RESEARCH ARTICLE

Blood-Feeding Ectoparasites as Developmental Stressors: Does Corticosterone Mediate Effects of Mite Infestation on Nestling Growth, Immunity, and Energy Availability?

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ABSTRACT

How resources are distributed to growth and self-maintenance early in life is likely to impact survival and reproduction. Early resource allocation decisions may be particularly critical in altricial birds, as they have rapid developmental trajectories, and may be highly susceptible to environmental factors that can perturb development. The aim of this study was to determine if blood-feeding ectoparasites act as developmental stressors in European starling (*Sturnus vulgaris)* nestlings, driving a trade-off between growth and immunity. We hypothesized that because ectoparasites compete for resources they would induce growth-immunity trade-offs in parasitized nestlings. We also tested the hypothesis that changes in plasma corticosterone mediate the effects of ectoparasites on growth and immunity. Throughout development we assessed between-nest variation in ectoparasite density, measured growth, and a variety of blood parameters, including plasma corticosterone. We also assessed immune function across development. We found that nestlings from nests with high levels of ectoparasites were smaller, had elevated blood glucose, lower hematocrit levels, and appeared to engage in compensatory growth prior to fledging. They also had elevated innate immune responses early, but reduced responses later relative to nestlings from nests with low levels of ectoparasites. Plasma corticosterone was not affected by ectoparasite load, but did increase with nestling age. Overall, we find evidence that ectoparasites are developmental stressors that affect growth-immunity trade-offs, but their effects do not appear to be mediated by changes in circulating levels of corticosterone. *J. Exp. Zool. 9999A: 1–12, 2015.* © 2015 Wiley Periodicals, Inc.

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Development is an energetically demanding phase of the vertebrate life cycle; organisms must have sufficient energy to support both structural growth as well as the maturation of physiological systems essential for survival and reproduction. The rearing environment significantly influences the outcome of developmental processes, and environmental perturbations during this time can delay or diminish growth (Bize et al., 2003). Such perturbations may be particularly important for altricial songbird nestlings that develop from helpless,

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unfeathered hatchlings into adult-sized, flight-capable fledglings in as little as 3 weeks (Brzek and Konarzweksi, 2007).

Environmental factors that can affect nestling development range from large-scale perturbations, such as drought that can influence entire populations, to smaller scale perturbations such as endo- and ectoparasitism that act at the level of colonies, nests or individual nestlings. Parasites can profoundly impact development because they often compete with hosts for resources that could otherwise be used for growth and maintenance (Møller, '97). Haematophagous ectoparasites can be common in the nests of birds, and when present compete with their hosts for nutritional resources by taking blood meals (Proctor and Owens, 2000; Wolfs et al., 2012) in some cases inducing anemia, reduced growth and diminished survival in nestlings reared in infested nests (Lehmann, '93). In addition to reducing nesting growth through competition for nutritional resources, ectoparasites may also alter other physiological processes such as immune function (Brommer et al., 2011; King et al., 2011; Hanssen et al., 2013). Moreover, average daily metabolic rate and mass-independent daily energy expenditure of nestlings are positively related to the intensity of hematophagous ectoparasite infestation (Møller et al., 1994). When available energy is insufficient to maintain optimal growth and self-maintenance, energy utilization shifts away from less essential processes, to those more critical for immediate survival (Wingfield et al., '98). Shifting energy away from energetically costly processes, such as immune function, may allow an organism to successfully cope with a stressor (Wingfield and Kitaysky, 2002).

In adult birds, corticosterone, an adrenocortical hormone associated with energy mobilization in response to predictable and unpredictable stressors, is involved in mediating trade-offs that promote survival (McEwen and Wingfield, 2003). Elevated levels of corticosterone have also been shown to suppress immune and inflammatory reactions in adults (Saino et al., 2001; Rubolini et al., 2005; Lobato et al., 2008). Although the role of corticosterone in mediating responses to environmental perturbations is well established in adults, its role in response to developmental perturbations in altricial avian nestlings remains less clear (reviewed in Schoech et al., 2011), but there is evidence that chronic elevation can lead to reduced growth in nestlings (Kitaysky et al., 2003; Spencer and Verhulst, 2008). Previous research has shown positive relationships between ectoparasite load and baseline or stress-induced corticosterone in altricial nestlings (Quillfeldt et al., 2004; Raouf et al., 2006; but see Eggert et al., 2009 for contrasting results). However, the precise mechanisms by which ectoparasites induce increased corticosterone are unknown. One possibility is that reduced energy availability due to competition with ectoparasites for nutritional resources may trigger an adrenocortical response, resulting in elevated corticosterone levels.

As a glucocorticoid, corticosterone regulates the availability of glucose, a vital molecule for survival (Nelson, 2011). Previous studies have shown increases in plasma corticosterone levels when birds are deprived of food (Kitaysky et al., 2001; Lynn et al., 2003); however, blood glucose has also been shown to decrease with food deprivation (Savory, '87). Because of its importance for survival, several hormones including corticosterone exert tight regulation over blood glucose levels. For example, if available blood glucose decreases due to reduced food intake, corticosterone is released and stimulates processes such as hepatic gluconeogenesis, muscle tissue break down, and diminished glucose uptake to allow continued glucose homeostasis (reviewed in Sapolsky et al., 2000). In nestlings, which cannot independently forage for food, ectoparasites may act as nutritional stressors by competing with their hosts for nutritional resources and increasing nestling need for nutrients to sustain normal growth.

The aim of this study was to experimentally determine if blood-feeding ectoparasites act as developmental stressors in European starling (*Sturnus vulgaris)* nestlings, driving a trade-off between growth and immunity. We hypothesized that because ectoparasites compete for resources they would induce growth-immunity trade-offs in parasitized nestlings. We predicted that nestlings from parasite-infested nests would have reduced growth, reduced immune function, or both during nestling development in comparison with those from less parasitized nests. We also tested the hypothesis that changes in plasma corticosterone mediate the effects of ectoparasites on growth and immunity. We predicted that if nestling European starlings have developed a reactive adrenocortical response to stressors, plasma corticosterone levels should be higher in nestlings of highly parasitized nests compared with those from less parasitized nests.

METHODS

Study Species, Study Site, and Experimental Treatment

This study was conducted in 2010 on a population of European starlings breeding in nest boxes in central Illinois. The European starling is a cavity-nesting songbird that is native to Europe and Asia and is a highly successful introduced agricultural pest throughout much of North America (Feare, '84). Sixty nest boxes were placed on the Illinois State University crop and livestock farm in Lexington, Illinois, and 35 nest boxes were placed at the Illinois State University crop farm in Normal, Illinois. The wooden nest boxes were constructed of 2 cm thick cedar boards, and had internal dimensions of $33 \times 13.5 \times 18.5$ cm (height $\times$ width $\times$ depth) with a 4.8 cm in diameter entrance hole located in the front wall. The nest boxes were mounted at least 8 m apart on farm buildings, fences, and utility poles at these locations. During the breeding season, boxes were checked daily to determine the beginning of nest construction, clutch initiation, and clutch completion. The first nest of the season was randomly assigned to a treatment group 4 days after the first egg was laid in the nest. Thereafter, nest treatment was alternated, to maintain equal

numbers of control and pesticide-treated nests. All vertebrate animal use in this research was in accordance with prevailing local, state, and federal standards and guidelines and approved by the Institutional Animal Use and Care Committee of Illinois State University.

In total, 78 nests had at least one starling egg laid in them during the course of the breeding season. Of those nests, 54 had active nests 4 days later and received either the experimental pesticide treatment ($n = 27$) or the inert control treatment ($n = 27$). A 2.0×10.5 cm permethrin-impregnated plastic strip (Die-No-Mite Strips, Smith Poultry and Game, Bucyrus, KS) was stapled to the bottom half of the front wall, just below the top of the nest in each pesticide-treated nest box. In the remaining nest boxes, an inert plastic strip of similar color and size, containing no permethrin, was stapled in the same location. After treatment, nests were monitored daily for hatching. Of the 54 nest boxes that were experimentally treated, 42 were observed to have at least one egg hatch, and of those nests, 29 (16 pesticide-treated nests and 13 control nests) had at least one nestling still alive at brood day 17. It is these 29 nests that were included in this study. The day that a majority of the eggs in a clutch hatched was designated as brood day 0. Once each nestling hatched, one of its toenails was clipped to permit unique within-brood identification.

Assessment of Mite Loads

Similar to other reports of starling colonies in North America (e.g., Boyd, '51; Fairn et al., 2014), in our colonies northern fowl mites (*Ornithonyssus sylviarum*), when present, are usually the most abundant hematophagous ectoparasite within nest boxes, although carnid flies (*Carnus hemapterus*) are also common but typically in lower abundance (Casto personal observations). Mite loads were assessed on brood days 5, 10, and 15 following the procedure of Møller (2002), which takes advantage of the fact that these mites increase locomotion in response to substrate vibration and exhibit positive thermotaxis (Owen and Mullens, 2004). A warm bare hand was placed in the nest for one minute then removed. We visually estimated the number of mites that were hand sampled to the nearest order of magnitude (0, 1–10, 11–100, 101–1,000, 1,001–10,000, 10,001–100,000) and then used that number to test for treatment and nestling age effects on the intensity of mite infestation. We also used the brood day 15 estimate of mite load to assign nests to dichotomous mite load categories for use in ex post facto quasi-experimental statistical analyses (see results).

Somatic Growth

Nestling growth was monitored by measuring mass, wing length, and tarsus length. All measurements were taken on brood days 5, 10, and 15. Mass was measured to the nearest 0.01 g using a portable electronic scale. Flattened wing length was measured to the nearest 0.1 cm using a metric wing ruler and tarsus length was measured to the nearest 0.1 cm using digital calipers.

Blood Collection and Processing

Blood samples were collected by puncturing the brachial vein with a 26-gauge needle and collecting blood into heparinized capillary tubes. Upto 150 μL of blood was collected from each nestling on each of the three sampling days (brood days 5, 10, 15). Immediately after collection, capillary tubes were sealed with clay, and stored on ice until transfer to the laboratory. Stopwatches were used to record time of initial disturbance, time of each nestling's removal from nest, time taken to collect the first capillary tube, and time to finish the bleed. From these measures blood collection latencies were calculated and used in assessing whether the measured plasma corticosterone titers reflected baseline or disturbance-induced levels. The first tube collected was marked to ensure that plasma from it was used to analyze plasma corticosterone, as it would be the least likely to be influenced by an adrenocortical response to disturbance (see Romero and Reed, 2005). Blood glucose was also measured at this time using a small amount of blood and a commercially available blood glucose meter and test strips (ReliOn microm, Arkray USA, Edina, MN) that provided rapid measurement of blood glucose via electrochemical detection. For each blood sample, capillary tubes were centrifuged at 13,300 rpm (17,000*g*) for 10 min in a micro-hematocrit centrifuge, hematocrit was measured with a micro-hematocrit scale, and plasma was harvested and its volume measured with a Hamilton syringe. Fresh plasma from all but the first capillary tube collected was refrigerated as needed (see below) and the remaining plasma was stored frozen at −20°C in microcentrifuge tubes for use in corticosterone immunoassays.

Plasma Corticosterone

Plasma corticosterone concentrations from each nestling on each collection day were measured using previously frozen plasma with Detect X Corticosterone Immunoassay Kits (Arbor Assays, Ann Arbor, MI; K0145-H5). We combined 10 μL of plasma with 10 μL of dissociation reagent and 380 μL of the supplied assay buffer to give a total volume of 400 μL of dilute plasma. Duplicate 50 μL samples were then assayed following the manufacturer's instructions. Plasma aliquots from a long-term plasma pool were used to quantify intraplate and interplate variability. The mean intraplate coefficient of variation (CV) was 10.7% (±2.1% SEM) and the interplate CV was 12.9%.

Immune Function

We used two assays to measure immunity, a delayed-type hypersensitivity assay and a bacterial killing assay. These assays were selected because each is an integrated measure of functional immunity. We performed an immunochallenge with phytohaemagglutinin (PHA). This test has often been used as an indicator of cutaneous inflammatory responsiveness caused by migration of numerous cell types to the site of injection (McCorkle et al., '80; Martin et al., 2006; Salaberria et al., 2013). We measured both the primary and secondary immune response to determine if there

was a significantly higher immune response (due to immune memory) elicited in nestlings previously exposed to PHA and nestlings in which PHA was a novel mitogen. On brood day 5, individual nestlings within a nest were randomly assigned to either the primary or secondary PHA response treatment. Individuals assigned the secondary response received a subcutaneous injection of 150 μg PHA in 30 μL PBS into the scapular apterium just below the nape of the neck, and individuals assigned the primary response received a subcutaneous injection of 30 μL of PBS in the same location. On brood day 16, individuals in both PHA treatments received an injection of 150 μg PHA in 30 μL of PBS in the left wing web (patagium). Wing web thickness was measured prior to injection on brood day 16 and at 24 hr post injection using a pressure-sensitive digital thickness gage (Mitutoyo Model 547–500). Change in wing-web thickness was used to quantify the magnitude of the immune response.

We also performed bacterial killing assays across nestling development. The bacterial killing assay measures the bactericidal ability of plasma and assesses the ability of natural antibodies to limit early infection, complement enzymes to lyse targeted cells, and lysozyme to enzymatically digest targeted cell walls (Matson et al., 2006). This particular method was chosen because, it is an in vitro test that can be used repeatedly on individuals, it is easily interpreted, and it measures the coordinated effects of multiple aspects of innate immunity (Matson et al., 2006). Fresh plasma collected from each nestling on brood days 5, 10, and 15 was used to characterize bactericidal ability across early and mid stages of nestling development. The methods for this procedure followed the protocol of Matson et al. (2006), with modifications by Forsman et al. (2008). Five microliters of fresh plasma was combined with 100 μL of cell culture medium (500 mL CO_2 independent media, 26.4 mL fetal bovine serum, 0.298 g L-glutamine) and 200 colony-forming units (CFU) of *Escherichia coli* (Microbiologics; ATCC 8739; 0483PEC) in 10 μL of medium, and then incubated at 41°C for 30 min. A control sample, without added plasma, was also run. For each sample, including the control, 50 μL was then plated in duplicate on tryptic soy agar plates and incubated at 37°C for 24 hr, after which bacterial colonies were counted. Bacterial killing was expressed as the percentage of colony-forming units killed relative to the control.

Statistical Analyses

To determine the effect of the permethrin and control treatments on mite loads and all measures of growth and immune function, we ran a series of repeated measures linear mixed models with nestling age as the repeated measure. Our experimental treatments were ineffective at producing nests with predictably different intensities of ectoparasite infestation. This lack of a treatment effect was likely due to a combination of sub-optimal placement of pesticidal strips within nest boxes and high heterogeneity in initial mite infestation among nests studied. However, since the pesticide treatment did not significantly influence any of the other dependent variables in which we were interested, we opted to replace the independent variable, treatment, with a dichotomized quasi-experimental variable, mite load at brood day 15 (see results for more detail). The effects of mite load at brood day 15 (hereafter referred to as mite load) and nestling age on plasma corticosterone, plasma bactericidal ability, PHA swelling response, mass, wing length and tarsus length, were assessed with a series of repeated measures linear mixed models similar to those described for the original independent variable, treatment. When appropriate, all models were initially run using brood size as a covariate; however, brood size was not a significant factor in any of the models. Multivariate approaches to these analyses were also considered, but we deemed them inappropriate given the limited variation that existed in the magnitude estimates of mite load. We also ran a series of linear regressions to determine if the various blood collection latencies and plasma corticosterone concentrations were correlated, as information on the temporal dynamics of the adrenocortical response of starling nestlings to handling and nest disturbance has not previously been reported. All analyses were performed using SAS Statistical Analysis (SAS Institute, Inc., Cary NC) and all data were analyzed as untransformed data using nest as a random variable when appropriate. In instances where LS means were used in statistical analyses, the LS means are reported in figures.

RESULTS

Experimental Treatment, Ectoparasite Density, and Alternative Quasi-Experimental Groupings

We found no effect of experimental treatment on mite loads in nest boxes; mite loads were similar in both control and permethrin treated nests ($F_{127} = 0.747$, $P = 0.395$). Mite loads tended to be undetectable or low in most nests when first sampled on brood day 5 and remained low in most nests, but increased with increasing nestling age in several nests regardless of treatment ($F_{254} = 14.93$, $P = <0.0001$; Fig. 1A). We also found no effect of our experimental treatment on any of the dependent variables we measured (all P values ≥ 0.120). Despite the lack of treatment effect on mite load, there was still substantial non-experimental variation in mite load among nests throughout the study. Ectoparasite density in nests on brood day 15 varied substantially when assessed by hand sampling, ranging from non-detectable to over 10,000 mites/hand sample (Fig. 1B). Because the experimental treatment did not provide differential exposure to mites in our treatment groups and did not appear to influence our measures of interest, we used an ex post facto quasi-experimental design based on brood day 15 mite loads, to analyze the effect of high (greater than 100 mites during hand sampling) or low (100 or fewer mites during hand sampling) mite

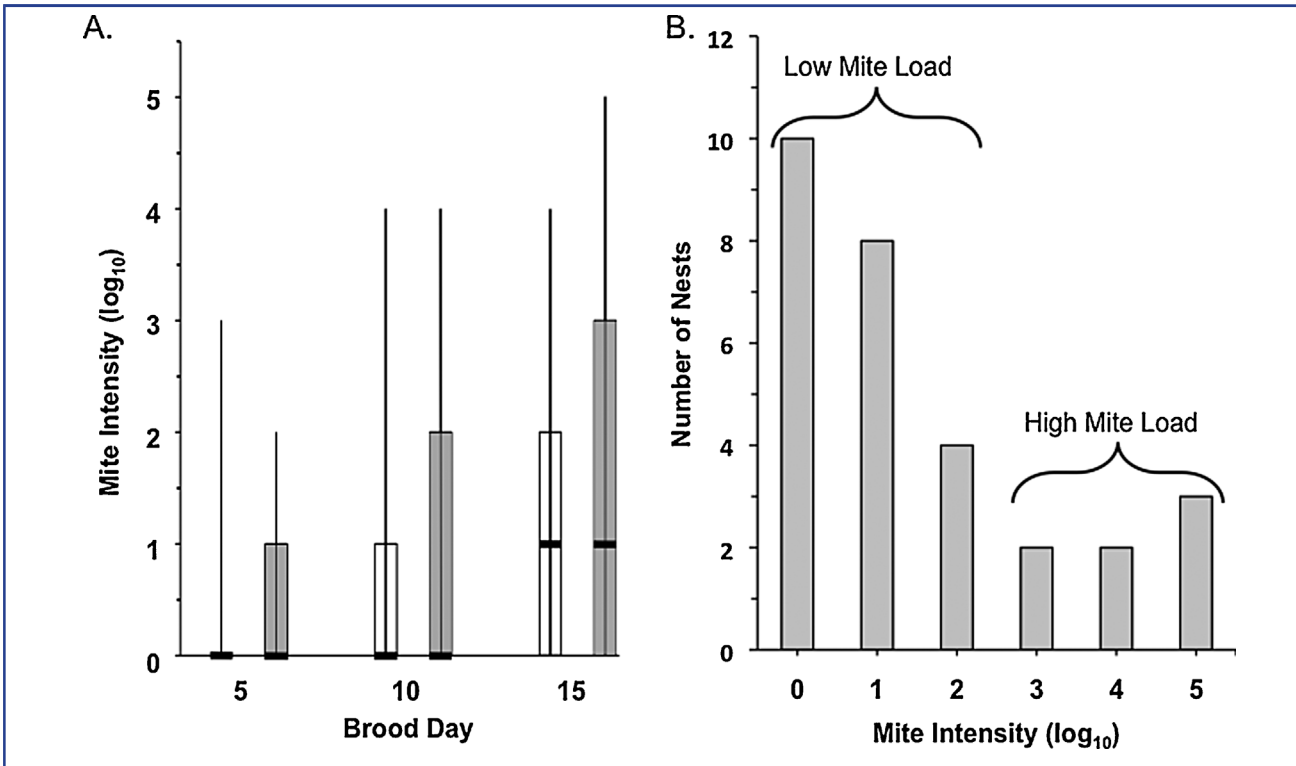

Figure 1. Experimental and non-experimental variation in mite loads among nests on brood days 5, 10, and 15. A: box-and-whiskers plot illustrating mite infestation intensities as a function of nestling age and pesticide treatment; horizontal bars indicate medians, boxes are first through third interquartile ranges, and vertical lines depict the range of mite intensity; permethrin treatment is indicated by unfilled boxes, and control treatment by filled boxes. B: frequency histogram depicting the number of nests in each mite infestation intensity level at brood day 15; nests included in the quasi-experimental high mite load and low mite load groups are indicated by the brackets.

loads on each of the dependent variables. It is the results of these quasi-experimental statistical analyses that are reported and discussed from hereon.

Growth and Development

We found significant effects of both mite load ($F_{129.1} = 5.58$, $P = 0.025$) and age ($F_{2,211} = 1{,}333.64$, $P < 0.0001$) on nestling mass as well as an age by mite load interaction ($F_{2,211} = 5.30$, $P = 0.0057$). Although there were no apparent differences in nestling mass on brood day 5, young from nests with high mite loads had lower mass than those from nests with low mite loads on both brood days 10 and 15 (Fig. 2A). There was a significant effect of both mite load ($F_{127.4} = 6.37$, $P = 0.018$) and age ($F_{2,200} = 1{,}907.84$, $P < 0.0001$) on tarsus length, but no significant interaction ($F_{2,200} = 2.18$, $P = 0.115$; Fig. 2B). Finally, we found a significant effect of both mite load ($F_{129.7} = 4.34$, $P = 0.046$) and age ($F_{2,219} = 6333.25$, $P < 0.0001$), as well as an age by mite load interaction on wing length ($F_{2,219} = 4.40$, $P = 0.0134$). Nestlings from nests with high mite loads had shorter wing lengths at brood days 10 and 15 than those from nests with low mite loads (Fig. 2C). Figure 2D depicts the percentage differences ([(High mite load − Low mite load) Low mite load^{-1}]100) in growth rates in body mass, tarsus length and wing length between nestlings raised with low and high mite loads, illustrating that high mite loads were associated with patterns of slower growth (indicated by increasing differences) relative to low mite loads for mass and tarsus length between brood days 5 and 10 and more rapid growth (indicated by decreasing differences) between brood days 10 and 15. In the case of wing length, compensatory growth began earlier, after brood day 5, and continued through brood day 15.

Blood Parameters

Mite load significantly affected blood glucose levels of nestlings ($F_{126.2} = 10.81$, $P = 0.0029$), such that nestlings from nests with high mite loads had higher levels. Blood glucose levels also increased significantly with age ($F_{2,213} = 15.68$, $P < 0.0001$), with no significant interaction ($F_{2,213} = 2.41$, $P = 0.0919$; Fig. 3A). Mite load ($F_{128.1} = 32.74$, $P = <0.0001$) and age ($F_{2,200} = 9.78$,

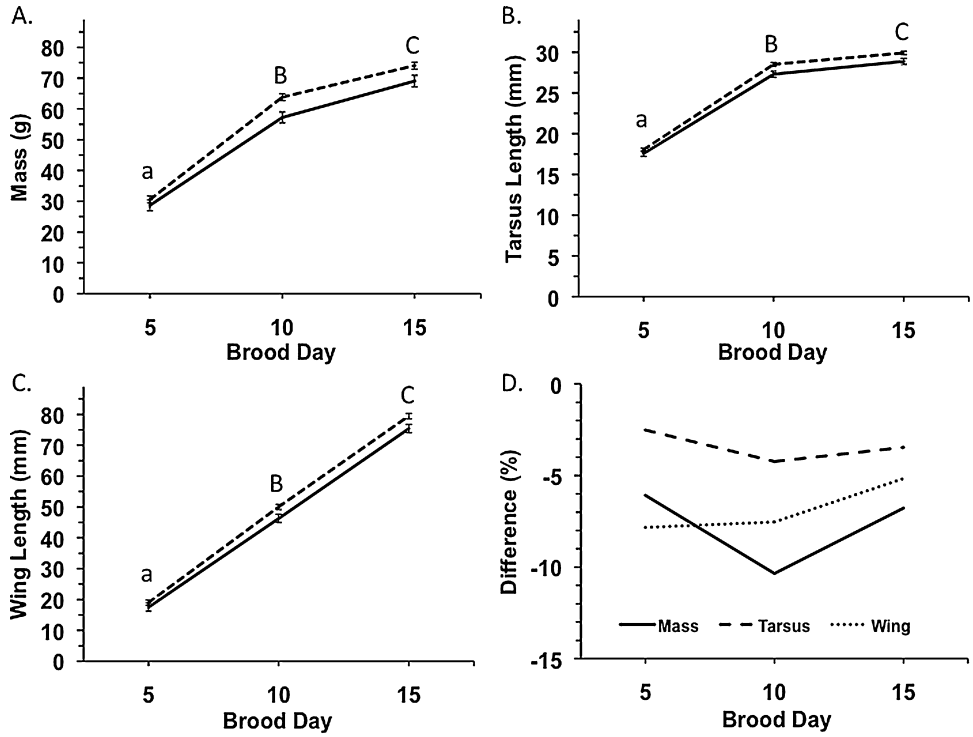

Figure 2. Growth patterns in brood day 5, 10, and 15 nestlings raised in nests with either high or low mite loads of (A) mean body mass (g ± SE); (B) mean tarsus length (mm ± SE); (C) wing length (mm ± SE); and (D) percent mean differences in mass, tarsus, and wing, between nestlings that experienced low and high mite loads. In panels A–C dotted lines indicate low mite loads and solid lines indicate high mite loads. Uppercase superscript letters indicate ages at which high and low mite load groups differed; different superscript letters indicate differences among ages ($P < 0.05$).

$P < 0.0001$) also significantly affected nestling hematocrit, with no significant interaction ($F_{2,200} = 1.17$, $P = 0.314$). Nestlings from nests with high mite loads had lower levels of hematocrit, which irrespective of mite load was lower on brood day 10 than brood days 5 and 15 (Fig. 3B).

Immune Function

Nestlings that were primed with an injection of PHA on brood day 5 did not have significantly different swelling responses induced by a PHA challenge than nestlings that were primed with saline on brood day 5 ($F_{168} = 0.15$, $P = 0.6985$). There was also no significant effect of mite load ($F_{168} = 2.01$, $P = 0.161$) or an interaction between mite load and priming injection on PHA-induced swelling ($F_{168} = 0.02$, $P = 0.902$). Although mite load did not significantly affect bactericidal ability ($F_{125.6} = 0.00$, $P = 0.978$), there was a significant age effect ($F_{2,212} = 52.34$, $P < 0.0001$), as well as a significant interaction of age and mite load ($F_{2,212} = 18.75$, $P < 0.0001$). Bactericidal ability increased with age; however, nestlings in nests with high mite loads had higher bactericidal ability at brood day 5 of development, and lower bactericidal ability at brood day 15 of development, compared with nestlings in nests with low mite loads (Fig. 4).

Plasma Corticosterone

There were no significant correlations between plasma corticosterone and collection latency (characterized as either time from initial nest disturbance or time to collect the first blood sample) on days 5 and 10 (all P values ≥ 0.062; Table 1). We did find a significant correlation between plasma corticosterone and time from initial disturbance on day 15 nestlings ($F_{196} = 35.80$, $P < 0.0001$), but not between plasma corticosterone and the time to collect the first blood sample ($F_{198} = 1.50$, $P = 0.224$; Table 1).

Mite load did not affect plasma corticosterone concentrations; nestlings from nests with high mite loads did not have significantly different concentrations of corticosterone than nestlings from nests with low mite loads ($F_{1,25.2} = 1.15$, $P = 0.293$). There was, however, a significant effect of age ($F_{2,173} = 3.27$, $P = 0.0405$), but no age by mite load interaction ($F_{2,173} = 1.86$, $P = 0.159$). Regardless of mite category,

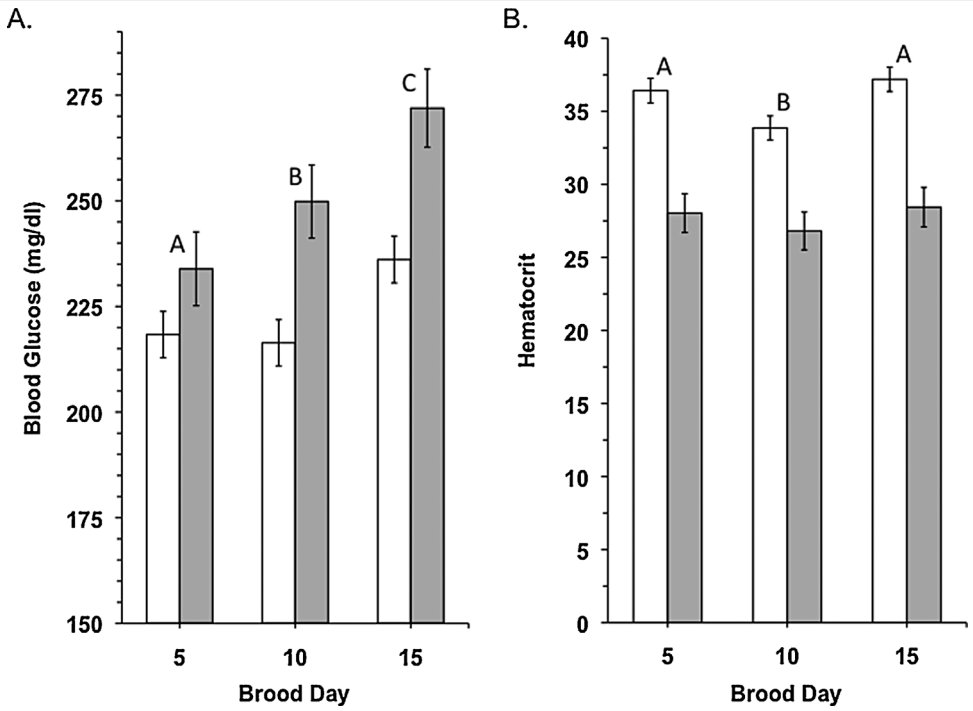

Fig. 3. Blood parameters in brood day 5, 10, and 15 nestlings raised in nests with either high (filled bars) or low (unfilled bars) mite loads of (A) mean blood glucose levels (mg/dl ± SE); (B) mean hematocrit levels (±SE). Uppercase superscript letters indicate ages at which high and low mite load groups differed; different superscript letters indicate differences among ages ($P < 0.05$).

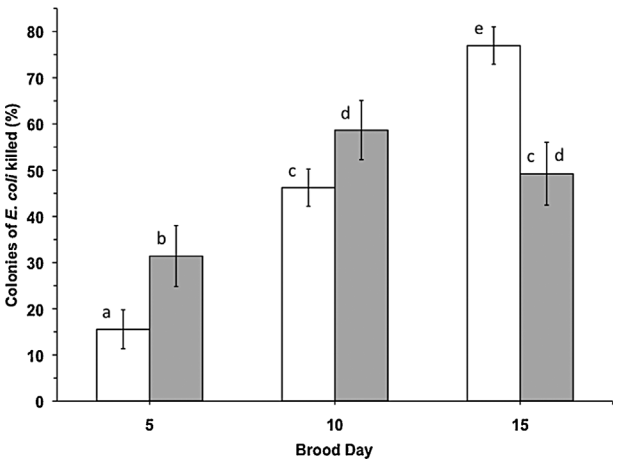

Fig. 4. Mean percentage (±SE) of *Escherichia coli* colonies killed by plasma from brood day 5, 10, and 15 nestlings raised in nests with high (filled bars) and low (unfilled bars) mite loads. Bars with differing superscript letters indicate ages or mite load categories that differed significantly ($P < 0.05$).

concentrations of plasma corticosterone were higher in day 15 nestlings than in day 5 or 10 nestlings (Table 1).

DISCUSSION

We set out to examine the effects of blood-feeding ectoparasites on the physiology and immunology of nestling European starlings, and tested a set of hypotheses related to these aims. We first hypothesized that ectoparasites would induce growth-immunity trade-offs in parasitized nestlings, and predicted nestlings experiencing high mite loads would exhibit reduced growth or reduced immune function relative to those from less parasitized nests. As discussed below, we found evidence in support of ectoparasite-related decreased growth and increased immunity at early nestling ages and the exact opposite pattern in older nestlings.

We found significant effects of mite load on mass, wing, and tarsus length of nestlings. As illustrated in Figure 2D, these differences were greatest when assessed at brood days 5 (wing length) and 10 (mass and tarsus length), and subsequent reduction in the size of these differences indicates accelerated or prolonged growth in all three measures by nestlings in nests with high mite loads, although the pattern is most pronounced for nestling mass. Such compensatory growth would allow nestlings

that experience nutritional stress during early stages of growth to achieve near normal adult size prior to fledging, which may explain why other studies in European starlings have not found effects of ectoparasites on body size when collecting measurements just prior to fledging (Fauth et al., '91; Gwinner and Berger, 2005). However, there can be costs associated with compensated growth occurring late in nestling development (Metcalfe and Monaghan, 2001; Monaghan, 2008). Previous work has found both decreased lifespan and reduced adult competitiveness in individuals that experienced compensatory growth in a commonly studied songbird, the Zebra finch (*Taeniopygia guttata*; Birkhead et al., '99; Spencer and Verhulst, 2008). In addition to costs later in life, more immediate trade-offs between compensated growth and other important developmental process such as maturation of immunity or perhaps the HPA axis may also result.

We found that differences in mite load were related to blood glucose levels, and that blood glucose levels increased with nestling age. Nestlings from high mite load nests had significantly higher levels of blood glucose than those from low mite load nests. It is possible that nestlings in the high mite load nests were elevating blood glucose to compensate for resources lost through the feeding of the ectoparasites, or they may have been mobilizing additional resources to permit increased growth. The age-related increase in blood glucose is consistent with a previous report in developing starlings in which blood glucose levels increased between 1 and 8 days of age after which they were maintained at adult-like levels until fledging (Jurani et al., 2004). As in that earlier study, the developmental increase in glucose reported here may be attributed to increases in the capacity for gluconeogenesis or may relate to developmental changes in metabolic rate and body mass (Jurani et al., 2004).

The positive relationship between mite load and blood glucose levels was unexpected. Although blood glucose in passerine nestlings has been shown to vary with brood size (Bańbura et al., 2008), individual nestling size, weather conditions and nesting habitat (Kaliński et al., 2014), the physiological mechanisms that regulate blood glucose levels in passerine nestlings are as yet unknown. Glucose levels in birds are known to be higher than levels in other vertebrate taxa and the hormone glucagon typically exerts a greater influence than insulin does on blood glucose (Hazelwood, 2000). With respect to the endocrine stress response in birds, plasma glucose levels increase in response to exogenous corticosterone administration in chickens but not in turkeys, the latter which may rely more on sympathoadrenal regulation of glucose during stress (Thurston et al., '93). Given the distinct differences in adrenal regulation of blood glucose in these relatively closely related precocial species and the paucity of research in more distantly related altricial passerine nestlings, the likely mechanism(s) by which mite load might influence blood glucose in the starling nestlings studied here remains elusive, but given the current results, may be corticosterone independent.

An alternative explanation for the positive relationship between mite load and blood glucose is that parents at nests with high mite loads may alter the nestling diet, feeding a higher proportion of sugar-rich fleshy fruits such as cherries and mulberries, which are rapidly reflected by increases in blood glucose (Martinez Del Rio, 1988). Similarly, as parasite burdens often tend to increase in our colony throughout the breeding season due to exponential growth, highly parasitized nests may have been fed at times when fleshy fruits were more readily available than soft-bodied invertebrates. We have observed accumulation of fruit pits in nests as first broods near fledging and during provisioning of replacement nests following failure of first nests (Casto unpublished data), suggesting that the diets of nestlings in or study population do include these fruits and vary across the breeding season; however, nestling diet was not assessed in this study. This possible alternative, as well as others, underscores the need for more experimental investigations of ectoparasite load to control for potential confounds in future studies. Given that glucose is essential for survival and its concentration in the blood is tightly regulated (Lill, 2011), the disregulation of glucose in response to blood-feeding ectoparasites might be an important factor to consider in addition to basal corticosterone levels when characterizing the physiological response of nestlings to ectoparasitism.

Unlike blood glucose, hematocrit showed an opposite pattern with respect to mite loads, as it was 20% lower in nestlings raised in nests with high mite loads than in those from nests with low mite loads. Our findings are consistent with other studies that have shown significant decreases in hematocrit associated with blood-feeding ectoparasite infestation (e.g., Simon et al., 2004; Brommer et al., 2011), and suggest that erythropoiesis does not keep pace with the amount of red blood cells lost to mites. While lost blood volume may be quick to recover in birds compared to mammals due to enhanced ability to mobilize extravascular fluids (Ploucha and Fink, '86), recovery of red blood cells via erythropoiesis is likely a slow process. Perhaps due to the dramatic increases in blood volume that accompany rapid nestling growth (Fair et al., 2007), young appear limited in the amount of additional erythropoiesis they can accommodate. Alternatively, limited resources may get shunted away from erythropoiesis and toward immunity or other physiological functions in young exposed to high mite loads. Irrespective of its proximate cause, the anemia found in highly parasitized nestlings and any associated reductions in aerobic performance in young upon nest leaving (O'Brien et al., 2001) would be expected to persist until hematocrit recovers.

Nestlings from nests with low mite loads exhibited steady increases in bactericidal ability of plasma across development, a pattern similar to that reported for another cavity nesting species, the tree swallow (*Tachycineta bicolor*; Stambaugh et al., 2011). In comparison to the starling nestlings from low mite load nests, those from nests with high mite loads exhibited enhanced

bactericidal capacity of plasma at brood days 5 and 10, yet significantly reduced capacity at brood day 15. Other studies have also found that ectoparasitism is associated with enhanced innate immunity (De Coster et al., 2010; Pitala et al., 2010), but the dampened response exhibited by the brood day 15 nestlings exposed to high mite loads likely indicates a trade-off between immunity and compensatory growth. Nestlings may compensate for the inhibitory effects of ectoparasites on early growth by investing less in immunity and more in growth during later nestling development. Experimentally manipulating ectoparasite load in future studies should allow a more clear assessment of the effects of ectoparasites on these types of developmental trade-offs.

Unlike with bacterial killing, we found no effect of mite load on our test of delayed-type hypersensitivity, the PHA-induced wing web swelling response. Similar responses to PHA in nestlings infested with hen fleas (*Ceratophyllus gallinae*) have been reported in both Tree swallows (Harriman et al., 2014) and in Blue tits (*Cyanistes caeruleus*; Brommer et al., 2011; but see Pitala et al., 2009 for contrasting results in the same system). However, a number of previous studies have shown significant reductions in nestling PHA-induced swelling associated with ectoparasite infestation (reviewed in Owen et al., 2010). It may be that either the magnitude of mite infestation does not effect this response during nestling development in starlings, or that they were not yet fully capable of responding to a PHA priming exposure at brood day 5 or similarly a PHA challenge at brood day 16. Further investigation of the effects of blood-feeding ectoparasites on the induction of immune memory in nestlings may clarify these alternative explanations.

We also tested the hypothesis that changes in plasma corticosterone mediate the effects of ectoparasites on growth and immunity, and predicted that plasma corticosterone levels should be higher in nestlings of highly parasitized nests than in those from less parasitized nests. Corticosterone levels did not differ in nestlings regardless of mite load, a finding that suggests the effects of mite load on nestling growth and immunity reported here are not mediated via changes in plasma corticosterone. It is possible that starling nestlings are not yet competent to respond to ectoparasites with an elevated adrenocortical response. Although we did not directly assess the ability of nestlings to produce an adrenocortical response to handling stress by administering an experimental stress procedure (sensu Lynn et al., 2013), we did assess the relationship between handling duration during blood collection and plasma corticosterone concentration and between the time elapsed from first nest disturbance to blood collection for each nestling in a nest and its plasma corticosterone concentration. We found no evidence of a detectable adrenocortical response to these putative stressors for 5 or 10 day-old nestlings, but did detect an elevated response in 15 day-old nestlings related to the time elapsed from first nest disturbance (see Table 1). While the lack of an adrenocortical response to handling may in part be due to very rapid blood collection in most older nestlings (brood day 10 and brood day 15 nestlings were typically bled within 2 min of being removed from the nest) that precluded detection of the relatively slow adrenocortical response (Romero and Reed, 2005), at all ages nestlings bled subsequent to the first nestling in a nest experienced substantially greater than 5-min durations between first nest disturbance and collection of the first capillary tube of blood for corticosterone assessment. The fact that there was no significant relation between the time elapsed and plasma corticosterone in day 5 or 10 nestlings is surprising, but could indicate that nestling handling or nest disturbance by researchers are not stressors (i.e., something that induces an adrenocortical response) in younger starling nestlings, or that the adrenocortical response to stressors is not very well developed prior to brood day 15 in this cavity-nesting species.

Research in other species does indicate that nestlings are capable of mounting adrenocortical responses during earlier phases of

Table 1. Baseline plasma corticosterone levels and their associated correlations with blood collection latencies: range, (mean ± SE).

	Baseline corticosterone (ng/mL)	First nest disturbance until first tube collected (sec)	Removal from nest until first tube collected (sec)
Brood day 5	5.66–65.91 (17.57 ± 1.18)	46–6087 (905.27 ± 113.04) $r^2 = 0.0244$	39–260 (98.77 ± 5.96) $r^2 = 0.0551$
Brood day 10	3.54–86.67 (16.87 ± 1.45)	38–2040 (729.53 ± 55.88) $r^2 = 0.0149$	18–425 (61.24 ± 4.17) $r^2 = 0.0048$
Brood day 15	3.08–127.84 (22.80 ± 2.01)	37–1939 (652.96 ± 51.21) $r^2 = 0.272$*	27–123 (60.53 ± 1.87) $r^2 = 0.0151$

*$P < 0.01$.

nestling development, including in response to ectoparasites (Quillfeldt et al., 2004; Raouf et al., 2006; Lynn et al., 2013). The fact that we did not find an effect of ectoparasite load on plasma corticosterone levels in the present study may indicate that starling nestlings engage in an alternative response to what may be considered a chronic developmental stressor, that is, rather than activating the HPA axis, they dampen this response, protecting limited energetic resources. In a recent study on song sparrows (*Melospiza melodia*), another developmental stressor, food restriction during the nestling period, also did not result in elevated levels of corticosterone relative to controls, nor did it alter final body composition (Schmidt et al., 2012). Thus, there appear to be species-specific differences in early responses to a variety of developmental stressors. Direct manipulation of corticosterone and ectoparasite load may help clarify whether nestling starlings are indeed engaging in alternative responses to developmental stressors that do not directly involve activation of the HPA axis.

CONCLUSIONS

We are cautious not to over interpret the data given the quasi-experimental nature of our statistical analyses. Our original experimental design proved untenable as the experimental treatment designed to alter ectoparasite burdens in nests was ineffective due to the low initial levels of parasites in the nests (new nest boxes were used in this breeding season), and perhaps suboptimal placement of pesticidal strips within nest boxes. Given that other studies have had similar experiences with the efficacy of experimentally treating nests with pesticides (e.g., Eggert et al., 2009), we view these results as somewhat preliminary and in need of future experimental corroboration. Despite these setbacks, we found support for the idea that hematophagous ectoparasite infestation in the nests of European starlings is associated with growth-immunity trade-offs in nestlings prior to fledging. These trade-offs appear first as decreased growth and increased innate immunity at early stages of nestling development and then as compensatory growth and decreased innate immunity at later stages. While we found developmental increases in plasma corticosterone concentration regardless of ectoparasite burden, we found no indication that nestlings increased corticosterone in response to ectoparasite density. Nevertheless, the ectoparasite-associated, growth-immunity trade-offs reported here suggest that northern fowl mites may be acting as developmental stressors in starling nestlings (Brommer et al., 2011). A fully experimental approach, that increases as well as decreases blood-feeding ectoparasite burden, is needed to further address these questions and to test whether other forms of immunity are involved in this developmental trade-off.

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