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Development of the Innate Immune Response in Nestling Tree Swallows (*Tachycineta bicolor*)



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Abstract

*The innate immune system provides an immediate, short term, first line of defense from pathogens; its appearance early in development in vertebrates is evidence of its critical importance. Even so, few studies have investigated the development of the immune response as juveniles transition into adults. Ultimately, the ability to respond to pathogens confers fitness benefits in terms of health, survival, and reproductive success, and it follows that functions such as rapid growth cannot be fully met simultaneously since energy is a limiting resource. As a result, defense mechanisms are compromised at an early age due to energy allocation to rapid growth; therefore, immunity should increase as individuals mature. I studied the development of innate immunity in nestling Tree Swallows using microbicidal assays which were conducted in vitro to assess the ability of the immune system to kill *E. coli* via lysis. This research may provide insight into patterns of disease susceptibility, which in turn influence evolutionary fitness and population dynamics.*

Introduction

Ecoimmunology seeks to explore and understand various immune defense strategies observed among species. The ecological study of the development of immune function in birds and other vertebrates is in its infancy [1], and age-specific variation in immune function has been understudied in regard to innate immune development in wild animals [2, 1].

The vertebrate immune system consists of two components: innate and acquired immunity [3]. Innate immunity is the nonspecific response to pathogens through complement, a cascade of proteins acting in sequence to produce biological effects concerned with inflammation and the lysis of cells, and phagocytosis, macrophages that ingest foreign particulate matter by enveloping them into the cell, occurring in the blood. Unlike acquired immunity, which develops over the lifetime of an organism from previous encounters with pathogens, innate immunity is present at birth. Innate immunity provides an immediate, short-term, first line of defense that

develops throughout the juvenile stage. Because these components have a genetic basis, they are directly subjected to natural selection, thus favoring the evolution of immune competence, and observations that the components of the innate immune system appear early in development in vertebrates [2, 4], including Tree Swallows, *Tachycineta bicolor* [1], are evidence of its critical importance.

Immune function involves life-history trade-offs influenced by differences in energy allocation to growth, maintenance and reproduction [5], all of which are energetically costly [6]; thus these three processes cannot be fully met simultaneously. Juveniles use energy for growth and maintenance, whereas adults use energy for maintenance and reproduction. Ultimately, the ability to respond to pathogens confers fitness benefits in terms of health, survival, and reproductive success [7]. Selection should favor those individuals that are able to budget their energy so as to balance the demands of growth and maintenance. This demand results in strong selection pressures on developing songbirds, which must grow rapidly to be able to leave the nest and avoid predation [8] while at the same time develop a competent innate immune response.

Most ecoimmunological studies have not focused on the development of innate immunity in immature, wild animals, but rather have used domesticated species such as chickens with precocial development [9, 10] as opposed to altricial development found in wild Tree Swallows and other passerines. Furthermore, chickens have been artificially bred for increased body growth for meat production or egg-laying, which may alter their lifespan, thus possibly altering natural, innate immune defense mechanisms [11, 12]. Additionally, in those studies that do exist on wild species, measurements were primarily taken at one specific age (e.g., 12 days after hatching), neglecting developmental stages as juveniles transition into adults.

Studies on the development of immune defense in nestling Tree Swallows may provide insight into patterns of age-related disease susceptibility, which in turn influences evolutionary fitness and population dynamics [13]. I studied the development of innate

immunity in a free-living, altricial bird, the Tree Swallow. Microbicidal assays [14] were used to examine age-specific variation in the innate immune function of nestlings at different stages of development, and I predicted that immune function would increase over the course of the nestling period.

Methods

Tree Swallows are a common, aerially foraging, passerine species distributed throughout North America that readily accept nest boxes as breeding sites. Females usually lay clutches of 5-6 eggs which hatch after approximately 15 days of incubation displaying. Hatchlings are altricial, and both parents feed the nestlings up until the time they fledge, which is about 20 days [15]. I studied Tree Swallows that nested in wooden nest boxes arranged in grids in an old field on the campus of Grand Valley State University (GVSU) (42°57'N, 85°53'W), Ottawa County, Michigan in June and early July, 2009. Nests were monitored closely to determine clutch completion date, exact hatch date, and, therefore, exact nestling age.

I collected blood samples from 36 nestlings. Due to the small size of Tree Swallows, I obtained only a single blood sample from each individual nestling to prevent jeopardizing its health. Tree Swallow nestlings undergo three basic stages of development: their eyes open 3-4 days after hatching; they develop endothermy 8-9 days after hatching [16]; and, they fledge approximately 20 days after hatching [15]. I examined the innate immune system at nestling days (ND; ND-0 = the day the first egg in a clutch hatched) 6, 12, and 18, which fall in the range of these developmental stages. At ND-3-4, nestlings were too small to obtain blood samples of adequate volume to perform *in vitro* assays, so I began drawing blood from ND-6 nestlings, which weigh 10-12 g, about half as much as adult swallows weigh [15]. At ND-12, Tree Swallow nestlings weighed as much or more than adults and then dropped in weight as they began to allocate their energy into feather growth [16, 15]. In addition, on ND-18 nestlings, I measured nestling mass to the nearest 0.2 g with a Pesola spring, and flattened wing chord of the right wing to the nearest 1 mm using a ruler with a stop fixed to one end.

Blood samples were collected within 3 min. of handling because the stress hormone

corticosterone, which can affect immune response, remains at or near baseline levels in birds for up to 3 min. following handling [17]. Blood was collected into sterile heparinized capillary tubes (50-75 μ L capacity) directly from the brachial vein in the wing after venipuncture using sterile lancets or needles. Prior to venipuncture, the area on the wing was sterilized with 70% ethanol. Venipuncture and blood collection occurred after the ethanol completely evaporated because ethanol can cause hemolysis, which can complicate immune assays [14]. Capillary tubes were sealed with clay and placed in sterilized containers for transport to the lab. Blood was transported to the laboratory usually within 60 min. of collection for the best results during immune assays [14].

The microbicidal assay produces an index of the capacity of the blood, measuring how rapidly it can thwart an invasion by potential pathogens [14]. This assay is a general *in vitro* measurement of the effectiveness of the innate immune system because it evaluates the ability of multiple elements in the blood (e.g., white blood cells, natural and specific antibodies, lysozymes, and opsonins [molecules that help white blood cells to bind to potential pathogens during phagocytosis]) to kill microbes, and the assay integrates many of the important components of the innate immune system [14].

This assay is ideal for examining innate immunity in small birds for two reasons. First, it was optimized to evaluate the immunocompetence of the innate immune system using very small blood volumes (< 100 μ L) with species-non-specific reagents [14]. Second, it was developed to be a rapid, simple, and reliable measure of innate immune function and does not require elaborate training or specialized equipment.

Microbicidal Assay—This assay evaluated the ability of constituents found in whole blood to kill microbes following the methods described in Millet et al. (2007). Assays were performed on samples of less than 100 μ L. Briefly, I tested the ability of whole blood to kill microbes *Escherichia coli* (ATCC#8739, Microbiologics Inc., Saint Cloud, MN) supplied as 10 organisms per lyophilized pellet. These are common microbes and did not need special treatment beyond normal microbiological methods. All lab work took place inside laminar flow hoods. Microbe pellets were reconstituted according to manufacturer's instructions in 40 mL of sterile,

endotoxin-free Phosphate Buffered Saline (PBS) and held at 4° C. Each day that assays were run, the stock culture was diluted with cold PBS to make a working culture with approx. 50,000 microbes/mL. Working cultures were kept on ice.

Whole blood was diluted to 1:4 with pre-warmed (41° C) CO₂-independent media (#18045; Gibco-Invitrogen, California, CA) plus 4mM L-glutamine in a sterile 1.5 mL capped tube. Ten microliters of the working culture (200 microbes) was added per 100 μ L of diluted blood, vortexed, and incubated for 45 min. at 40.5° C. After incubation samples were removed from the incubator, vortexed, and duplicated, 50 μ L aliquots were pipetted onto agar plates, spread, inverted, and incubated at 37° C for 24 hours. *E. coli* colonies were counted after 24 hours. The number of microbes in the initial inoculum were determined by diluting microbes in media alone (10 μ L of working culture per 100 μ L of media) and then immediately plated. Negative controls in which no microbes were added were also prepared. Microbicidal activity of the blood was defined as the percent of the inoculum killed which equal 1- (viable microbes after incubation/number inoculated) [14].

Responsible Conduct of Research—The methods used in this study were in accordance with suggestions for the ethical handling of wild birds [18]. We obtained permission to conduct this study from the GVSU Institutional Animal Care and Use Committee (IACUC).

Results

Total numbers of agar plated *E. coli* bacteria were measured in nestlings at ND-6 (n = 10), ND-12 (n = 16), and ND-18 (n = 22). Lysis increased as birds matured; the ability of constituents in the blood to kill this pathogen increased from approximately zero at ND-6 to 22% at ND-12 to 43% at ND-18 (ANOVA, F = 12.25, df = 2,50, P < 0.001, Fig. 1). Neither wing chord nor mass showed any significant correlation in the amount of lysis that occurred in ND-12s and ND-18s (Fig. 2 & 3). Even though ND-18 nestlings became independent when they fledged, their innate immune systems were not fully developed. In comparison, adult Tree Swallows (n = 79) (B. Houdek et al., unpublished data) at my study site displayed significantly greater lysis (mean = 82.6% \pm 24.6%) than did nestlings

at ND-18 (ANOVA, $F = 62.12$, $df = 3,123$, $P < 0.001$). Though body weight remained nearly the same (ND-18 mean mass = 21.0 g, $n = 22$; adult mean mass = 20.8g, $n = 79$), wing length was significantly shorter (second year female = $110.3\text{mm} \pm 2.6\text{mm}$, $\mu \pm \text{sd}$, $n = 77$; after second year female = $112.3\text{mm} \pm 3.1\text{mm}$, $\mu \pm \text{sd}$, $n = 216$; second year male = $116.7\text{mm} \pm 4.0\text{mm}$, $\mu \pm \text{sd}$, $n = 51$; after second year male = $116.2\text{mm} \pm 3.4\text{mm}$, $\mu \pm \text{sd}$, $n = 114$) and percent lysis (fig. 4) was significantly less in ND-18s than in adults (Mann-Whitney $U = 267.5$, $P < 0.001$).

Discussion

These data show evidence of life-history trade-offs. At ND-6, nestlings possessed little to no immunity (Fig. 1) while presumably expending considerable amounts of energy on body growth [5]. At ND-12, Tree Swallow nestlings showed increased immunity over that of ND-6 nestlings (Fig. 1) and weighed as much or more than adults and then dropped in weight as they began to allocate their energy into feather growth [16, 15]. As they transitioned to ND-18, there was another significant increase in immunity (Fig. 1) while body mass increased to a weight similar to that of adults. Energy allocation to innate immunity appeared to differ between the three stages of development in nestlings as well as between ND-18 nestlings and adults. Though body weight remained nearly the same, percent lysis (Fig. 4) was significantly less in ND-18s than in adults. This suggested that a large amount of energy was still being used by ND-18s for wing growth, thus less was devoted to developing immunity, further suggesting differences in energy allocation with an immunological cost during growth and development, which is consistent with findings in previous studies [19, 5].

In addition to trade-offs between growth and immune development, it has been shown that increased reproductive effort reduces humoral immunocompetence [20] in both adult female Barn Swallows, *Hirundo rustica*, and her nestlings, showing decreased immunity as brood size increases due to lower per capita feeding rate with limited resources [21]. This was supported with the findings of Nordling et al., [22] which showed that female Collared Flycatchers, *Ficedula albicollis*, raising enlarged broods, had lower humoral immunocompetence and increased parasit-

ism, and that parasitism was associated with a reduction in survival probability. Because the immune system is designed to prevent and control infection by pathogens and parasites, a reduction in immunocompetence might lead to an increased risk of infection, thus reduced fitness and chances of survival [7].

Finally, there are data suggesting that leaving the nest upon a predation attempt could be part of the normal behavioral repertoire of altricial nestlings [23] and is associated with adaptations in their ontogenesis and morphology [24]. Research on Meadow Pipits, *Anthus pratensis* [25], suggests patterns of growth in nestlings with directional selection for high growth rates, where the mean growth rate of nestlings in entirely depredated broods was lower than the population mean while that for partially depredated broods was higher. In broods that grew extremely slowly, the number of nestlings was reduced by either nestling competition for food or by predation, or by a combination of both. Magrath [26] showed that the post-fledging survival of slowly growing young is low, suggesting that mortality due to lack of food and by predation were synergistic, yet both acted independently [25] and differed with range. All broods in Halupka's study, with the exception of those birds that grew extremely fast, were susceptible to predation, and only the slowest growing nestlings suffered from mortality associated with the food shortage. Thus, it appears that nest predation is the most important factor selecting for rapid growth in altricial bird species, including Tree Swallows.

In summary, I studied the development of the innate immune response in nestling Tree Swallows at different stages of development via blood samples using microbicidal assays. Since there is strong selection pressure to develop a competent innate immune response, as expected, there was a steady increase in the ability of the innate immune system to kill bacteria as juveniles transitioned into independence along with transitions in energy allocation. Energy is limited, and because of this, there are physiological trade-offs within an energy budget in terms of costs and benefits. Juveniles appeared to allocate more energy to maintenance and rapid growth, with apparently little energy towards immunity, as clearly seen with the ND-6 nestlings. Energy allocation to immunity appeared to increase throughout each subsequent stage

of development, with full immune potential not being reached until after fledging. This trade-off may reflect the balance between predation and pathogen pressures on nestlings favoring selection on rapid growth.

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Figure 1. Mean percent lysis (\pm SE) measuring the ability of the blood to kill bacteria *E. coli* at different stages of development in nestling Tree Swallows (ANOVA, $F = 12.25$, $df = 2,50$, $P < 0.001$).

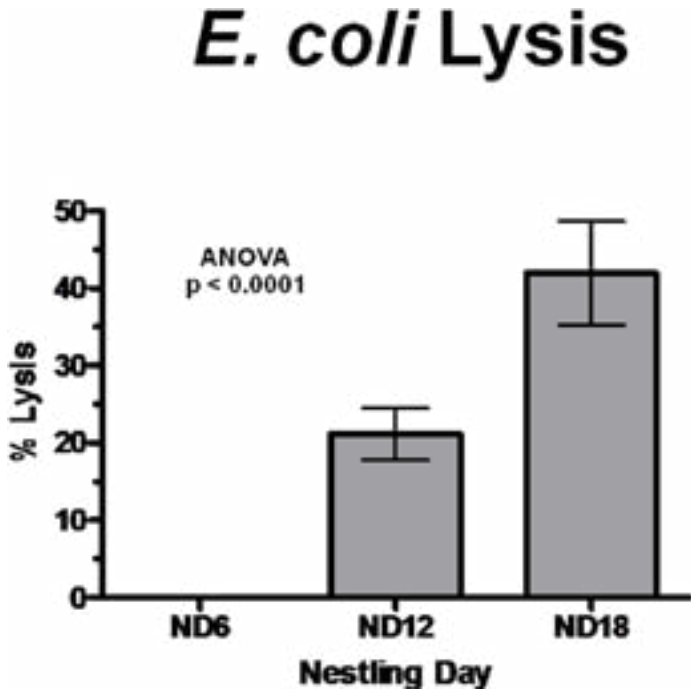


Figure 2. Measurements of wing chord and mass showing percent lysis assessing the ability of the blood to kill bacteria *E. coli* for each nestling day 12 Tree Swallow sampled.

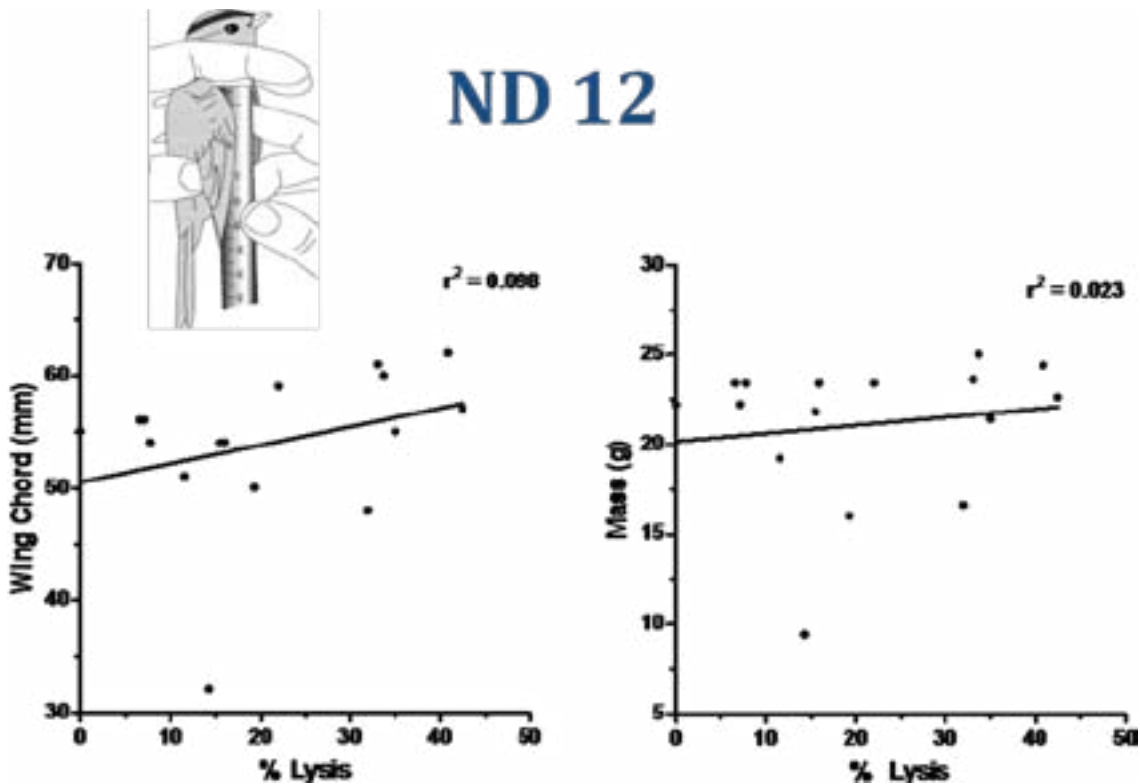


Figure 3. Measurements of wing chord and mass showing percent lysis assessing the ability of the blood to kill bacteria *E. coli* for each nestling day 18 Tree Swallow sampled.

ND 18

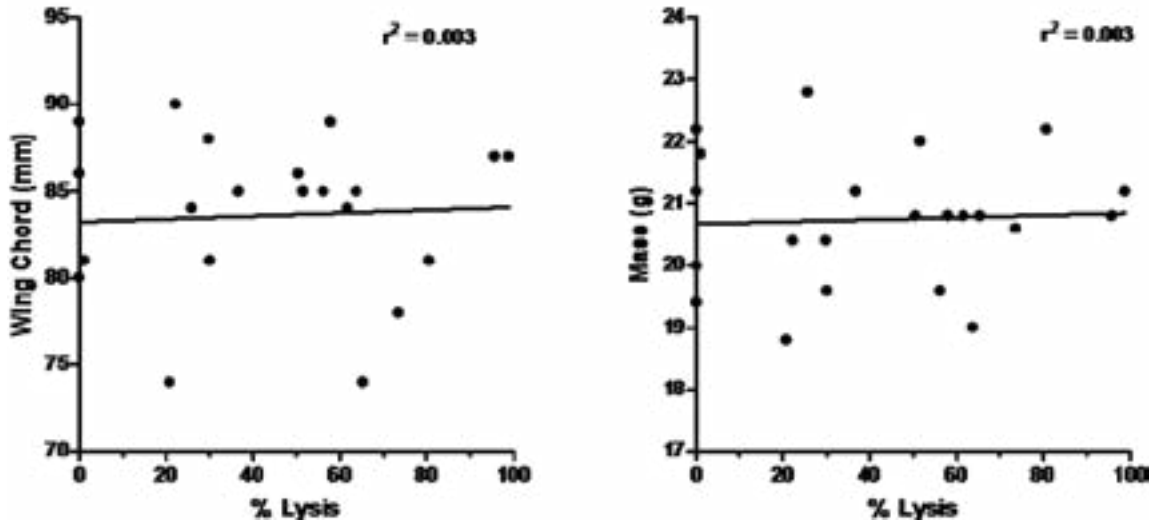
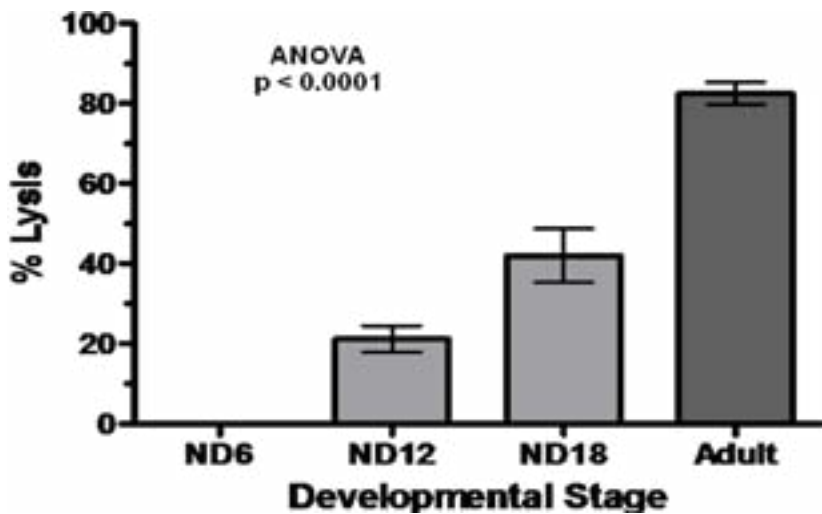


Figure 4. Mean percent lysis (\pm SE) measuring the ability of the blood to kill *E. coli* bacteria at different stages of development, including adults, in Tree Swallows (ANOVA, $F = 62.12$, $df = 3,123$, $P < 0.001$).



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