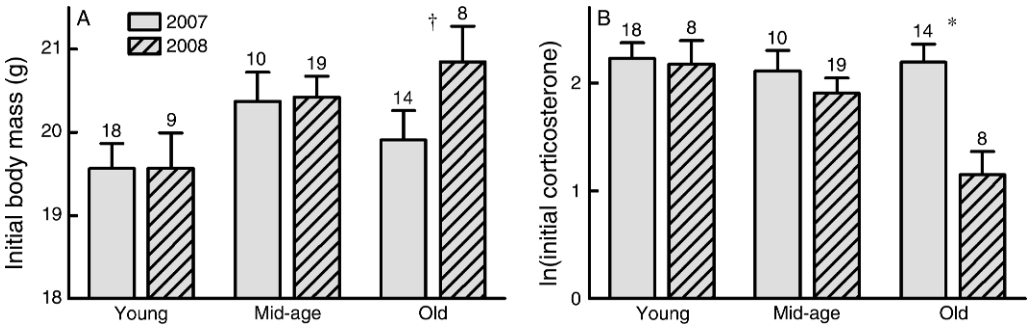

FIG. 6. Comparison of female Tree Swallow measurements before the onset of the experiment between study years. (A) Initial (pre-treatment) body mass, (B) initial (pre-treatment) corticosterone levels (measured as ng/mL). Data are least-square means and SE, and sample sizes are depicted at the tips of the error bars. Asterisks (*) show statistically significant ($P < 0.05$) between age groups, and daggers (†) indicate marginally significant ($0.1 < P < 0.05$) differences between age groups.

adaptive immune response to the challenge. Alternatively, LPS injection might have resulted in higher levels of pro-inflammatory cytokines (Th1 type) in older, immunosenescent females (Godbout et al. 2005), leading to their blunted specific antibody responses compared to younger ones, given that these cytokines typically down regulate Th 2 responses (Mosmann and Moore 1991).

Immunosenescence and fitness costs.—Despite the more extreme sickness responses in old LPS females, this did not translate into immediate fitness-related costs. One exception might be the effects on nestling growth, which tended to be more pronounced for the offspring of old females (Fig. 5A), although the trend was not statistically significant, likely due to the small sample size. On the other hand, there was no evidence of an effect of LPS on fledging success or survival of females. This dearth of immediate fitness costs in response to LPS challenge could be attributed to several factors. First is the moderate dose of LPS we used (0.5 mg/kg). Because we wanted to monitor sickness behavior and physiological responses of females, we selected a dose of LPS that would not cause nest abandonment (our pilot study showed nest abandonment by some females that received 1 mg/kg of LPS; M. G. Palacios, *unpublished data*). Had we used a higher dose for our experiment, we might have found that older females were more likely to abandon their broods than younger females, but this remains to be tested. Another factor might be that the negative effects of LPS challenge are of relatively short duration and presumably less costly than those of real pathogens. Real pathogens can replicate inside their host, and in addition to activating the immune system, they can also utilize host resources for nutrition and cause extensive tissue damage. Therefore, it is likely that a real infection would result in higher fitness costs to old, immunosenescent Tree Swallow females than did our simulated infection with a non-replicating antigen. Finally, our study was performed in breeding females because we wanted to document potential costs of immunosenescence in an ecologically relevant context. If individuals that did not manage to breed (likely those in worse condition or of lower quality) had been included in our study, the fitness costs would probably have been higher.

Differences between study years

Results in 2008 differed radically from those in 2007; old individuals did not suffer greater costs of immune defense than did young and mid-age individuals in 2008. This lack of higher costs to older individuals is what we would predict (based on our hypothesis of the costs of immunosenescence) in the absence of immunosenescence, which is the pattern that we observed in Tree Swallow females sampled in 2008. In contrast to the immunosenescence pattern described initially in this population in 2003 (Haussmann et al. 2005) and subsequently documented in 2005 (Palacios et al. 2007) and 2007 (this study), the response to PHA in 2008 tended to be higher in older than in younger females rather than lower. What could have caused this discrepancy between years?

One difference between the two years of study was apparent in the pattern of nest failure during incubation (i.e., egg failure) in the population (Appendix D), which might have resulted in the 2008 sample being biased towards higher quality females compared with the 2007 sample. Interestingly, comparison of our 2007 and 2008 initial measurements (i.e., capture 1) shows that differences between years occurred exclusively within the old age group. Old females in our experimental sample for 2008 not only did not show immunosenescence (Fig. 2), but they also tended to have higher initial body mass (~9%) and lower levels of basal corticosterone (~65%) than the old females in 2007, suggesting that they were of better quality and/or in better condition, while mid-age and young females did not differ between years in either body mass or corticosterone levels (Fig. 6). To test whether these differences in female quality could have driven the disparate patterns between years, we attempted to mimic the results for female body mass in 2008 (Fig. 3C) by removing the lower quality (i.e., smaller initial body mass) old females

PLATE 1. Adult female Tree Swallow from our study population in Tompkins County, New York, USA. Notice the aluminum band, used to uniquely identify individuals, on the left leg. Photo credit: Paulo E. Llambias.

from the 2007 data set (both from LPS and control groups), therefore biasing the data towards higher quality old females. Limiting the analysis to these higher quality old females reduced the difference in body mass observed 24 h after the injection between LPS and control females, effectively making the response of old females resemble that of mid-age females (as observed in 2008). Thus, differences in the quality of old females between study years is a likely explanation for the disparate results obtained in 2007 and 2008.

In addition to the different patterns of incubation failure and female quality, food availability during the nestling rearing period also differed between study years, with the number of flying insects being significantly higher ($t = 4.632$, $P < 0.0001$) in 2008 (mean $\pm$ SE $=$ 305.4 $\pm$ 40.22 insects/d) than in 2007 (109.5 $\pm$ 13.06 insects/d). This difference in food availability might have further contributed to the reduced costs of LPS challenge observed in 2008 compared to 2007. Finally, it is also possible that the LPS (or the LPS water–oil emulsion) used in 2008 was of lower potency than that used in 2007, resulting in a weaker challenge, and therefore lower costs in the second year of study. An indication that this might have been the case comes from the overall weaker specific anti-LPS antibody responses mounted by females of all ages in 2008 compared to 2007 (Fig. 5E). If this was correct, then the differences between years could partly mimic how the responses might differ in the case of real bacterial infections with strains differing in pathogenicity.

Although we cannot know for sure the cause of the discrepancy between years, our results are consistent with the suggestion that in 2008 the sample of old females was biased towards better quality old individuals that had higher levels of food available and did not suffer reduced immune function compared to younger ones, and therefore, did not show increased costs compared to younger birds when challenged with LPS.

CONCLUSION

Studies within the past 10 years suggest that immunosenescence is a common phenomenon in wild animal populations. We provide the first evidence of costs associated with the immunosenescent phenotype in free-living animals. As observed in laboratory rodents, immunosenescence in free-living Tree Swallows is associated with an exaggerated sickness response to infection. This implies that if infection occurs during a critical stage of the life cycle, the exaggerated sickness response is likely to interfere with important fitness-related activities. Our results also suggest that the costs of immunosenescence for wild animals are likely to depend on environmental factors such as weather patterns and food availability, such that greater costs are likely to be paid in bad years compared to good years. Importantly, our study also highlights the fact that old age per se does not necessarily imply immunosenescence, even in populations where the pattern of immune system aging can be repeatedly detected. In summary, this study contributes to the understanding of aging in the wild and provides a solid foundation for future research on the costs of immunosenescence in free-living animals. A better understanding of immune system function in an ecological context is

central for wildlife disease ecology and conservation (Hawley and Altizer 2010), especially in the face of accelerated challenges that result from greater mobility of zoonotic pathogens, increased incidence of emerging wildlife diseases, and global climate change.

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APPENDIX A

Models for female and nestling response variables showing year effects (*Ecological Archives* E092-080-A1).

APPENDIX B

Models for female response variables one week posttreatment (*Ecological Archives* E092-080-A2).

APPENDIX C

Models for female and nestling responses in 2008 after removal of "single mothers" (*Ecological Archives* E092-080-A3).

APPENDIX D

Differences in incubation failure between the two study years (*Ecological Archives* E092-080-A4).