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RESEARCH ARTICLE

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Ectoparasites as developmental stressors: Effects on somatic and physiological development

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Abstract

Developmental stress can alter resource allocation in early life, and in altricial birds with rapid developmental trajectories and high resource demands, nestlings may adjust early resource partitioning to cope with challenging environments. We experimentally manipulated ectoparasite levels in nests and assessed whether ectoparasites affected somatic and physiological development in European starling (*Sturnus vulgaris*) nestlings. We hypothesized that mites act as developmental stressors in nestlings and predicted that nestlings from infested nests would exhibit either reduced somatic growth, or reduced physiological development, including impaired innate immunity, and would have elevated corticosterone concentrations. We either added ≈200 mites to nests during early incubation, or treated nests with a pesticide, permethrin, to reduce mites and possibly other arthropods. We assessed treatment effects on egg spottiness and mite abundance, and monitored offspring hatching and survival. We also measured somatic growth (mass, tarsus length, and feather growth), hematocrit, immune-related metrics (bacterial killing ability [BKA] and spleen mass), and baseline corticosterone concentrations in response to treatment. Compared with mite treatment, permethrin reduced egg spottiness and mite abundance in nests. Relative to nestlings in mite-reduced nests, nestlings in mite-enhanced nests had lower survival, hematocrit, and corticosterone concentrations. Early in development, nestlings from both treatments exhibited similar rapid somatic growth, yet mite-treated nestlings exhibited lower BKA. Nestlings in both treatments increased BKA across development, despite nestlings in mite-treated nests exhibiting lower mass as nest leaving neared. Overall, we found evidence that mites can act as development stressors, but contrary to our prediction, mites decreased corticosterone concentrations.

1 | INTRODUCTION

Development is a very energetically costly process for most organisms (Brzek and Konarzweksi, 2007). While there is variation in the amount of time organisms take to develop, in general they must have enough energy to allow for both somatic growth and the development of physiological systems, such as the circulatory, endocrine, and immune systems, in a relatively short period of time. The energetic demands of rapid growth coupled with the external constraints influencing growing individuals often force trade-offs between key physiological processes (McCarty, 2001). Both somatic and physiological development must compete for limited resources within an individual (Møller, 1997). Issues associated with resource limitations tend to be accentuated in organisms that must grow rapidly within a short window of time, such as altricial nestlings (Brzek & Konarzewski, 2007).

Developmental stress can affect many different key processes in the body including growth and immune function. Both of these processes are costly, and as a result when resources are limited, one or both may exhibit developmental deficiencies. Several studies have shown a negative relationship between immune function and growth rates such that when investment in immune function is low, growth rates are significantly higher and vice versa. For example, chicks reared in germ-free environments have substantially lower metabolic rates, and greater rates of growth as compared with conventionally reared chicks (Lochmiller & Deerenberg, 2000). In contrast, magpie nestlings (*Pica pica*) that were supplemented with methionine (an immunoenhancing supplement) had significantly higher T-cell-mediated immune responses and significantly lower growth rates (Soler, Neve, Perez-Contreras, Soler, & Sorci, 2003). While these types of trade-offs naturally occur due to limited resources and high energetic demand, the environment in which a nestling is reared can strongly influence how they respond.

External factors, such as sibling competition, predation risk, food supply, disease, and parasitism, that influence survival are potential

key mediators of offspring fitness, and can strongly affect which physiological process is favored. Sibling competition is particularly important with regards to altricial nestlings in which parental provisioning is the sole source of food. Asynchronous hatching often leads to size hierarchies in which some nestlings are significantly smaller than their siblings (Amundsen & Stokland, 1988; Martinez Padilla and Vinuela, 2011; Stouffer & Power, 1991). When resources are low and overall provisioning rates are diminished, smaller nestlings often die due to an inability to compete with their larger siblings (reviewed by Werschkul & Jackson, 1979). Reaching a size that allows adequate competitive ability may be a key determinate between life and death, and as a result, early nestling growth may be favored at the expense of immune function. Growth is also often favored over immune function when rates of predation are high and relatively quick dispersal from the nest is adaptive. Predation risk is important in determining growth rates in many passerine species (reviewed in Remes & Martin, 2002). If predation risk is high during development, nestlings may benefit by investing more energy in growth in order to leave the nest sooner to reduce predation risk.

Unlike the case with increased predator pressure, increased parasite pressure during development may direct energy away from growth. Ectoparasites are common in the nests of birds and can affect both nestling growth rates and survival, and might be expected to influence the ability of individuals to cope with increased resource limitation. Ectoparasites compete with their hosts for resources that could otherwise be used for growth and self-maintenance. Previous research commonly reports anemia in vertebrate hosts infested with hematophagous ectoparasites and numerous studies have shown a significant decline in host fitness associated with ectoparasite infestation (reviewed in Lehmann, 1993). Ectoparasites can also vector disease and induce costly immune responses in their hosts, which can lead to even higher energetic demands diverting even more energy from growth and development (Lochmiller & Deerenberg, 2000; Owen et al., 2010; Pryor & Casto, 2015).

Ectoparasites might also affect their developing avian hosts by elevating their baseline and stress-induced levels of corticosterone (Eggert, Jodice, & O'Reilly, 2009). Short-term increases in corticosterone may benefit nestlings, allowing them to mobilize stored energy and increase behaviors such as begging that might increase parental provisioning and hence food availability (Kitaysky et al., 2002; Loiseau, Sorci, Dano, & Chastel, 2008). Protracted increases in corticosterone, however, can be very detrimental to birds, resulting in suppression of the immune system and catabolism of muscle in adult birds, and impaired cognitive development and reduced growth in nestlings (Kitaysky, Kitaiskaia, Piatt, & Wingfield, 2003; Spencer & Verhulst, 2007). The functions of corticosterone in nestlings have not been as well studied and are less well understood, but the adrenocortical response to stress is thought to help maintain homeostasis by shifting energy away from non-essential processes and activities to those more essential to survival (Spencer & Verhulst, 2008). The mechanism by which ectoparasites induce a corticosterone response is unclear; however, reduced energy availability due to competition with ectoparasites for nutritional resources may induce increased corticosterone signaling in nestlings.

The European starling (*Sturnus vulgaris*) is an excellent model to study the effects of ectoparasites during development. In North America, the European starling is an introduced species that nests in secondary cavities (Mazgajski, 2007). They are invasive pests throughout most of their introduced range, but are experiencing substantial population declines across northern Europe and Britain (Freeman, Robinson, Clark, Griffin, & Adams, 2007). Throughout their native and introduced range, European starlings have been documented as having heavy parasite burdens that differ with region (Boyd, 1951; Fairn, Hornsby, Galloway, & Barber, 2014; Mitchell & Turner, 1969; Powlesland, 1977). Much research focusing on starlings and ectoparasites has studied the hypothesis that adult starlings incorporate green, aromatic plant material into nests to reduce nest ectoparasite numbers or otherwise improve nestling growth and immunity, and while this hypothesis has received some support (Clark & Mason, 1985) other studies find beneficial effects on nestling outcomes, but no effect on ectoparasite numbers (Fauth et al., 1991, Gwinner, Oltrogge, Trost, & Nienaber, 2000; Gwinner & Berger, 2005). Relatively little early research focused on the effects of heavy parasite burdens nestling survival or offspring phenotype. This paucity of information may be due to the high fledging success rates of starlings despite large parasite loads (Powlesland, 1977), and the idea that a lack of easily identifiable effects on survival is uninteresting (Linz, Homan, Gaukler, Penry, & Bleier, 2007). However, understanding how starlings compensate for high parasite abundance may help to explain how invasive species, such as starlings, cope with a wide range of parasites and are able to be so successful in new environments.

Recent work has focused on the effects of ectoparasites on potential fitness correlates in young starlings, with findings such as the first pre-basic molt is delayed and shortened when young are raised in nests naturally infested by ectoparasites (Pirrello, Pilastro, & Serra, 2015), and that ectoparasite infestation does not reliably alter integument coloration used by nestlings to signal need to parents (Pirrello et al., 2017). A recent study conducted on starling nestlings assessed the effect of varying magnitudes of natural blood-feeding ectoparasite infestation on somatic growth, immune function, and several other physiological variables and found that high parasite loads were associated with decreased growth and increased immune function in early nestling development followed by the inverse pattern in later nestling development (Pryor & Casto, 2015). Because mite abundance in infested nests tends to increase across the breeding season (Garvin, Scheidler, Cantor, & Bell, 2004) and cavity-nesting birds are known to have high among nest variation in natural mite loads (Cantarero et al., 2013; Pryor & Casto, 2015), we used an experimental approach to more directly isolate the effects of ectoparasites on development. The aim of the current study was to experimentally determine if blood-feeding ectoparasites directly affect the development of European starling nestlings by competing with their hosts for resources, and in doing so, affect somatic growth and immunity or other physiological measures. We hypothesized that blood-feeding ectoparasites act as developmental stressors in starling nestlings and predicted that due to competition with ectoparasites for limited resources, nestlings from nests infested with ectoparasites would exhibit either reduced growth or reduced physiological development, including immune function, and

that nestlings infested with ectoparasites would exhibit increases in circulating plasma corticosterone.

2 | METHODS

2.1 | Study site, species, and experimental treatment

This study was conducted in 2011 on a population of European starlings breeding in nest boxes in central Illinois (described in detail by Pryor & Casto, 2015). All 39 nests on the study sites, which commenced egg laying between the April 28 and May 22 and remained active until nest treatments were applied, were included in the study. Nest boxes were checked daily to determine the start of nest building, clutch initiation, clutch completion, and hatching. Three days after the last egg was laid, each nest was randomly assigned to a treatment. Permethrin-treated nests ($n = 17$) were sprayed with approximately 5 mL of a 0.1 % solution of the pesticide permethrin (Durvet Inc., Blue Springs, MO) to reduce ectoparasite numbers. Permethrin was reapplied once every 2 weeks throughout the study. Both eggs and nestlings were removed temporarily while the treatment was applied to reduce direct exposure. The remaining nest boxes ($n = 22$) were inoculated with ≈200 adult northern fowl mites (*Ornithonyssus sylviarum*) that were obtained from nests of the 2010 field season, in order to increase mite numbers to the upper range of their natural abundance based on numbers seen in previous field seasons. The mite treatment was also administered 3 days after the last egg was laid, but was not repeated throughout the study, as mite populations increased naturally as breeding progressed. We did not include a control group with untreated nests because of the high level of natural among-nest variation in mite numbers at our study sites (Pryor & Casto, 2015) that might have confounded mite load with season (Garvin et al., 2004). Once each nestling hatched, one of its toenails was clipped to permit unique within-brood identification until USGS leg bands were applied later in nestling development.

2.2 | Mite maintenance and nest inoculation

Mites were obtained from nest material from the 2010 field season. Nest material from the previous breeding season was collected during the winter and kept refrigerated in zipper lock bags until the 2011 field season to ensure ectoparasites remained dormant. During the field season, nest material was kept at room temperature to allow mites to emerge from dormancy. Mites were collected for transfer by placing a latex gloved hand in the nest material and allowing ≈200 mites (predominantly unfed protonymphs) to crawl onto the glove. The glove was then placed in the nest box and the mites were allowed to crawl off into the nest material.

2.3 | Egg spottiness

Hematophagous ectoparasites often feed on the highly vascularized brood patch of incubating adult starlings. This leaves blood spots on the pristine blue eggs, which can indicate the level of ectoparasite infestation (Aviles, Perez-Contreras, Navarro, & Soler, 2009). To assess the effectiveness of our pre-hatch treatments, we assessed egg spottiness in a subset of nests that successfully hatched young. Clutches of eggs from these nests were photographed 1 day before their expected hatch day. While blind to treatment, photographs were used to assign an egg spottiness score to each clutch, based on the following scale: Eggs were assigned a score of 0 if no spots were present on the eggs, a score of 1 if there were a small number of spots on the eggs, a score of 2 if the eggs were moderately spotted, and a score of 3 if the eggs were largely covered with spots (for examples of egg spottiness scores, see Figure 1).

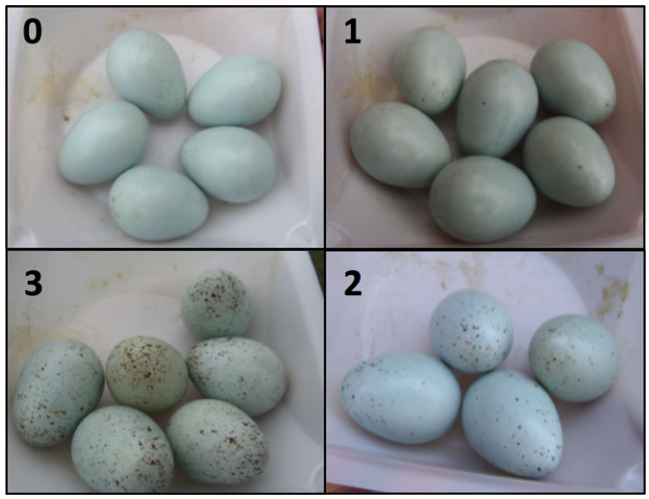

FIGURE 1 Example of spottiness scoring of starling eggs. Top left spottiness score 0, clockwise to spottiness score 3 [Color figure can be viewed at wileyonlinelibrary.com]

2.4 | Mite load in nests

In order to assess treatment effects on mite load, a subset of nests was removed from nest boxes 1 week after nestlings were collected on brood day 17 (*vide infra*). Upon collection, nests were placed in Berlese funnels suspended above glass collection jars containing approximately 200 mL of 95% ethanol. The interior top circumference of the funnels was coated with petroleum jelly, as were the exterior bottom circumferences in order to confine mites to the funnel interior and the collection jar. Mites were driven from the funnel to the collection jar by a continuous 1-week exposure to a 40-watt incandescent light suspended 15 cm above each funnel. Jars were then removed from the funnels and ethanol was added to each jar to ensure that each jar had 275 mL of ethanol. While blind to nest treatment, we used a modified counting method (after Pacejka, Gratton, & Thompson, 1998) to estimate the number of mites in each jar. An initial visual assessment of each jar yielded a gross estimate of the number of ectoparasites. Jars were then placed on a magnetic stir plate to produce a homogenous mixture of ectoparasites throughout the jar. Using a micropipette, four 1 mL or 5 mL samples were then collected from the jars depending upon the gross estimate of ectoparasites (1 mL samples were taken from jars with relatively high mite densities and 5 mL samples were taken from jars with relatively moderate and low densities), and each sample was vacuum filtered onto a separate filter paper. Ectoparasite numbers were then determined under a dissecting microscope. Mean

mite counts from the four samples from each nest were extrapolated to estimate the total number of mites in each collection jar.

2.5 | Somatic growth

Somatic growth was determined by measuring mass, wing length, and tarsus length. The day on which a majority of the eggs in a clutch had hatched was designated as brood day 0. All measurements were taken on brood days 5, 10, and 15 after hatching. Mass was also assessed on brood day 17 to the nearest 0.01 g using a portable electronic scale. 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 mm using digital calipers.

2.6 | Blood sampling and processing

Blood samples were collected by puncturing the brachial vein with a 26 g needle. Blood was allowed to pool on the surface of the wing at the puncture site and collected using 70 μL heparinized capillary tubes. Up to 150 μL of blood was collected from each bird on brood days 10 and 15. Nestlings were removed from the nest one-at-a-time and immediately bled, and then somatic growth measures were collected (*vide infra*). Stopwatches were used to record time of initial nest disturbance, time of nestling removal from the nest, time taken to collect the first capillary tube, and time to finish the bleed. The first tube collected was marked to ensure that plasma from it was used to assess plasma corticosterone, as it would be the least likely to be influenced by an adrenocortical response to disturbance. Blood samples that were used to assess baseline corticosterone levels were collected within 2 min of a nestling's removal from its nest. Previous data from our laboratory suggest that there is no detectable adrenocortical response in the blood of nestlings at brood days 10 and 15 within 2 min of removal from the nest, irrespective of time since the nest was first disturbed (Pryor & Casto, 2015). Immediately after collection, capillary tubes were sealed on one end with clay, stored in coolers with icepacks, and transported to the laboratory. For each blood sample, capillary tubes were centrifuged at 13,300 rpm (17,000 $\times$ *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 was refrigerated at 4°C as needed (*vide infra*) and the remaining plasma was stored frozen at −20°C in microcentrifuge tubes until used to assess plasma corticosterone concentrations.

2.7 | Plasma corticosterone assay

Plasma corticosterone concentrations were analyzed using plasma from the first capillary tube collected after nestling removal from the nest, and measured using 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. For plasma samples, within plate coefficients of variation (CV) ranged from 3.51 to 17.51, and the between plate CV was 5.33.

2.8 | Bacterial killing ability

To measure innate immunity, we performed a bacterial killing ability (BKA) assay. The assay measures the ability of nestling plasma to kill bacteria and as a result allows the study of the ability of natural antibodies to limit early infection, complement enzymes to lyse targeted cells, and lysosyme to enzymatically digest targeted cell walls (Matson, Tieleman, & Klasing, 2006). This particular assay was chosen because it is easily interpreted and measures the coordinated effects of multiple aspects of innate immunity (Matson et al., 2006). Fresh plasma was collected from each nestling on brood days 10 and 15 and used to characterize bactericidal ability across nestling development. The methods for this procedure followed the protocol described in Matson et al. (2006), with modifications by Forsman, Vogel, Sakaluk, Grindstaff, and Thompson (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, then incubated at 41°C for 30 min. A control sample without added plasma was also run to serve as a daily standard to which levels of plasma-induced bacterial killing could be compared. Fifty microliters of each sample, including the control, was plated in duplicate on tryptic soy agar plates and incubated at 37°C for 24 h, after which, bacterial colonies were counted. Bacterial killing is expressed as the proportion of CFU killed relative to the control. Mean colony counts of duplicates were calculated and reported as a proportion of control means to yield an estimate of BKA for each plasma sample.

2.9 | Spleen mass and sex determination

Nestlings were removed from their nest boxes on brood day 17, and were held in ventilated plastic transport chambers before they were euthanized. Time spent in the chambers before euthanasia was standardized between each field site. All nestlings were weighed to the nearest 0.01 g with an electronic balance following euthanasia by carbon dioxide asphyxiation. Spleens were immediately harvested, weighed, and processed for use in unrelated assay development, and sex was determined by visual inspection of the bilateral testes in males and unilateral ovary in females. Despite existing controversy as to whether spleen mass is a useful index of immune function in wild birds (Smith & Hunt, 2004), spleen mass was assessed and is reported here because, in starlings, nestlings with large spleens mount strong adaptive immune responses to the plant lectin phytohemagglutinin (Ardia, 2005), a mitogen commonly used in ecoimmunology research (Demas, Zysling, Beechler, Muehlenbein, & French, 2011).

2.10 | Statistical analysis

We analyzed the effect of nest treatment on egg spottiness and final mite abundance using *t*-tests, and evaluated the effect of nest treatment on nest success, both during incubation and post hatching, using Cox proportional hazard survival regression (Lawless, 1982). We analyzed the effects of nest treatment and, in most cases age, on

measures of somatic development and physiology (i.e., hematocrit, baseline corticosterone concentration, BKA, and spleen mass) with repeated measures linear mixed models, in which nesting age was included as a repeated measure and nest identity was included as a random effect. When necessary, differences among least squares means were analyzed using Fisher's least significant difference to further probe significant main and interaction effects. All models were initially run with sex, hatch date, and brood size as covariates; if any of these covariates were significant, they were retained in the model and are reported in the results, and non-significant covariates were removed from the model and are not reported in the results. The corticosterone data were transformed using an inverse square root transformation and group means and standard errors were back-transformed for graphical display. All other statistical analyses and graphs used untransformed data. Analyses were performed using SAS 9.4 Software (SAS Institute Inc., Cary, NC).

3 | RESULTS

3.1 | Egg spottiness, mite load estimates, and nest success

Nest treatment significantly affected egg spottiness. The eggs from eight mite-treated nests had significantly higher mean spottiness scores than the eggs from 11 permethrin-treated nests ($t_{18} = 4.86$, $P = 0.0006$; Figure 2A). Based on a subsample of nine mite-treated and 11 permethrin-treated nests from which nestlings were collected on brood day 17, mite-treated nests had significantly higher estimated mean mite numbers than did permethrin-treated nests ($t_{19} = 2.24$, $P = 0.038$; Figure 2B).

Nest treatment significantly affected hatching success during incubation (Likelihood ratio, $\chi^2 = 5.863$, $P = 0.016$; Figure 3A), as mite-treated nests had significantly higher rates of abandonment/nest failure (six of 22) than permethrin-treated nests (0 of 17). Although not significant, during post-hatching development, there was a similar trend for a higher failure rate in mite treated nests (Likelihood ratio, $\chi^2 = 3.665$, $P = 0.056$; Figure 3B), as five of 16 mite-treated nests and one of 17 permethrin treated nests failed prior to collection of nestlings on brood day 17.

3.2 | Nesting growth

Unlike the other somatic growth measures, mass was collected on brood days 5, 10, and 15, as well as on brood day 17. Nest treatment did not significantly affect nestling mass ($F_{1,28.8} = 0.18$, $P = 0.671$), but nestling age did ($F_{3,312} = 1158.38$, $P < 0.0001$), as did the interaction of nest treatment and nestling age ($F_{3,312} = 8.75$, $P < 0.0001$). As depicted in Figure 4A, nestlings from mite-treated and permethrin-treated nests tended to be of similar weight on brood days 5 and 10 ($P = 0.187$, and $P = 0.168$, respectively). As development progressed, nestlings from mite-treated nests tended to weigh less than those from permethrin-treated nests on brood day 15 ($P = 0.061$), and weighed significantly less on brood 17 ($P = 0.033$). Tarsus length increased with

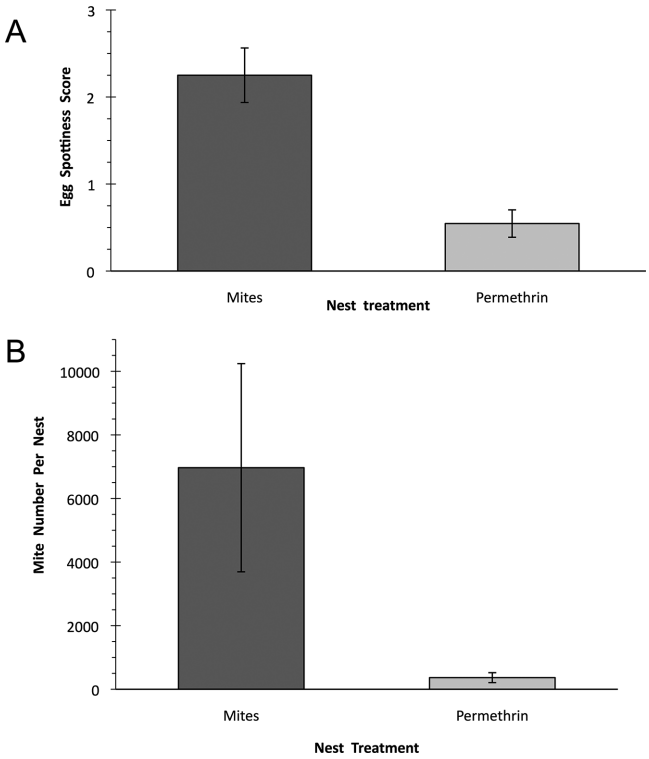

FIGURE 2 Treatment effects on ectoparasite metrics. (A) Mean eggshell spottiness scores of eggs 1 day before expected hatch (±SE). There was a significant difference between nest treatments ($P = 0.0006$). (B) Estimates of mite numbers collected from mite-treated or permethrin-treated nests during development (±SE). There was a significant difference between nest treatments ($P = 0.038$)

nestling age ($F_{2,241} = 479.12$, $P < 0.0001$; Figure 4B); but was not significantly influenced by nest treatment ($F_{1,29.1} = 3.70$, $P = 0.064$) or the interaction of nestling age and nest treatment ($F_{2,241} = 1.92$, $P = 0.149$). Wing length also increased significantly with nestling age ($F_{2,233} = 2954.67$, $P < 0.0001$; Figure 4C), but did not differ significantly between nest treatments ($F_{1,28.7} = 0.710$, $P = 0.759$) nor was it significantly influenced by the interaction of nestling age and nest treatment ($F_{2,233} = 1.45$, $P = 0.236$).

3.3 | Hematocrit

Nest treatment and nestling age significantly affected hematocrit ($F_{1,30.1} = 18.18$, $P = 0.0002$; $F_{1,108} = 17.87$, $P < 0.0001$, respectively). Similarly, nest treatment and nestling age interacted to significantly affect nestling hematocrit ($F_{1,111} = 7.17$, $P = 0.0085$). As illustrated in Figure 5A, in addition to having significantly higher hematocrit values regardless of age, nestlings from permethrin-treated nests exhibited significant increases in hematocrit between brood days 10 and 15 ($P < 0.0001$), while nestlings from mite-treated nests did not ($P = 0.295$).

3.4 | Plasma corticosterone concentration

Nest treatments significantly affected baseline corticosterone concentrations ($F_{1,23} = 5.66$, $P = 0.026$; Figure 5B). Nestlings from

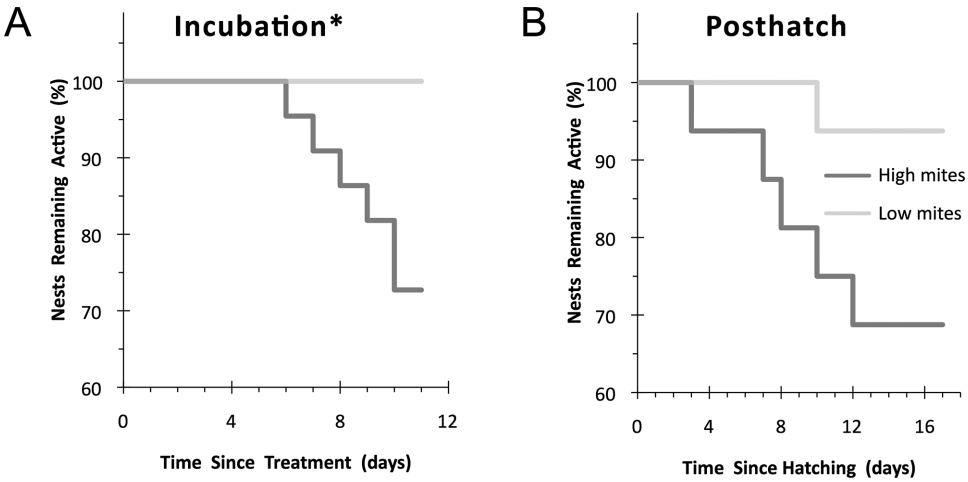

FIGURE 3 Success of mite-treated (high mites) and permethrin-treated (low mites) nests. (A) Percentage of nests that remained active during incubation (from nest treatment until hatching). (B) Percentage of nests that remained active during the post-hatching period (from hatching until nestling collection on brood day 17). The dark gray lines indicate mite-treated nests and the light gray lines indicate permethrin-treated nests. Asterisk indicates significant difference in nest success due to nest treatment during the stage of nesting ($P = 0.016$)

mite-treated nests had significantly lower baseline concentrations of plasma corticosterone than nestlings from permethrin-treated nests. Neither nestling age ($F_{1,107} = 3.05$, $P = 0.084$) nor the interaction of nest treatment and nestling age ($F_{1,107} = 0.30$, $P = 0.586$) significantly affected plasma corticosterone concentrations.

3.5 | Bacterial killing ability

Hatch date had a significant effect on BKA ($F_{1,25.1} = 33.7$, $P < 0.0001$), so that covariate was retained in the model. Nest treatment did not significantly affect BKA of nestling blood plasma ($F_{1,23.6} = 1.46$, $P = 0.2391$). Nestling age ($F_{1,115} = 40.17$, $P < 0.0001$), as well as the interaction of nest treatment and nestling age ($F_{1,119} = 8.41$, $P = 0.0045$) did significantly affect the BKA of nestling plasma. As depicted in Figure 5C, nestling plasma from permethrin-treated nests had significantly greater BKA than that of mite-treated nests on brood day 10 ($P = 0.004$), but not on brood day 15 ($P = 0.246$).

3.6 | Spleen mass

There was no effect of treatment on spleen mass at brood day 17 ($t_{23} = 0.72$, $P = 0.406$). Spleen masses ranged from 0.04 to 0.37 g (mean = 0.119 g, ±0.013) for nestlings from mite-treated nests, and from 0.05 to 0.44 g (mean = 0.104 g, ±0.012) for nestlings from permethrin-treated nests.

4 | DISCUSSION

We set out to experimentally assess the effects of blood-feeding ectoparasites on somatic and physiological development in European starling nestlings. We first hypothesized that mites act as developmental stressors in nestlings, and predicted that nestlings from mite-enhanced nests would exhibit either reduced somatic growth, or reduced development of physiological response variables such as BKA of nestling plasma, nestling hematocrit, and spleen mass relative to those of nestlings from mite-reduced nests. We found that relative to mite reduction, mite enhancement led to lower BKA of plasma, moderately lower nestling mass after the period of rapid growth, a greatly diminished developmental rise in hematocrit, and no effect on tarsus length, wing length or spleen mass. We also hypothesized that the experimental mite enhancement would be perceived physiologically by nestlings as a developmental stressor, and predicted that nestlings from infested nests would exhibit increases in circulating corticosterone. We found no evidence of increased plasma corticosterone concentrations in response to mite enhancement, yet, surprisingly, found the exact opposite pattern. Permethrin-induced mite reduction produced nestlings with significantly higher baseline corticosterone concentrations than those from mite-enhanced nests. Taken together these data suggest that experimentally induced variation in mite infestations differentially affects somatic growth, physiological development, and innate immune function.

4.1 | Egg spottiness, mite abundance, and hatching success

Our nest treatments were highly effective in inducing dramatic differences in ectoparasite infestations in the nests we manipulated, as we found that relative to permethrin treatment, adding ≈200 northern fowl mites to nests significantly increased the density of blood-colored spots on the surface of starling eggs during incubation, as well as the number of mites collected from nest material after nestlings were removed from nests. Together these findings suggest that ectoparasite burdens in mite-enhanced nests were significantly elevated throughout incubation and post-hatching development in comparison with those of permethrin-treated nests. The mite-induced increases in egg spottiness provide support for the somewhat contentious idea that ectoparasites are the cause of egg spots in European starlings (Feare

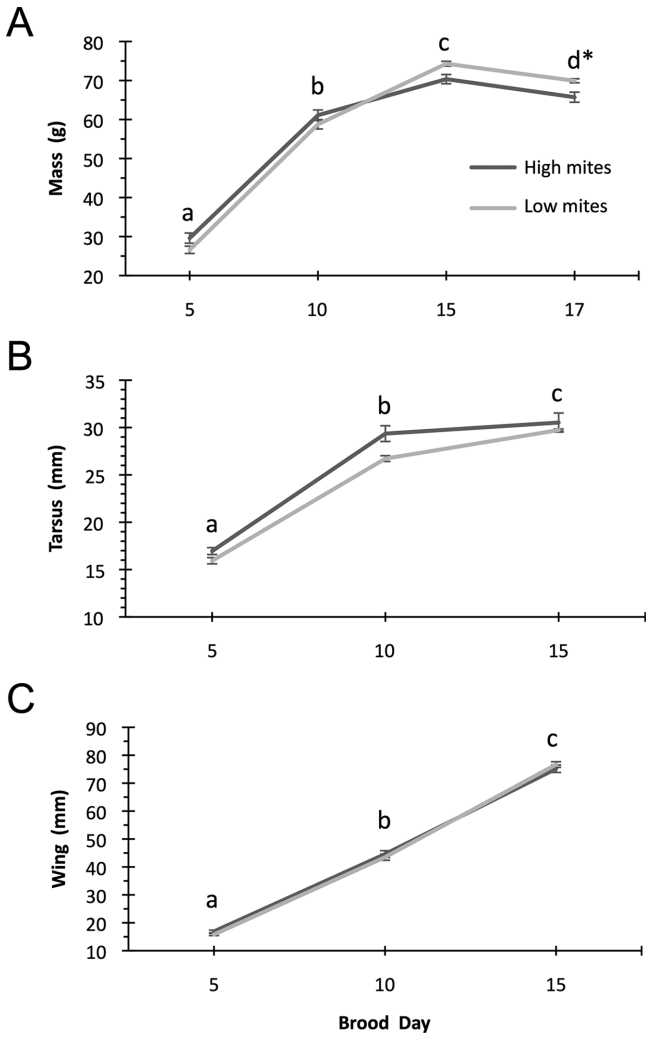

FIGURE 4 Somatic growth in brood day 5, 10, 15, and 17 nestlings raised in mite-treated (high mites) or permethrin-treated (low mites) nests. (A) Mean body mass (g ± SE); (B) mean tarsus length (mm ± SE); (C) mean wing length (mm ± SE). Asterisks indicate ages at which nest treatments differed significantly ($P = 0.033$); different superscript letters indicate significant differences among ages ($P < 0.05$)

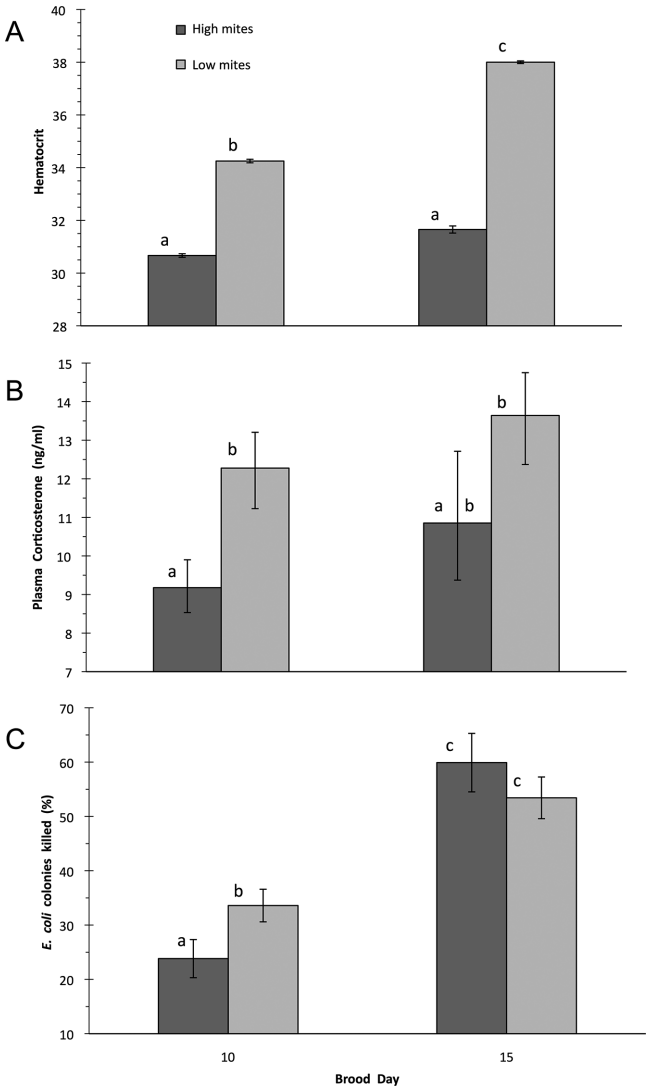

FIGURE 5 Physiological and immune metrics in brood day 10 and 15 nestlings raised in mite-treated (high mites) or permethrin-treated (low mites) nests. (A) Mean hematocrit levels (±SE). (B) Back-transformed mean plasma corticosterone concentrations (ng/mL ± SE). (C) Mean percentage (±SE) of *E. coli* colonies killed by plasma. Bars with differing superscript letters indicate ages or nest treatments that differed significantly ($P < 0.05$)

& Constantine, 1980; Hornsby, Fairn, & Barber, 2013; Jackson, 1970). Similar increases in egg spottiness have been reported in the spotless starling (*Sturnus unicolor*) in response to parasitism by the carnid fly, *Carnus hemapterus* (Lopez-Rull, Gil, & Gil, 2007), but it was not clear how those spots were formed; and it was suggested that the spots may actually be fly droppings (Lopez-Rull et al., 2007). This may also be the case with the spottiness induced by the mites in our study, or, alternatively, the spots may be dried blood from female or male starlings caused by the bleeding of bites to the brood patch. Although it is unclear exactly how these spots are formed, they may significantly impact nest success by influencing the parental behavior exhibited by adults tending the nest. For example, in adult male, but not female spotless starlings, hatchling provisioning rates were reduced in nests containing highly spotted eggs (Aviles et al., 2009). While similar reductions in nestling provisioning in response to egg spottiness have not been detected in a North American population of European starlings (Hornsby et al., 2013), it is possible that such a cue could alter male or female likelihood of continued incubation of eggs and the resulting nesting success. We found mite-treated nests, had higher rates of failure during incubation than nests treated with permethrin (27% and 0% failure, respectively), and research on great tits (*Parus major*) shows similar effects of hen fleas on hatching success (Oppliger, Richner, & Christe, 1993). Our data on hatching success during incubation are generally consistent with this proposed influence of ectoparasite-induced egg spottiness on hatching success, but direct experimental manipulation of egg spottiness would be required to rule out alternative interpretations.

The reasons for the high level of pre-hatching nest failure we report for mite-treated nests are unresolved. We added mites to nests after eggs were laid; however, in a more natural nesting sequence females might have detected high mite loads early and abandoned an infested

nest prior to laying eggs. In fact, we often find recently built nests that are abandoned prior to egg laying, and while we have not sampled these nests to determine if mite loads are high, this is one aspect of nest site quality to which females might attend. Mite abundance in nests typically varies across the breeding season, being somewhat low early, and substantially higher as the breeding season progresses (Garvin et al., 2004; Lehman, 1993). The high rates of nest abandonment/failure we noted in mite-treated nests may be a response to unnatural mite infestation magnitudes in the early breeding season, or to highly synchronized blood feeding by the introduced mites that had overwintered or hatched without access to blood meals and that were likely all seeking simultaneous blood meals. The timing of egg laying in some birds may be an adaptation to avoid high ectoparasite numbers during nesting. Adaptive timing of laying has been found in great tits that delay their laying to avoid high levels of hen fleas (Oppliger et al., 1993). Further studies relating rates of nest abandonment/failure and natural mite variation are needed to understand better the relationship between these variables, which should then allow experimental studies to better approximate natural infestation dynamics.

4.2 | Effects on nestlings

Our results indicate relatively few significant effects of nest treatment on nestling growth. Throughout development, wing and tarsus lengths were not affected by nest treatments, and nestling mass differed between treatment groups only on brood days 15 and 17 when, relative to nestlings from permethrin treated nests, it was 5%–6% lower in nestlings of mite-treated nests. While this difference in somatic growth is in the direction we predicted, it was somewhat surprising in its timing, as it occurred after the species-typical period of rapid growth (Feare, 1984). In a previous study conducted on starlings at these same study sites, Pryor and Casto (2015) found that natural variation in mite burden led to earlier reductions in somatic growth in nestlings exposed to high mite loads relative to those exposed to low mite loads, such that wing, tarsus and body mass were all significantly smaller by brood day 10 and differences tended to diminish by brood day 15. While there are many aspects of these two studies that might account for the differences in the effects of mite infestation on somatic growth, we believe one warrants particular attention. Relative to other mite reduction techniques, experimental pyrethroid (the class of pesticides that includes permethrin) treatment of nests has recently been found to underestimate the effects of ectoparasites on somatic growth in cavity-nesting Pied flycatchers (*Ficedua hypoleuca*) (López-Arrabé, Cantarero, Pérez-Rodríguez, Palma, & Moreno, 2014). In that study, pyrethroids inhibited wing, tarsus and body mass, likely in part via related disruption of glutathione metabolism, an important intracellular antioxidant pathway. While we cannot confirm that a similar effect occurred in nestlings from our permethrin treated nests, we did find unexpectedly higher basal corticosterone levels on brood days 10 and 15 in nestlings from permethrin-treated nests relative to those of mite-treated nests, and elevated corticosterone has been associated with reduced somatic growth in nestlings (Muller, Jenni-Eiermann, & Jenni, 2009; Spencer & Verhulst, 2007; Wada et al., 2008). It is unclear whether our permethrin treatment increased basal corticosterone concentrations, or whether increased mite burden decreased those concentrations; however, in a previous study that assessed the effects of natural variation in mite exposure on starling nestlings without pyrethroid exposure, mite burden did not affect plasma corticosterone concentrations (Pryor & Casto, 2015). Whether pyrethroid pesticides are associated with potentially adverse endocrine-disrupting effects in developing birds and other vertebrate young, is a question that warrants further investigation. This is especially so, given the demonstrated effectiveness of pyrethroids in countering the effects of larvae of the introduced parasitic fly, *Philornis downsi*, on nestlings of various land bird species of conservation concern in the Galapagos Islands (Fessl, Kleindorfer, & Tebbich, 2006; Knutie, McNew, Bartlow, Vargas, & Clayton, 2014), and their considered more widespread use in related conservation efforts (Causton, Cunninghame, & Tapia Aguilera, 2013; Koop, Kim, Knutie, Adler, & Clayton, 2017).

We found that nestlings from mite-treated nests had significantly lower hematocrit (10% lower at brood day 10, and 20% lower at brood day 15) than nestlings from permethrin-treated nests. Ectoparasite-induced anemia has also been reported in Eastern blue bird (*Sialia sialis*) nestlings from nests infested with blowflies (Hannam, 2006). Although hematocrit has its limitations as a sole measure of an individual's condition due to many sources of variation (Fair, Whitaker, & Pearson, 2007), anemia can lead to decreased oxygen transport to tissues that impact flight performance, and fledglings that continue to suffer from anemia could have reduced abilities to evade predators and reduced foraging abilities (O'Brien et al., 2001). A recent study in starling nestlings found that physiological maturity as indexed by both hematocrit and hemoglobin concentration lags behind somatic maturity at nest leaving, that there is greater variability in physiological traits associated with oxygen-carrying capacity than with somatic measures of growth, and that both somatic and physiological maturity contribute significantly to models of initial flying ability at nest leaving (Cornell, Gibson, & Williams, 2017). We expect that any deficits associated with anemia in nestlings would persist until normal hematocrit levels are achieved, which might take several weeks given the slow renewal rates of red blood cell in birds (Rondan et al., 1957). On average, adult European starlings have a hematocrit of 43 during the summer (Hill & Murray, 1987). If nestlings from nests with high hematophagous mite densities are slow to reach these adult levels, it could negatively affect fledgling survival and eventual recruitment into the breeding population.

Regarding innate immune function, we found that plasma from nestlings of mite-enhanced nests had lower BKA on brood day 10, but due to a relatively greater developmental increase in bactericidal activity over the next 5 days, exhibited similar BKA at brood day 15, relative to plasma from nestlings reared in mite-reduced nests. While these results confirm our prediction of reduced immune function in response to experimental mite enhancement (at least through the first half of nestling development), they are quite different from previous results in developing starlings tested under natural mite burdens (Pryor and Casto, 20015). In that study, relative to low mite burdens, high mite burdens led to increased plasma bactericidal activity on brood days 5 and 10 followed by decreased plasma bactericidal

activity on brood day 15. Reconciling these two sets of findings is challenging. In the earlier quasi-experimental study, relative to nestlings raised under low natural mite burdens, nestlings under high natural mite burdens appeared to sacrifice growth for increased innate immunity during the early half of nestling development and vice versa during the latter half. Such a pattern is unexpected in starlings, as smaller nestlings are prone to brood reduction during early nestling development (Feare et al 1984; Korpimäki, 1978). However, the current experimental data suggest that nestlings respond to mite infestation early in post-hatching development by maintaining rapid somatic growth and reducing innate immunity, and only later in development do they reduce body mass in favor of increasing BKA. Differences in overall mite burdens, alterations in baseline corticosterone concentrations and possible growth inhibiting effects of permethrin in nestlings raised under low mite burdens, are all possible explanations for the dissimilar findings from these two studies. Yet another is the possibility that the manipulative experimental approach used in the current study minimized the effects of potential covariation between seasonal increases in northern fowl mite populations (Garvin et al., 2004; Mašán, 1997), and seasonal changes in nutritional resources (Feare, 1984; Pryor & Casto, 2015) and nestling quality (Cornell & Williams, 2016; Serra et al., 2012) that may have been unavoidable in the quasi-experimental study.

5 | CONCLUSIONS

Using experimental enhancement or reduction of northern fowl mites in the nests of starlings, we found significant effects of nest treatment on the spottiness of eggs in late incubation, number of mites in the nest after nestling development, and the likelihood of hatching success and nestling survival. We also found that relative to nestlings from mite-reduced nests, those from mite-enhanced nests exhibit decreased BKA during early nestling development, a stage characterized by rapid nestling growth. This is followed in later nestling development by decreased body mass and compensated BKA. Such a pattern may indicate a mite-induced prioritization of early nestling growth over innate immunity, perhaps to avoid the brood reduction common to early nestling development. Unexpectedly, we found significant treatment effects on baseline corticosterone, such that nestlings in permethrin-treated nests had higher baseline corticosterone concentrations than nestlings in mite-treated nests. Future experiments that not only manipulate ectoparasite burden, but also other aspects of nestling phenotype shown to be affected by ectoparasites, may help clarify the complex relationship between ectoparasitism and nestling development.

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