

INNATE IMMUNITY IS NOT RELATED TO THE SEX OF ADULT TREE SWALLOWS DURING THE NESTLING PERIOD

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Abstract. Evolutionary theory predicts that exposure to more diverse pathogens will result in the evolution of a more robust immune response. We predicted that during the breeding season the innate immune function of female Tree Swallows (*Tachycineta bicolor*) should be more effective than that of males because (1) the transmission of sexually transmitted microbes during copulation puts females at greater risk because ejaculates move from males to females, (2) females copulate with multiple males, exposing them to the potentially pathogenic microbes in semen, and (3) females spend more time in the nest than do males so may be more exposed to nest microbes and ectoparasites that can be vectors of bacterial and viral pathogens. In addition, elevated testosterone in males may suppress immune function. We tested our prediction during the 2009 breeding season with microbicidal assays *in vitro* to assess the ability of the innate immune system to kill *Escherichia coli*. The sexes did not differ in the ability of their whole blood to kill *E. coli*. We also found no significant relationships between the ability of whole blood to kill *E. coli* and the reproductive performance or the physical condition of males or females. These results indicate that during the nestling period there are no sexual differences in this component of the innate immune system. In addition, they suggest that there is little association between this component of innate immunity and the reproductive performance and physical condition during the nestling period of adult Tree Swallows.

Key words: *ecoimmunology, immune function, microbicidal assay, Tree Swallow.*

La Inmunidad Innata no está Relacionada al Sexo del Adulto de *Tachycineta bicolor* durante el periodo de Pichones en el Nido

Resumen. La teoría evolutiva predice que la exposición a una mayor diversidad de patógenos daría como resultado la evolución de una respuesta inmune más robusta. Predijimos que durante la época reproductiva la función inmune innata de las golondrinas hembra *Tachycineta bicolor* debería ser más eficaz que la de los machos debido a que (1) la transmisión de microbios transmitidos sexualmente durante la cópula pone a las hembras en mayor riesgo debido a que la eyacuación pasa de los machos a las hembras; (2) las hembras copulan con varios machos, exponiéndolas a los microbios potencialmente patógenos que se encuentran en el semen; y (3) las hembras pasan más tiempo en el nido que los machos, por lo que pueden estar más expuestas a microbios y ectoparásitos que pueden ser vectores de patógenos bacterianos y virales. Además, la testosterona elevada en los machos puede suprimir la función inmune. Evaluamos nuestra predicción durante la temporada de cría de 2009 con ensayos microbicidas *in vitro* para determinar la capacidad del sistema inmune innato de matar a *Escherichia coli*. La capacidad de la sangre de matar a *E. coli* no varió entre los sexos. Además, no encontramos una relación significativa entre la capacidad de la sangre de matar a *E. coli* y el comportamiento reproductivo o la condición física de los machos o de las hembras. Estos resultados indican que durante el período de pichones no existen diferencias sexuales en este componente del sistema inmune innato. Además, sugieren que hay poca relación entre este componente de la inmunidad innata y el comportamiento reproductivo y la condición física durante el período con pichones de las golondrinas adultas.

INTRODUCTION

Ecoimmunology examines immune function in an ecological context. Constitutive innate immunity provides a rapid, nonspecific, first line of defense against invading microbes (Rickert 2005, Greives et al. 2006, Lochmiller and Deerenberg 2000) via inflammation, the lysis of cells, phagocytosis by macrophages and heterophils, and the action of soluble plasma proteins (Roitt et al. 1998). One aspect of

ecoimmunology explores sex differences in immune function (Stoehr and Kokko 2006, Pap et al. 2010) but in natural populations these investigations are relatively uncommon and have produced mixed results. For example, while some studies have not detected sex differences in immune function (Råberg et al. 2003, Lee et al. 2006, Martin et al. 2006), other studies have shown male birds to have lower immune function and greater parasite loads during breeding than

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female conspecifics (Poulin 1996, Møller et al. 1998, Tschirren et al. 2003, McGraw and Ardia 2005, Pap et al. 2010). At a proximate level, sex differences in immune function have frequently been attributed to the modulating effects of steroid hormones (Grossman 1989, Folstad and Karter 1992, Olsen and Kovacs 1996). Sex differences in immune function may also be caused by asymmetrical exposure to parasites and sex-biased differences in tradeoffs between pathogen defense and other costly life-history traits (Reisen and Hahn 2007, Nunn et al. 2009, Restif and Amos 2010).

We set out to determine if there are sex differences in the *in vitro* ability of the whole blood of breeding Tree Swallows (*Tachycineta bicolor*) to kill the Gram negative bacterium *Escherichia coli*, a potentially pathogenic bacterium that has been isolated from the cloacae of adults (Lombardo et al. 1996) and nestlings (Mills et al. 1999) and from semen (Lombardo and Thorpe 2000). The microbicidal ability of whole blood *in vitro* provides a measure of constitutive immune function (e.g., Tieleman et al. 2005, Matson et al. 2006, Millet et al. 2007). In some species of birds it may vary among individuals and be highly repeatable within an individual (Tieleman et al. 2010).

We predicted that during the breeding season the whole blood of female Tree Swallows should show greater ability to kill *E. coli* than that of males for both ultimate and proximate reasons. From an evolutionary perspective, females may experience greater selection on immune defenses because they may be exposed to a greater risk of infection than are males for at least two reasons. First, females participate in extra-pair copulations with multiple males (e.g., Barber et al. 2005), exposing them to more sexually transmitted microbes (STMs) such as bacteria, fungi, and viruses (Lombardo et al. 1999) contained in semen (Lombardo and Thorpe 2000) because the transmission of STMs during copulation is asymmetrical; ejaculates move from males to females. Second, females may be more exposed to nest microbes and ectoparasites because they spend more time than do males in the nest cavity during nest building, incubation, brooding, and nestling care (Robertson et al. 1992). Female-biased ectoparasite and microbe exposure may be further amplified because Tree Swallows commonly reuse cavities from year to year (Robertson et al. 1992), potentially exposing them to parasitic mites (*Dermanyssus hirundinis*) that may have overwintered in their nests (Burt et al. 1991). *Dermanyssus hirundinis*, the northern fowl mite, is a blood-feeding mite that may act as vector of bacterial and viral pathogens (Valiente Moro et al. 2005). From a proximate perspective, males may experience more immunosuppression by testosterone than do females (Folstad and Karter 1992). For example, in wild breeding Dark-eyed Juncos (*Junco hyemalis*), Greives et al. (2006) found testosterone levels were negatively correlated with two measures of innate immune function, immunoglobulin-G (IgG) levels and complement activity.

Differences between individuals in immunity may also be affected by general physical condition (e.g., mass, structural size), so we also investigated the association between

the microbicidal ability of whole blood and physical condition, including the ability to resist ectoparasite damage as estimated by the number of holes chewed in wing and tail feathers by lice. Late-arriving male Barn Swallows (*Hirundo rustica*) that mounted weaker T-cell-mediated immune responses were more heavily infested with chewing lice (order Phthiraptera) than were males that arrived earlier (Møller et al. 2004). Tree Swallows that begin breeding earlier in the season lay larger clutches and produce more fledglings and recruits (Winkler and Allen 1996, Robertson and Rendell 2001, Shutler et al. 2006) and thus are more likely to be in better physical condition than swallows that begin breeding later, so we predicted that they should have greater microbicidal ability.

METHODS

STUDY SITE AND FIELD METHODS

The Tree Swallow is a cavity-nesting aerial insectivore with a socially monogamous breeding system characterized by high rates of extra-pair copulation. Females do most of the nest building with males adding most of the feathers that line the nest cup, only females incubate eggs and brood hatchlings, and both adults feed nestlings, remove fecal sacs from nests, and defend their nest from conspecific intruders and predators (Robertson et al. 1992).

We studied breeding Tree Swallows nesting on the campus of Grand Valley State University in Allendale, Michigan (42° 57' N, 85° 53' W), from May to July 2009. The study site was located in an old agricultural field and consisted of a 10 × 10 grid of 100 standard wooden nest boxes mounted on metal poles. Nest-box holes were about 1.5 m from the ground. Boxes were protected by predator guards and spaced 20 m apart.

We categorized breeding females as either second year (SY), if their dorsal plumage was mostly brown, or (b) after second year (ASY), if their dorsal plumage was mostly iridescent blue-green (Hussell 1983). Male Tree Swallows cannot be reliably aged by size or plumage characteristics (Dwight 1900, Robertson et al. 1992). We started monitoring breeding behavior on 1 May 2009. To evaluate the possible effects of timing of sampling on the ability of whole blood to kill *E. coli*, we recorded the sample's date, the time of day it was obtained, the number of days between clutch initiation and sampling, and nestlings' age the day the sample was obtained. We measured reproductive performance by recording at each nest the clutch-initiation date, clutch size, the number of hatchlings, the proportion of eggs that hatched, the number of fledglings, and the proportion of nestlings that fledged. In the Tree Swallow, clutch size, fledging success, and recruitment are negatively correlated with clutch-initiation date (Robertson et al. 1992, Winkler and Allen 1996, Shutler et al. 2006).

We used plastic box traps (Yunick 1990) to catch adult swallows tending nests between days 1 and 15 of the 20-day nestling period of 2009. We collected blood samples (~10–60 µL) from the brachial vein on the right wing of breeding

adults. To ensure a sterile site, we cleared the skin surrounding the brachial vein of interfering feathers, soaked it liberally with 70% ethanol, swabbed it with a fresh cotton ball, and allowed it to air dry 15–20 sec. We punctured the brachial vein with a sterile lancet and collected blood in heparinized capillary tubes (50- μ L capacity). We held tubes horizontally to ensure air-bubble formation at each end of the tube then sealed them with an ethanol-sterilized clay card and transported them to the laboratory in sterile 50-mL Fisher tubes. To avoid the effects of suppression of the immune response mediated by stress hormones such as corticosterone (Romero and Romero 2002) we collected all blood samples within 3 min of removing trapped birds from nest boxes.

After drawing a blood sample from each individual we measured its mass to the nearest 0.2 g with a spring scale, head–bill length with electronic digital calipers to the nearest 0.1 mm, length of the flattened right wing to the nearest 1 mm with a ruler with a stop fixed to one end, and depth of tail fork to the nearest 1 mm with a ruler by measuring the length of right tail feathers from the notch in the center of the tail to the tip of the outer rectrices on each side of the tail while the tail was held so that the outer edges of the outer rectrices were parallel to each other. Wings and tails with damaged feathers were not measured. In size, the Tree Swallow is sexually monomorphic, except that males have wings (Robertson et al. 1992) and tail forks (Lombardo, unpubl. data) longer than those of females. Following Palacios et al. (2007, 2009) we used the residuals of mass regressed on head–bill length to estimate body condition (Schulte-Hostedde et al. 2005). After making measurements, we counted the number of holes chewed by lice (order Phthiraptera, suborder Ischnocera) in the flight feathers, both wings and tail combined. Ischnoceran lice feed on the nonliving keratin of feather barbules (Janovy 1997). To preclude interobserver variation, Lombardo made all morphological measurements and counted louse holes.

LABORATORY METHODS

We performed a microbicidal assay of whole blood by following the methods of Millet et al. (2007). This assay measures the ability of components (e.g., complement proteins, lysozymes, natural antibodies) of the innate immune system within plasma to kill *E. coli*. These components presumably destroy foreign cells by lysing them, which in the case of lysozymes ruptures bacteria by cutting structural chains of polysaccharide in the cell wall (Alberts et al. 1998). We reconstituted pellets of lyophilized *E. coli* (ATCC#8739; concentration 10^7 or 10^8 per pellet; Epower Assayed Microorganism Preparation, Microbiologics, Inc., Saint Cloud, MN) according to manufacturer's instructions in 40 mL sterile tissue-culture-grade phosphate-buffered saline (PBS). Stock solutions of suspension concentrations and PBS were refrigerated at all times and were prepared every 5–7 days. As concentrations of a stock suspension may fluctuate, we plated a dilution series (e.g., 1:5, 1:10, 1:20) daily to determine

the concentration that would ensure 100 to 150 *E. coli* colonies per control plate. Both control and experimental plates were plated in duplicate. To ensure sterility, we carried out all laboratory procedures inside a laminar-flow hood. All solutions were kept on ice at all times.

We processed samples of whole blood within 2 hr of collection to avoid the possibility of immune-component deactivation (Millet et al. 2007). Experimental solutions consisted of 5 μ L whole blood, 10 μ L *E. coli* stock suspension of appropriate concentration, and 95 μ L CO_2 -independent media supplemented with 4-mM L-glutamine, pre-warmed to 41 °C, all in a sterile 1.5-mL tube. Negative control solutions were identical to experimental ones, except that whole blood was replaced with 5 μ L sterile PBS. Both experimental and control solutions were incubated at 40.5 °C for 45 min. Following incubation, solutions were vortexed, pipetted in 50- μ L aliquots onto plates of tryptic soy agar, spread, inverted, and allowed to sit at room temperature for 1 hr, followed by overnight incubation at 37 °C. Colonies were counted 18–24 hr after incubation.

STATISTICAL METHODS

Following Millet et al. (2007), we calculated the mean proportion *E. coli* killed (MPK) as $[1 - (\text{mean number of colonies on duplicate experimental plates} / \text{mean number of colonies on duplicate control plates})]$.

We analyzed data with PASW 18.0 (SPSS 2010) after data exploration (cf. Zuur et al. 2009) to determine when to use parametric or nonparametric statistical analyses. We used *t*-tests, paired *t*-tests, and the Kruskal–Wallis ANOVA to compare means, Pearson's correlation coefficient (*r*) to compare the MPK of mates and the MPK and numbers of louse holes in males and females, and a stepwise multiple linear regression to examine the relationship between the dependent variable MPK and measures of (1) time of sampling (sample date, time of day sample was obtained, nest-completion date, the number of days between clutch initiation and sample date, and nestling age on sample date), (2) reproductive performance (clutch-initiation date, clutch size, number of eggs that hatched, proportion of eggs that hatched, number of fledglings produced, proportion of hatchlings that fledged), and (3) physical condition (mass corrected for date of measurement, right wing length, length of tail fork on right side, number of louse holes in wings, number of louse holes in tail, total number of louse holes, and body condition). The criterion for an independent variable's entry into the stepwise linear regression was set at $\alpha = 0.05$. The probability level for significance for comparisons of means and in correlation analyses was set at $\alpha = 0.05$. All tests are two-tailed. All values are reported as means \pm SD.

RESULTS

Second-year and ASY females did not differ significantly in MPK ($t_{39} = 0.11$, $P = 0.91$; Fig. 1), so we pooled their MPK data

for further analyses. The sexes did not significantly differ in mean MPK ($t_{72} = 0.64$, $P = 0.52$; Fig. 1). In addition, mates did not significantly differ in mean MPK (paired $t_{31} = 0.63$, $P = 0.54$; Fig. 1), and there was no significant correlation between mates in MPK ($r = -0.12$, $n = 32$, $P = 0.50$).

There were no significant differences in mean total louse holes among SY females (9.69 ± 9.76 , $n = 16$), ASY females (3.52 ± 4.59 , $n = 25$), and males (11.48 ± 17.45 , $n = 33$) ($F_{2,71} = 2.76$, $P = 0.07$) or between mates (females, 5.52 ± 7.76 ; males, 11.94 ± 18.24) (paired $t_{30} = -1.94$, $P = 0.06$). However, ASY females had significantly fewer mean total louse holes than did SY females ($U = 114.5$, $P = 0.02$), and there were no significant differences in mean total louse holes between males and SY females ($U = 224.0$, $P = 0.39$) or ASY females ($U = 310.5$, $P = 0.10$). When we considered each age/sex category separately, we found no significant correlations between MPK and total louse holes in SY females ($r_s = -0.35$, $n = 16$, $P = 0.18$), ASY females ($r_s = -0.30$, $n = 25$, $P = 0.14$), or males ($r_s = -0.01$, $n = 33$, $P = 0.94$).

Because the sexes did not differ significantly in MPK we pooled their MPK data to examine the relationship between MPK and timing of sampling, measures of reproductive performance, and physical condition. A stepwise multiple linear regression showed that none of the measures of time of sampling, reproductive performance, or physical condition had a statistically significant effect on MPK (all $P > 0.10$) in the 49 swallows for which we had data for all variables included in the model ($F_{17,31} = 0.55$, $P = 0.91$, $R^2 = 0.23$).

DISCUSSION

All animals are exposed to a myriad of parasites and pathogens, so maintenance of a robust immune system is critical to survival and reproduction (Zuk 1996). As immune function is shaped strongly by selection, variation in this trait is

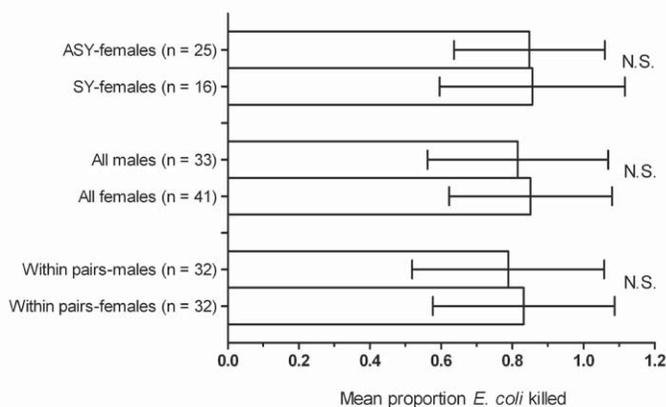


FIGURE 1. Category of breeding Tree Swallow and mean proportion *E. coli* killed (MPK). See text for definitions. Means \pm SD are illustrated.

predicted. However, ecoimmunologists have only recently developed an interest in better understanding the causes of variation in the immune response, both within and across species boundaries. In a within-species context, we searched for sex differences between breeding Tree Swallows in the microbicidal ability of their whole blood to kill *E. coli* in vitro. We predicted that the microbicidal ability of female Tree Swallows should be greater than that of males because females (1) may be more exposed to pathogens, resulting in stronger selection on females' immune systems (Reisen and Hahn 2007, Hahn and Reisen 2011) and (2) have lower levels of endogenous, immune-suppressing testosterone (Folstad and Karter 1992). Contrary to our expectations we found no differences between the sexes in ectoparasite damage. Therefore, it may not be surprising that, contrary to our prediction, we found no difference between the sexes in the ability of their whole blood to kill *E. coli* in vitro. We did not compare the levels of endoparasites, including microbes, of males and females, so the question of sex differences in parasite exposure and infection in the Tree Swallow remains open. Nevertheless, we are confident that our measures of MPK accurately represent the microbicidal ability of the whole blood of the Tree Swallows breeding at our study site because our sample is an accurate representation of the breeding population; our sample included 83% (44/53) of females that successfully fledged at least one young.

The literature on sex differences in immunity, including those within a species, contains conflicting results. Our results are consistent with other studies that failed to detect sex differences in immune function in adult birds, including the Blue Tit (*Cyanistes caeruleus*; Råberg et al. 2003), Red-winged Blackbird (*Agelaius phoeniceus*; Hasselquist et al. 1999), sparrows (*Passer* spp.; Lee et al. 2006, Martin et al. 2006), and Tree Swallow (Lifjeld et al. 2002, Hausmann et al. 2005). In contrast, sex differences have been detected in the Magellanic Penguin (*Spheniscus magellanicus*; Moreno et al. 2001), Zebra Finch (*Taeniopygia guttata*; McGraw and Ardia 2005) and House Sparrow (*Passer domesticus*; Pap et al. 2010). Sex differences in immune function have also been examined in nestlings with inconsistent findings: some studies have detected immune differences between the sexes, as for the Great Tit (*Parus major*; Tschirren et al. 2003), Blue Tit (Dubeic et al. 2006), and Common Kestrel (*Falco tinnunculus*) (Fargallo et al. 2002), while others have not, as for the American Kestrel (*F. sparverius*; Tella et al. 2000), Alpine Swift (*Apus melba*; Bize et al. 2005), and Barn Swallow (Saino et al. 2002).

Immune function in birds may be a highly plastic trait regulated in a sex-specific and context-dependent manner (Love et al. 2008, Pap et al. 2010). For example, Greives et al. (2006) showed that IgG levels of breeding male Dark-eyed Juncos varied significantly with stage of breeding whereas complement activity did not. Love et al. (2008) found that male Zebra

Finches had greater inflammatory responses than did females at fledging, but the sexes had similar inflammatory responses during prebasic molt and when reproductively mature. Love et al. (2008) concluded that male and female Zebra Finches have different immune responses during early development but similar ones in adulthood. Pap et al. (2010), examining sex differences in immune function with a variety of assays that measured eight immune variables in the House Sparrow, demonstrated that immune function varied through the annual cycle both within and between the sexes. Field studies that examine innate immune responses through the entire life cycle are likely to be fruitful.

Ecoimmunological studies of birds demonstrate that a complete picture of immune function can be formed only by simultaneously considering multiple components of the immune system across each stage of the annual life cycle (Pap et al. 2010). This is because the components of the immune system vary in their relative costs and benefits (Klasing and Leshchinsky 1999, Klasing 2004, Lee 2006) and respond differently to environmental pressures (Norris and Evans 2000), life-history stages (Greenman et al. 2005), and phases of development (Martin 2005). Therefore, the immune function of the sexes may vary (1) on a component-by-component basis, (2) at different ages, (3) during different parts of the annual life cycle (e.g., breeding, nonbreeding, migration), and (4) on a life-history basis (e.g., different species may differ in immunity by sex). Thus, only through consideration of all components at different times may a larger pattern of the relationship between sex and immune function emerge. The use of increasingly sophisticated assays of immune function by behavioral ecologists (e.g., Millet et al. 2007, Hahn and Reisen 2011) is also expected to increase the accuracy of our assessments of immune function in natural populations.

We found no evidence suggesting a relationship between the *in vitro* microbicidal ability of the whole blood of breeding male and female Tree Swallows and correlates of fitness. However, the relationship between MPK and male reproductive performance may be difficult to detect in the field because of the high frequency of extra-pair paternity in the Tree Swallow (Robertson et al. 1992). Our results were inconsistent with those of other studies that examined the relationship between immune function and correlates of fitness. For example, in a sample of 18 species of passerines, individuals with smaller spleens, a possible sign of diminished immune function, suffered greater rates of predation than did those with larger spleens (Møller and Erritzøe 2000). Greives et al. (2006) showed that in wild breeding male Dark-eyed Juncos IgG levels and complement activity were positively correlated with body mass whereas tarsus length, a measure of skeletal size, was positively correlated with complement activity but not IgG levels. The positive correlation between mass and IgG and complement activity suggests that energetic mechanisms may have an important effect on innate immune function in the junco (Greives et al. 2006). Other studies (e.g.,

Sorci et al. 1997) have demonstrated a positive correlation between nutritional condition and cell-mediated immunity. Cell-mediated immunity does not involve complement or antibodies but instead relies on cytokines, macrophages, natural killer cells, and T-cell lymphocytes (Roitt et al. 1998). Lower-quality late-arriving breeding Chinstrap Penguins (*Pygoscelis antarcticus*) had leukocyte counts and T-cell mediated responses significantly lower than those of higher-quality individuals that arrived on breeding grounds earlier in the season (Moreno et al. 1998). Similarly, later-arriving male Barn Swallows mounted weaker T-cell-mediated immune responses and were more heavily infested with chewing lice (order Phthiraptera) and blood parasites than were males that arrived earlier (Møller et al. 2004).

In Tree Swallows, Hasselquist et al. (2001) found by experiment that humoral immune function, specifically, the ability to mobilize antibodies against specific antigens (Roitt et al. 1998), may indicate overall quality of females because it was negatively correlated with date of egg laying, which is itself negatively correlated with reproductive success (DeSteven 1980, Robertson et al. 1992) and recruitment (Shutler et al. 2006). Lifjeld et al. (2002) found that T-cell-mediated immune function as estimated by local skin swelling in response to phytohaemagglutinin (PHA) injection was condition dependent; energetically stressed adult Tree Swallows feeding nestlings showed suppressed immune responses.

The lack of statistically significant relationships between MPK and our measures of physical condition are consistent with (1) Haussmann et al. (2005), who found no effect of body mass on PHA response of Tree Swallows that were either incubating eggs or tending nestlings, and (2) Palacios et al. (2007), who studied the same population of Tree Swallows as did Haussmann et al. and found that body condition was not correlated with levels of natural antibodies and complement-mediated cell lysis, two different measures of innate immunity, in breeding females.

In conclusion, we found no sex differences between breeding Tree Swallows in the ability of their whole blood to kill *E. coli* *in vitro* and that this component of the innate immune system was not associated with the timing of sampling, reproductive performance, or physical condition during the nestling period. We did not measure nestlings just prior to their fledging so do not know if there was an association between MPK in adults and condition of young at fledging. Finally, our results reinforce the idea that a complete understanding of immune function in natural populations can be formed only by simultaneously considering multiple components of the immune system across different stages of the annual life cycle.

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