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Skin decision: Northern fowl mite infection alters diversity of the skin microbiome in European Starling nestlings differentially with age

by

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Introduction

The skin microbiome communicates with and influences the physiology of both host and neighboring microbes through metabolite-mediated interactions (Villageliũ and Lyte, 2017, Swaney and Kalan, 2021) and may encode fitness-relevant information such as disease risk, offspring growth rate, adult predation rates, and cognitive skills of birds (Engel et al., 2020, van Veelen et al., 2018, Tomás et al., 2018). Furthermore, mouse studies have shown that the skin microbiome can be beneficial for host health by enhancing host immune system activation by promoting cytokine expression and pathogen inhibition (Swaney and Kalan, 2021). The surrounding environment has major impacts on the avian skin microbiome (Engel et al., 2020), especially in altricial nestlings that are born blind, with no feathers or thermoregulatory ability, as these obligate nest dwellers are in continuous contact with the nest environment, siblings, and parents.

Parasitic infection may negatively affect commensal bacteria of the skin microbiome, because it can alter the development, physiology, and behavior of the host (Addesso et al., 2020). The northern fowl mite (*Ornithonyssus sylviarum*) is a common hematophagous ectoparasite that affects songbird species worldwide, particularly in temperate regions (Pryor and Casto, 2017, Knee et al., 2021). In addition to infesting the nests of many wild bird species, northern fowl mites are the most widespread and damaging poultry parasite in the United States (Mullens et al., 2012) and have been known to cause pruritic dermatitis in humans (Knee et al., 2021). Climate change is projected to increase the range and abundance of ectoparasites such as the northern fowl mite, therefore it is critical to study the potential consequences of mites on wild species to better understand their impact on host health and survival.

Mites compete with the host for resources (Pryor and Casto, 2017) and cause an immune response, further diverting resources from somatic growth and normal body functions (Mullens et al., 2012). One of the most important host immune responses is inflammation, as this has been shown to physically prevent mites from accessing blood and to neutralize mite salivary compounds that can inhibit host immune responses (Owen et al., 2010). Factors such as the host's immune response, genetics, body condition, and other host traits that interact with environmental conditions such as diet can also affect parasite susceptibility (Griebel and Dawson, 2020).

To better understand the effect of ectoparasites on host species, experimental manipulations of parasite abundance are needed. However, one of the most common insecticides used to control northern fowl mites (pyrethroids) can have negative impacts on songbird nestlings, causing decreased body mass and fledgling success (Bulgarella, 2020). Another concern with using permethrin to combat ectoparasites is pesticide resistance. For example, mites collected from a field population with no pesticide exposure for over 30 years were able to walk on high concentration visible permethrin residues with no negative effects on survival (Mullens et al. 2004). An alternative to permethrin may be sulfur dust. In one chicken study, mite populations were dramatically reduced one week after a sulfur treatment was introduced, and completely eradicated three weeks post-treatment (Murillo and Mullens, 2016). I investigated sulfur dust bags as a non-toxic and cost-effective pesticide treatment for ectoparasite infection by attaching sulfur bags to the inside of nest boxes, allowing self-treatment of parent birds when they entered and exited the nest box.

In this study, I used sulfur bags (n=16) or empty control bags (n=19) in an attempt to alter northern fowl mite abundance in European starlings (*Sturnus vulgaris)* breeding in nest

boxes and study the impact of mites on the skin microbiome of nestlings. In chickens, northern fowl mites are obligate ectoparasites that complete most of their life cycle on the host with a generation time of 5 to 12 days (Mullens et al., 2012, Knee et al., 2021). However, in songbirds, this species has become adapted to living in the nest (McCulloch et al., 2020). I hypothesized that mite presence may alter the nest environment through mechanisms such as feeding and defecation, which may accumulate and increase nutrient availability for certain microbial strains (Tomás et al., 2018). Increased nutrients may alter the growth and colonization of bacteria on the skin, including pathogenic strains (Tomás et al., 2018), leading to altered bacterial richness and diversity. Northern fowl mites specifically have been shown to mechanically introduce pathogens such as Eastern equine encephalitis and St. Louis encephalitis virus (Knee et al., 2021), and it is possible that mites may introduce microbes to hosts as well. Furthermore, the host inflammatory response to ectoparasites could also potentially impact the microbial population on the skin, leading to an altered microbiota. Little is known of the effects of ectoparasites on host bacterial composition and diversity (Tomás et al., 2018), so one of the major goals of this study was to obtain a better understanding of how host-parasite interactions may affect the skin microbiome.

In addition to parasitism, nestling age and other aspects of the nest environment may play a role in shaping the microbiome. While age can affect the gut microbiome of birds, this has yet to be shown for the skin microbiome (Engel et al., 2020). One study in wild zebra finches (*Taeniopygia guttata*) found that age effected bacterial composition, but not bacterial diversity of the skin microbiome when comparing adults to nestlings within a family (Engel et al., 2020). Because nestlings are constantly exposed to each other and their nest environment, in this study I predicted that microbial diversity of nestling skin would be similar to that of the nest. I also

predicted I would find a significant effect of nestling age on bacterial diversity, as has been seen for the gut microbiome. Further, I predicted that chicks from nests with mites would show different patterns of skin bacterial diversity than chicks from nests without mites. In this study, I collected skin swabs at two different time points to characterize skin microbiomes using 16S rRNA gene amplicon sequencing, and measured body mass and size alongside ectoparasite abundance of European starling nestlings to gain a better understanding of the effects of host-parasite interactions, age, and environment on the avian skin microbiome.

Methods

Study site and sulfur treatments

Field experiments were conducted from March to July 2021 at an established field site of European starling nest boxes (n=35) at an LSU Agricultural Research Station in East Baton Rouge Parish, Louisiana. All protocols were approved by the LSU Institutional Animal Care and Use Committee (protocol #20-034) and birds collected under a Louisiana State Scientific Collecting Permit for starlings. Care was taken to minimize animal distress, appropriate anesthesia/analgesia was used for all procedures causing more than momentary discomfort, and all protocols and procedures complied with the Ornithological Council's Guidelines for the Use of Wild Birds in Research (Fair et al., 2010). Starling nest boxes were monitored daily after nest building began for the initiation of new clutches. On the first day an egg was found in each nest box, I stapled a cotton cloth bag (~20 μm openings; Hobby Lobby, Inc, Oklahoma City, OK) containing either a sulfur treatment of 60 g of 90% wettable sulfur dust (Hi-Yield, Snake Eyes Brand, Bonham, TX) (n=16) or an empty cotton cloth bag as a control (n=19) above the entrance of each nest box (Figure 1). The order of sulfur vs. control treatments was alternated. After

nestlings had fledged from boxes, bags were taken down and re-weighed to determine the amount of sulfur lost.

Mite quantification

Nine days after a clutch was complete, photos were taken of both sides of the eggs and the total number of egg spots quantified by a researcher using ImageJ, and egg spots were averaged per nest box (Schneider et al., 2012). Egg spottiness is thought to result from ectoparasites feeding on the highly vascularized brood patch of the brooding female, and the number of spots on starling eggs has been correlated with the number of mites collected from nest material (Pryor and Casto, 2017). Therefore, egg spots indicate infection of the parent starlings and nest material prior to the nestlings' introduction to the environment. The observer was blind to treatment to avoid potential bias, and egg spots were averaged across eggs in each nest.

After hatching, each nestling's left wing web was visually scored for parasite load 3 and 9 days post-hatch (Figure 2). Mites were scored from 0 to 4 (0=no mites seen; 1=1-10 mites; 2=11-50 mites; 3=51-100 mites; 4=101-500 mites) in the field based on a visualization tool created to reflect different levels of infection (Figure 3). The scorer was blind to sulfur treatment and remained consistent throughout the experiment. On day 14, the largest and the smallest nestling from each nest was euthanized for tissue collection for a co-occuring experiment. At this time, nestlings were dusted with ~6 g of powdered permethrin and ruffled by hand for 30 s (Koop and Clayton, 2013, Vivas et al., 1997). Birds remained in the bag for 60 s, then bags were immediately placed on dry ice; later, 10% (by mass) of the permethrin dust from each bag was manually sifted and contents visualized using a stereomicroscope (Olympus SZ61) to quantify

mites and other arthropods. I validated the use of 10% of the total by also quantifying 100% of arthropods from both a high yield mite bag and a low yield mite bag.

Morphological measurements and microbiome swabs

Body mass, wing chord, and tarsus length was measured for each nestling on days 3, 6, 9, and 12 post-hatch to evaluate nesting body condition, where the day of first hatch was classified as day 0. For mass, individual nestlings were measured to the nearest 0.1 g using a digital scale (AWS AC-650). Wing chord was measured to the nearest 0.5 mm using a wing ruler (Avinet) and average tarsus length was calculated from three measurements to the nearest 0.01 mm with calipers (SPI). All measurements were taken by the same researcher for consistency.

On days 3 and 9 post-hatch, a sterile polyester swab (Puritan Medical Products Company, Guilford, ME) was dipped in a fresh 1.6 mL tube of DEPC-treated water for 1 s before swabbing for the skin microbiome. Starting at the inner crease of the bird's left wing, the swab was twisted 360° while moving the swab ~2.5 cm down the flank under the wing toward the tail, then rotated 360° in the opposite direction, bringing the swab back to the starting position. This was repeated three times on each wing web (6 rotations total). The nest material was also swabbed with a sterile polyester swab dipped in a 1.6 mL tube of DEPC-treated water ~2.5 cm using a sterile plastic guide (2.5 cm × 2.5 cm hole cut from the plastic with a razor blade), then rotated in the opposite direction to bring the swab back to the starting position, and this was also repeated three times. Therefore, the same total surface area was swabbed for skin samples and for nest samples. Swab tips were cut off into a 1.6 mL tubes and flash frozen on dry ice. These tubes were stored at -80ºC until processing in the lab.

Microbial DNA extraction and validation

I extracted microbial DNA using NuceloSpin Soil kits (Macherey Nagel, Allentown, PA, USA) according to the manufacturer's protocol with some modifications to optimize DNA extraction from polyester swabs as follows. All swabs (nestling skin samples, nest samples, and control swabs that had not been used on nestlings or nests), were aseptically transferred to bead tubes, and sample lysis performed using a TissueLyser II (Qiagen) for a total of 4 min at 25 Hz, then the swab was aseptically removed. During the final elution step, the elution buffer was also heated to 70ºC and incubated on the DNA column for 3 min prior to the elution spin to maximize yield.

The resulting DNA concentrations were measured using a Nano Drop 2000 (Thermo Scientific; average 260/280=1.77). However, due to low yields and potential interference from kit components, the presence of microbial DNA was confirmed using 16S PCR amplification with a C1000 Touch Thermocycler (Bio-Rad) using 10 µl reaction volumes that contained: 2 µl of genomic DNA, 5 µl of DreamTaq Green PCR Master Mix (Thermo Fisher Scientific K1081), 2.88 µl of D/RNAse free water, 0.06 µl forward primer (Bakt341F), and 0.06 µl reverse primer (Bakt805R) (5'-CCTACGGGNGGCWGCAG-3' and 5'-GACTACHVGGGTATCTAATCC-3', respectively) (Herlemann et al., 2011, Kelly et al., 2022). Thermocycling conditions were: 94ºC for 3 min, then 30 cycles of: 94ºC for 30 s, 50ºC for 30 s, 72ºC for 1 min, followed by a final extension of 72ºC for 5 min and a holding temperature of 4ºC until removal. All amplicons were visualized on a 1% agarose gel stained with 2.5 µl RedSafe Nucleic Acid Staining Solution (Bulldog Bio) under UV light using the Bio-Rad Gel Doc System after electrophoresis at 100V/2A for 30 min. All samples showed clear 16S rRNA amplification; all negative controls showed very faint bands or no bands.

For amplicon library preparation and Illumina sequencing, 15 µl of genomic DNA was aliquoted into 96-well plates and sent to Michigan State University Genomics Core Research Technology Support Facility (sample sizes were as follows: 201 nestling samples, 73 nest samples, and 9 negative control samples). One set of samples (n=68) were unable to be sequenced because of failed DNA extractions when one kit component did not work, and 22 of those samples were from a second attempt of extracting DNA from the same swabs after the first failed extraction. Amplicon library preparation was completed using 16S-V4 amplicons using the primer pair 515f/806r (5'-GTGCCAGCMGCCGCGGTAA-3' and 5'-GGACTACHVGGGTWTCTAAT-3', respectively) with 1 µl of genomic DNA. Sequencing was completed using the MiSeq System with a v2 Standard run type for paired-end reads at a read length of 250 bp. A mock community with known microorganisms was included in one plate to serve as a positive control for Illumina sequencing.

Sequence data processing and analysis

As in Kelly et al. 2022, raw reads (5,989,889 total) were processed using a mothur pipeline (v.1.47.0) to filter reads for quality, create contigs, and reduce noise (Kozich et al., 2013, Schloss et al., 2013). I aligned sequences with the SILVA database (v.138) (Quast et al., 2013), and identified and removed chimeras using the 'chimera.vsearch' command in mothur. I removed sequences from skin and nest swabs that were identical to sequences present in DNA extraction negative controls and excluded samples with < 2000 sequences. 46 total samples were excluded for low reads, including 39 nestling samples (n=26 day 3; n=9 day 9) and 7 nest samples (n=7 day 3). Reads were classified in mothur using a Bayesian classifier according to taxa ('classify.seqs' command), which also filtered out mitochondrial and chloroplast sequences; this removed 2.2% of sequences. The SILVA database (v.138) was used to classify

representative sequences and operational taxonomic units (OTUs) defined at an evolutionary distance of 0.03 (97% sequence similarity) using mothur's 'opticlust' algorithm. Although the mothur package generates OTUs rather than amplicon sequence variants (ASVs), a consensus has not yet emerged on which approach better assesses host microbiome diversity and taxonomy. The use of OTUs aligns with several recent studies of avian gut and cloacal microbiomes (e.g., Escallón et al. 2019, Murray et al. 2020, Capunitan et al. 2020). After these steps, the number of retained reads per sample ranged from 2,016 to 51,800 (n=160; average 14,636 reads/sample). Final sample sizes included in statistical analyses were as follows: 120 nestling skin swab samples (n= 56 day 3; n= 64 day 9), where 83 nestlings did not have mites (n= 44 day 3; n= 39 day 9) and 37 were infected with mites (n=12 day 3; n= 25 day 9), and 40 nest swab samples (n= 20 day 3; n= 20 day 9).

Statistical analyses

I conducted all statistical analyses for sulfur treatments in R Studio version 1.4.1717 (RStudio Team, 2021). I generated two correlation matrixes, one for day 3 and one for day 9, using the 'rcorr' command from the *Hmisc* package (Harrell et al., 2021) to test for relationships between the different methods of mite quantifications (day 3, 9, and 14 mite counts and egg spot quantifications). To test whether the sulfur treatment reduced ectoparasite abundance, general linear mixed models with a Poisson distribution for count data ('glmer.nb' command in the *lmerTest* package) (Kuznetsova et al., 2017) were used with egg spottiness on day -9 and mite abundance on days 3, 9, and 14 as dependent variables (4 models total), sulfur treatment as a main effect, and box number and date as random effects for each model. To test whether sulfur treatment affected nestling morphology, linear mixed models ('lmer' command in the *lmerTest* package) tested for the effect of sulfur treatment on day 3 and 9 mass, wing length, and tarsus

length, with box number and date as random effects. To test for negative impacts of ectoparasite infection on nestling morphology, the effect of mite abundance on day 3 and 9 morphological measurements was examined using general linear mixed models with a Poisson distribution (‘glmer.nb’ command in the *lmerTest* package) (Kuznetsova et al., 2017), including box number and date as random effects.

Measures of alpha diversity were computed in mothur using the ‘summary.single’ command. The resulting non-parametric Shannon diversity was left-skewed, so I reflected the data with a constant value and log transformed to achieve normality. Therefore, in the statistical analysis (but not visualizations), a low value indicates high diversity. Chao diversity was right-skewed and was log transformed. No transformations were necessary for inverse Simpson diversity. To test the hypothesis that alpha diversity of the skin microbiome was similar to that of the nest microbiome, I conducted three linear mixed-effect models with box number, nestling ID, and date as random effects, and an interaction of sample type (nestling or nest microbiome) and age (day 3 and 9) using the ‘lmer’ command in the *lmerTest* package (Kuznetsova et al. 2017). These models differed in which measure of alpha diversity was used as the dependent variable (non-parametric Shannon, Chao1, or inverse Simpson). I performed pairwise comparisons using the ‘emmeans’ command in the *emmeans* package (Lenth 2021) to examine differences in skin swab vs. nest swab samples by age while controlling for box number, nestling ID, and date. To investigate if mite infection significantly altered alpha diversity of the nestling skin microbiome, I ran three linear mixed models with nestling ID, nest box number, and date as random effects with an interaction of infection status and age. These models differed in which measure of alpha diversity was used as the dependent variable (non-parametric Shannon, Chao1, or inverse Simpson). Pairwise comparisons were again performed using the ‘emmeans’ command in the

emmeans package (Lenth 2021) to observe differences in infected and not infected chicks, while controlling for age. I visualized alpha diversity using the phyloseq (McMurdie and Holmes 2013) and ggplot2 (Wickham et al. 2016) packages in R Studio.

Results

Sulfur dust bag treatment and mite measures

Of the four measures of ectoparasite abundance, only day 9 and 14 mite quantifications were correlated with one another (Tables 1, 2). Sulfur treatment had no effect on day 3, 9, or 14 mite measurements, but had a positive effect on the number of egg spots (Table 3, Figure 4). Sulfur treatment did not affect nestling body condition measures (i.e., mass, wing length, and tarsus length) on day 3 or 9 (Table 4). There was also no effect of mite abundance on day 3 or 9 mass, wing length, or tarsus length (Table 5).

Alpha diversity of the skin microbiome

The mean Matthew's correlation coefficient (0.89) estimated high quality of OTU assignments of 16S rRNA sequences from European starling nestling skin swabs. The resulting OTU table contained 16,381 unique OTUs. Alpha diversity was not significantly different between nestling skin and nest samples at day 3 or day 9 for any of the three alpha diversity metrics (Table 6a, Figure 5). Although linear mixed-effect models detected a significant relationship between age and diversity in nestling samples, this relationship was only significant for one metric (Inverse Simpson), where bacterial alpha diversity decreased in nestling skin swab samples from day 3 to day 9 (Table 6a). Alpha diversity had a significant relationship with mite infection status, but only for one metric (Chao), where there was an increase in diversity with mite infection (Table 6b). However, there was an interaction between mite infection status and age that affected alpha diversity for all three diversity measures (this was significant for non-

parametric Shannon and Chao, and marginal (p=0.056) for Inverse Simpson). In this interaction, alpha diversity of the skin declined over time in mite-infected individuals, but there was no significant difference in alpha diversity between day 3 and day 9 skin swabs in uninfected nestlings (Table 6b, 6c, Figure 6).

Discussion

As predicted, alpha diversity of the nest microbiome and the skin microbiome of European starling nestlings did not differ at the two ages we examined. Though it is not entirely known what contributes to the skin microbiome of free-living animals, environmental factors such as nesting materials and transgenerational transmission from parents are considered to play a large role in its development (van Veelen et al., 2018, Engel et al., 2020), so it is not surprising that the microbiome of the skin did not differ from that of the nest. Across all three diversity metrics, there was no consistent effect of age alone on alpha diversity of the skin microbiome. Although there are not many studies of the skin microbiome in wild nestlings, existing research has also not found a clear age-related increase in skin alpha diversity (Engel et al., 2020). This is contrary to results seen in the gut microbiome, where it is well supported that there are effects of age on alpha diversity (Engel et al., 2020, Ran et al., 2021, Barbosa et al., 2016). Other studies have shown differences in skin bacterial composition (beta diversity) with age (Engel et al., 2020, Lucas et al., 2005), so future work could investigate the development of both alpha and beta diversity of the skin microbiome from nestling hatch to fledge.

In support of my hypothesis that northern fowl mites would affect the nestling skin microbiome, the presence of mites interacted with age to affect alpha diversity, where diversity declined over time in mite-infected nestlings but remained relatively constant in uninfected nestlings. This is the first study to report effects of ectoparasites upon the skin microbiome of

nestling birds. Ectoparasites can affect the bacterial composition of the nests of wild birds, possibly due to accumulations of blood remains from biting hosts and parasite feces (Tomás et al., 2018), which in turn, may modify the skin microbiome of nestlings and alter host health. Commensal bacteria in a healthy skin microbiome help protect the host from pathogens through the production of antimicrobial proteins, modulate behavior with volatile compounds, and communicate fitness-relevant information such as genetic similarity (Ross et al., 2019, Addesso et al., 2020, Engel et al., 2020). Parasites can affect commensal organisms on a host directly or indirectly through consumption and competition (Addesso et al., 2020), which can affect host health. Parasites can also affect host immunity by interfering with immune cells like granulocytes that can disrupt the skin microbiome (Addesso et al., 2020).

Alpha diversity of spotless starling (*Sturnus unicolor*) eggshells has also been shown to be affected by ectoparasitism, with differential effects over time. The introduction of carnid flies increased bacterial richness on spotless starling eggshells four days after parasite addition, but this effect was reduced by the end of incubation (Tomás et al. 2018). The reduced richness later in incubation may be due to adaptive behavioral or physiological defenses of birds to reduce parasite loads that may also affect bacteria loads. Likewise, if nestlings increase inflammation in the skin in response to ectoparasites, this might also have negative effects on microbes living on the skin and be partly responsible for the decreased alpha diversity I observed in day 9 skin swabs relative to day 3 swabs. However, further analyses are required to elucidate how the skin microbiome is affected by parasite exposure by investigating the composition of microbes present prior to, during, and after ectoparasite infection at different life history stages. Future work should also investigate if the reduction in diversity at day 9 is a permanent or transient

effect by sampling at more time points, such as near fledgling (~day 21 in European starlings) or even at adulthood.

Some, but not all, of the measures we used to quantify mites were correlated. Measures taken later in development (day 9 and day 14) were positively correlated, but earlier measurements (egg spots and day 3) were not correlated with each other or the later measures. When 3 days old, only 18% of nestlings had detectable mite infections; by 9 days old, 38% of nestlings had detectable infections. Thus, mites may require more than 3 days to colonize nestlings, and quantifications prior to this may not accurately reflect the final ectoparasite burden experienced by nestlings. Alternatively, day 3 ectoparasite loads of nestlings may be driven by passive immunity (i.e., antibodies) obtained from the mother via the yolk. For example, adult tree swallows (*Tachycineta bicolor*) with higher levels of antibodies to blowflies had fewer blowfly larvae in their nest (DeSimone et al., 2018). Future work should use a cross-fostering design to investigate relationships between mite abundance in starling nests and the levels of anti-mite antibodies present in parents and nestlings to clarify the relationship between parental and nestling responses to mites.

Sulfur bags were not effective in reducing ectoparasite infections. Sulfur treatment was not related to any measure of mite abundance except for an unexpected *positive* effect of the sulfur bag treatment on egg spottiness. This positive effect was driven by a few nests in which egg spot counts were very high, suggesting that sulfur treatment could not compensate for the high parasite loads of the brooding parents. This finding contradicts the literature, where sulfur dust bag treatments were shown to be highly effective for reducing mite populations in chickens (Murillo and Mullens, 2016). The sulfur bags we used might have been ineffective for two reasons: the wettable nature of the powder and/or the location of the sulfur bag in the nest box.

As the breeding season progressed, temperature, humidity, and rainfall increased, which caused clumping of sulfur within the bags that may have impacted the ability of sulfur to penetrate through the cloth. Therefore, the ineffectiveness of our sulfur treatment may be because inadequate amounts of sulfur were lost from bags, as on average only 9% (5.3 g) was dispensed. Placement of the sulfur bag can also significantly alter the effectiveness of the treatment, where hanging sulfur bags resulted in complete ectoparasite eradication after two weeks, but sulfur bags clipped to the front of a poultry cage did not significantly reduce mite populations (Murillo and Mullens 2016). My method of hanging a sulfur dust bag at the entrance of the nest box may not have allowed for adequate release of sulfur dust onto parent and nestling starlings. A final consideration is that nest boxes were not subject to a standardized number of ectoparasites; mite distribution can be highly aggregated due to temperature, humidity, and host condition (Dube et al., 2018). Thus, many boxes remained uninfected for the duration of the experiment, including 47% of the control boxes and 50% of the sulfur boxes, which may have biased my data. Future research should eradicate ectoparasites from nest boxes prior to introducing a standardized number of ectoparasites to control for such variation (Dube et al., 2018).

Nestling morphology was not impacted by sulfur treatment or by northern fowl mites, and this was true for nestling body mass, tarsus length, and wing length. Although ectoparasites can negatively affect nestling growth and survival, negative impacts of nest parasites on their hosts are not always observed (Fessl et al., 2018, Pryor and Casto, 2017). Similar to our study, American Kestrels (*Falco sparverius*) from nests in which northern fowl mites and other parasites were removed did not differ from unmanipulated controls in nestling mass or wing length (Lesko and Smallwood, 2012). This was also the case in tree swallows exposed to blowflies; blowfly-infected nestlings did not differ from uninfected nestlings in body mass,

primary feather length, or likelihood of fledging (DeSimone et al. 2018). Altricial nestlings like European starlings are immobile during development and cannot avoid ectoparasites until they fledge. Thus, nestlings may be able to tolerate mite infection due to a long coevolutionary history with ectoparasites, as infection is unavoidable.

Bacteria have critical roles in homeostasis, can prevent pathogen establishment, and can modulate host behavior (Stevens et al., 2021, Tomás et al., 2018, Engel et al., 2020, Addesso et al., 2020). Moving forward, I will test for differences in bacteria community composition (beta diversity) in mite-exposed and unexposed nestling skin, assess the relative abundance of different bacterial phyla and families in these groups, and test for possible indicator species of ectoparasitism. These investigations will help identify the functional roles of the bacteria that are gained and lost and may indicate the mechanisms affecting their presence. Understanding the impacts of reduced bacteria diversity on birds from the nestling stage to adulthood is an exciting avenue for future research and is especially important considering the shifts and expansions to ectoparasite ranges predicted with future climate change (Cumming and van Vuuren, 2006, Eeva and Klemola, 2013).

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Tables and Figures

Table 1. Correlation matrix of day 3 mite scores, day 14 mite count, and egg spot count with r^2, p-values, and sample size reported.

	D3 Mite Score	*Egg Spot Count*	*D14 Mite Count*
D3 Mite Score	-	r^2= -0.002 p= 0.71 n= 93	r^2= -0.001 p= 0.81 n= 49
Egg Spot Count	r^2= -0.002 p= 0.71 n= 93	-	r^2= -0.002 p= 0.77 n= 47

Table 2. Correlation matrix of day 3 mite scores, day 14 mite count, and egg spot count with r^2, p-values, and sample size reported.

	D9 Mite Score	*Egg Spot Count*	*D14 Mite Count*
D9 Mite Score	-	r^2= -0.005 p= 0.54 n= 80	r^2= 0.20 p= **0.0009** n= 51
Egg Spot Count	r^2= -0.005 p= 0.54 n= 80	-	r^2= -0.002 p= 0.77 n= 47

Table 3. Results of general linear mixed models for the effects of sulfur treatment on mite measurements using Poisson distribution and date and box number as random effects. Separate models were run for day 3 (n=100), day 9 (n=85), and day 14 (n=49) mite scores and for egg spots (n=30). Outputs listed are as follows: β estimate, standard error, z-value, and p-value.

	Estimate	*Std. Error*	*z value*	*p value*
D3 Mite Score	0.27	0.80	0.34	0.73
D9 Mite Score	-0.49	0.62	-0.78	0.43
D14 Mite Score	-2.70	1.80	-1.50	0.13
Egg Spot Count	0.61	0.13	4.80	**<0.0001**

Table 4. Results of linear mixed models to test for the effect of sulfur treatment on day 3 and 9 body measurements of mass, wing length, and tarsus length. The following outputs are listed: estimate, standard error, degrees of freedom, t-value, and p-value. Date and box number were included as random effects in each model. Sample sizes as follows: day 3 n=100 and day 9 n=85.

	Estimate	*Std. Error*	*df*	*t value*	*p*
D3 Mass	1.84	1.22	23.20	1.50	0.15
D3 Wing	0.59	0.60	28.90	1.00	0.33
D3 Tarsus	0.73	0.59	26.17	1.23	0.23
D9 Mass	2.18	3.36	28.56	0.65	0.52
D9 Wing	0.87	2.21	28.75	0.39	0.70
D9 Tarsus	0.28	0.50	28.31	0.55	0.59

Table 5. Results of general linear mixed models to test for the effect of mite infestation on nestling morphology using a Poisson distribution and including date and box number as random effects. Outputs listed are as follows: estimate, standard error, z-value, and p-value. Sample sizes as follows: day 3 n=100 and day 9 n=85.

	Estimate	*Std. Error*	*z value*	*p*
D3 Mass	-0.12	0.07	-1.68	0.09
D3 Wing	-0.06	0.06	-0.99	0.32
D3 Tarsus	-0.05	0.05	-0.86	0.39
D9 Mass	0.002	0.032	0.07	0.94
D9 Wing	-0.03	0.026	-1.11	0.27
D9 Tarsus	-0.005	0.021	-0.22	0.82

Table 6. Alpha diversity of European starling skin microbiome did not differ from alpha diversity of the nest, but was affected by interactions of infection status and age. **(a)** Results of linear mixed-effects models for sample type and age on bacteria alpha diversity. Results are reported for three different alpha diversity metrics: non-parametric Shannon (reflected and log transformed; low values indicate high diversity), Chao (log transformed), and inverse Simpson. Final samples sizes were as follows: 120 nestling skin samples (n=56 for day 3 and n=64 for day 9) and 40 nest samples (n=20 for day 3 and n=20 for day 9). Effects are estimated for nests (with respect to chicks) and day 6 samples (with respect to day 3 samples). **b)** Results of linear mixed-effects models for infection status and age on skin alpha diversity of nestlings. Final samples sizes were as follows: 120 nestlings, where 83 did not have mites (n=44 for day 3, n=39 for day 9) and 37 were infected with mites (n=12 for day 3, n=25 for day 9). **(c)** Tukey's HSD post-hoc tests using estimated marginal means. Effects are estimated for day 3 skin swabs with respect to day 9 skin swabs.

	Parameter estimate ± standard error	**df**	**t**	**p**	**95% confidence interval**
(a) nest and skin swabs					
Non-parametric Shannon					
Sample type	0.08 ± 0.08	149.0	0.96	0.3	-0.085 – 0.24
Age	0.08 ± 0.07	42.7	1.0	0.3	-0.066 – 0.22
Sample type x age	-0.05 ± 0.1	145.0	-0.43	0.7	-0.27 – 0.17
Chao					
Sample type	-0.3 ± 0.2	146.0	-1.6	0.1	-0.64 - 0.076
Age	0.2 ± 0.2	42.4	1.1	0.3	-0.14 - 0.50
Sample type x age	0.4 ± 0.3	142.0	1.6	0.1	-0.10 - 0.89
Inverse Simpson					
Sample type	-4.3 ± 7.0	148.0	-0.65	0.5	-17.0 - 8.8
Age	-15 ± 5.0	41.1	-2.8	**0.006**	-26.0 – (-5.0)
Sample type x age	0.49 ± 9.0	141.0	0.054	0.9	-17.0 – 18.0

(b) skin swabs ***Non-parametric Shannon***					
Infection status	-0.2 ± 0.1	108.0	-1.7	0.09	-0.38 - 0.025
Age	-0.02 ± 0.08	26.3	-0.28	0.8	-0.19 - 0.14
Infection status x age	0.3 ± 0.1	110.0	2.6	**0.01**	0.080 - 0.59
Chao					
Infection status	0.6 ± 0.2	113.0	3.2	**0.002**	0.23 - 0.98
Age	0.3 ± 0.2	18.0	2.0	0.07	0.011 - 0.59
Infection status x age	-0.7 ± 0.2	109.0	-2.7	**0.009**	-1.1 – (-0.14)
Inverse Simpson					
Infection status	7.0 ± 8.0	113.0	0.76	0.5	-10.4 - 23.6
Age	-8.0 ± 7.0	28.1	-1.2	0.25	-20.8 - 4.99
Infection status x age	-21.0 ± 11	108.0	-1.9	*0.056*	-42.2 - 0.215
(c) post hoc comparisons for skin swabs ***Non-parametric Shannon***					**Estimated marginal means**
Not infected: age 3-9	0.02 ± 0.09	31.0	0.27	0.8	D3: 0.84 D9: 0.82
Infected: age 3-9	-0.3 ± 0.1	80.0	-2.6	**0.01**	D3: 0.67 D9: 0.98
Chao					
Not infected: age 3-9	-0.3 ± 0.2	31.0	-1.8	0.07	D3: 6.02 D9: 6.32
Infected: age 3-9	0.4 ± 0.2	83.0	1.6	0.1	D3: 6.63 D9: 6.28
Inverse Simpson					
Not infected: age 3-9	8.0± 7.0	23.0	1.1	0.28	D3: 47.6 D9: 39.9
Infected: age 3-9	29.0 ± 10.0	75.0	2.9	**0.004**	D3: 54.2 D9: 25.4

Figure 1. Cotton cloth bags stapled to the entrance of European starling nest boxes where sulfur treatment (left) contained 60 g of 90% wettable sulfur (n=16) or control bags (right) that were empty (n=19).

Figure 2. Images of 3 day old (left) and 9 day old (right) European starling nestlings for development comparisons. Three day old nestlings are blind, have minimal feather development, and had an average mass of 17 g. Nine day old nestlings have open eyes, growth of primary feathers, and an average mass of 53 g.

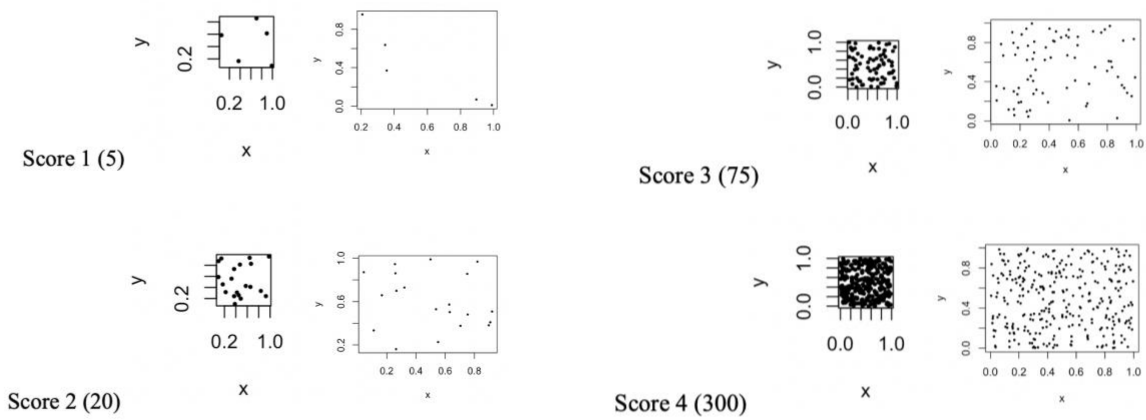

Figure 3. Visualization tool used to score mite abundance from 0-4, where 0=no mites seen; 1=1-10 mites; 2=11-50 mites; 3=51-100 mites; 4=101-500 mites. Images were created in R Studio with median values of abundance displayed.

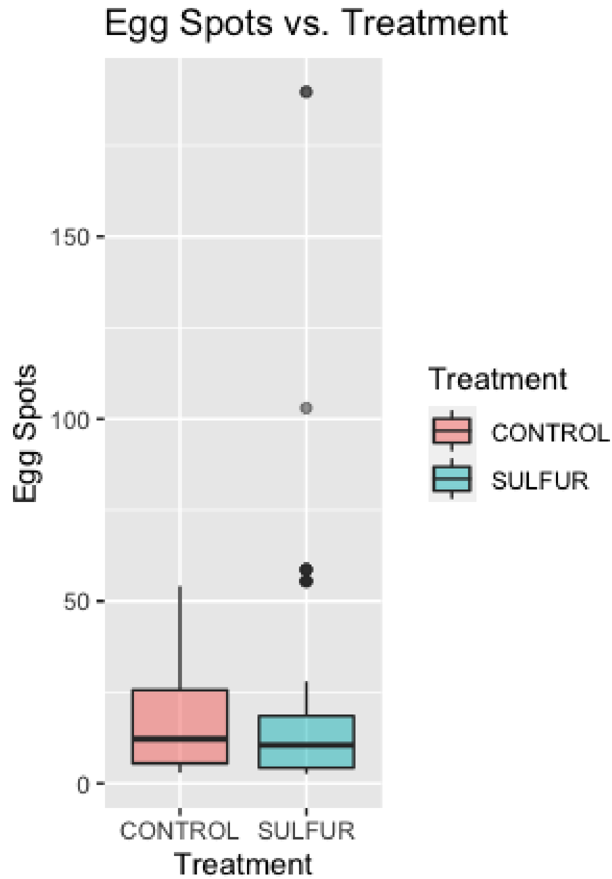

Figure 4. Egg spot quantifications of European starling eggs 9 days after the clutch was completed, averaged by box. Control bags are depicted in pink (n=17), and sulfur dust bags in blue (n=14). Box plots illustrate the median (bold line), 25th and 75th percentiles (interquartile range [IQR]; box ends) and 1.5 x IQR (whiskers). Outliers (dots) are identified for the sulfur treatment and are driven by six nest boxes. Due to the relatively small sample size, we elected not to drop the outliers from this model. Sulfur positively affected the number of egg spots ($p<0.0001$).

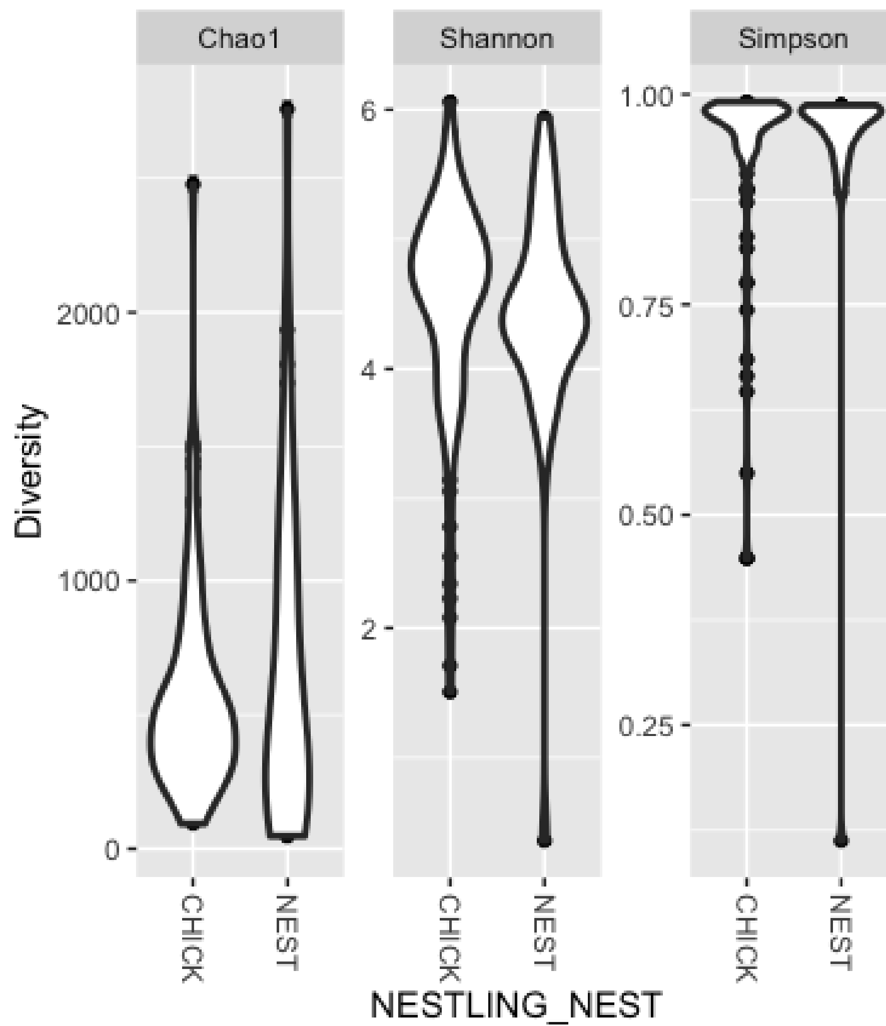

Figure 5. Violin plots that depict no significant difference in alpha diversity (Chao1, non-parametric Shannon, and inverse Simpson) between nestling skin and nest microbiomes (n=120 nestling samples and n=40 nest box samples). The width of the violin indicates more occurrences of the diversity value.

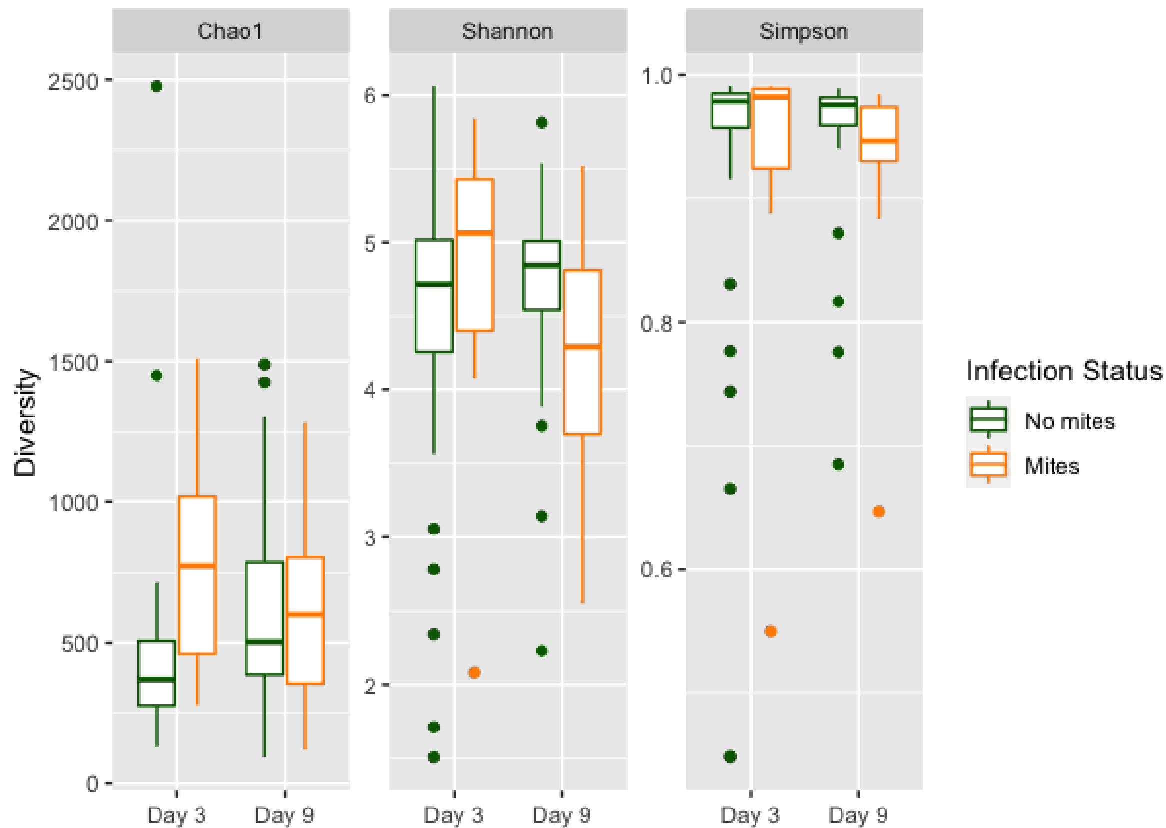

Figure 6. Alpha diversity significantly differs with infection status and age for all metrics. Day 3 skin alpha diversity was significantly higher infected nestlings than uninfected, and day 9 skin alpha diversity was significantly lower than in uninfected nestlings. Nestlings were sampled without northern fowl mite infection (green) and with mite infection (orange). Box plots illustrate the median (bold line), 25th and 75th percentiles (interquartile range [IQR]; box ends) and 1.5 x IQR (whiskers). Outliers (dots) are identified for infected and non-infected nestlings. Final samples sizes were as follows: 120 nestlings, where 83 did not have mites (n=44 day 3, n=39 day 9) and 37 were infected with mites (n=12 day 3, n=25 day 9).