Differential Effects of Elevated Nest Temperature and Parasitism on the Gut Microbiota of Wild Avian Hosts

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Differential effects of elevated nest temperature and parasitism on the gut microbiota of wild avian hosts

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ABSTRACT

Background:

Changes in wild animal gut microbiotas may influence host health and fitness. While many studies have shown correlations between gut microbiota structure and external factors, few studies demonstrate causal links between environmental variables and microbiota shifts. Here, we use a fully factorial experiment to test the effects of elevated ambient temperature and natural nest parasitism by nest flies (*Protocalliphora sialia*) on the microbiotas of two species of wild birds, the eastern bluebird (*Sialia sialis*) and the tree swallow (*Tachycineta bicolor*).

Results:

We find that bacterial communities from the nestlings of each host species show differential response to both heat and parasitism, with gut microbiotas of eastern bluebirds more disrupted by heat and parasitism than those of tree swallows. Thus, we find that eastern bluebirds are unable to maintain stable associations with their gut bacteria in the face of both elevated temperature and parasitism. In contrast, tree swallow gut microbiotas are not significantly impacted by either heat or nest parasitism.

Conclusions:

Our results suggest that excess heat (e.g., as a result of climate change) may destabilize natural host-parasite-microbiota systems, with the potential to affect host fitness and survival in the anthropocene.

Keywords: birds, climate change, gut microbiota, nest parasites, temperature

BACKGROUND

Host-associated gut microbiota are critical for host functioning and fulfill key roles such as provisioning nutrients for the host [1, 2], priming the host immune system [3, 4] and detoxifying xenobiotic compounds [5–7]. Over the past two decades, our knowledge of microbiota patterning as it relates to host evolution (e.g., [8, 9]) and ecology (e.g., [10, 11]) has increased. While these studies are useful for identifying important correlations between host intrinsic factors and gut community structure, few studies demonstrate causal links between external forces and changes in the gut microbiota.

Understanding how and why gut bacterial communities change in response to specific factors requires careful experimental manipulation. In many cases, such causal links have been inferred through the use of germ-free laboratory models that allow for the controlled testing of effector variables. For example, the inoculation of microbiota characteristic of patients suffering from rheumatoid arthritis was shown to produce the disease phenotype in germ-free mice [12]. Studies addressing causal factors influencing the gut microbiota in non-model animals are rare. Experimental studies performed on

free-ranging wild hosts are even rarer despite a growing body of research suggesting that animal-associated microbiotas may inform wildlife health and conservation [13–15].

An important variable with the potential to destabilize host-associated microbial communities is ambient temperature. Given predictions that rising global temperatures will lead to substantial biodiversity loss (e.g., [16–18]), it is reasonable to question how a warming climate might impact or disrupt associations between wildlife hosts and their symbiotic bacterial communities. To date, most studies examining the effects of ambient temperature on microbiota memberships have been performed on ectothermic animals, such as oysters [19], ticks [20], and corals [21]. Fewer studies have tested the effects of ambient temperature on endothermic homeotherms, such as mammals and birds (but see [22]). Furthermore, many field microbiome studies sample animals across different seasons and geographic sites, which may correlate with ambient temperature but may also be confounded by unmeasured variables associated with seasonality, such as variation in rainfall (e.g. [23, 24]). Field-based microbiome studies also rarely investigate the integration of temperature with other environmental stressors, including natural parasitism, the presence of environmental toxins, or habitat loss [25].

To address these knowledge gaps, we performed a fully-crossed field experiment to test the effects of elevated nest temperature and natural parasitism on the microbiotas of two free-ranging wild bird species, the eastern bluebird (*Sialia sialis*) and the tree swallow (*Tachycineta bicolor*) (Fig. 1). Unlike previous studies, our use of experimental heat manipulation during a single summer relieves the effect of confounding variables introduced by studying temperature changes over seasons. Both eastern bluebirds and tree swallows are box-nesting birds, making them a tractable and

easily manipulatable wild system. In addition, both species are parasitized by the same species of blowfly, *Protocalliphora sialia*. Adult *P. sialia* are non-parasitic but lay their eggs in the nests of birds soon after nestlings hatch. The larvae then feed externally on the blood of nestlings [26]. Larvae of *P. sialia* can be experimentally removed using insecticidal sprays that do not negatively impact bird nestlings survival [27–29]. In a related study, the interaction of elevated nest temperature and parasitism was shown to have physiological effects on eastern bluebirds and tree swallows. Bluebird nestlings, but not tree swallows, suffer higher parasite loads and lower body mass when exposed to heat and parasites [30]. This data suggests that hosts are capable of mediating the interaction between heat and parasitism. Because previous studies show that the early life microbiota may play a key role in mediating later parasitism outcomes (e.g., [31, 32]), it is possible that the microbiota may mediate the effects of heat and nest parasitism, probably via the immune system. We therefore tested whether heat and parasitism could cause a shift in the gut microbiota of eastern bluebirds and tree swallows. We hypothesize that tree swallows, which are generally more tolerant and resistant to both nest parasites and heat, should maintain their gut microbiota structure, while the more susceptible eastern bluebirds should show destabilized microbiotas.

RESULTS

Sequencing and Pre Processing

A total of 7,516,369 raw reads underwent DADA2 filtering, of which 6,489,278 passed the denoising step (86%). The average number of merged, non-chimeric reads was 45,673 across all samples (SE_{Mean} +/- 3,918). The total number of ASVs recovered

after contaminant filtering was 8,107. Analysis of contaminants with *decontam* identified only a single ASV as a potential contaminant, so we removed this ASV from the feature table prior to all statistical analyses.

Composition and Diversity of Eastern Bluebird and Tree Swallow Microbiota

The majority of taxa from both eastern bluebirds and tree swallows were represented by seven bacterial phyla. The most common members of the microbiota were the Proteobacteria, Bacteroidetes, and Firmicutes (Fig. 2). In general, eastern bluebirds had a higher relative abundance of Bacteroidetes and Planctomycetes compared to the tree swallows.

The microbiota of both nestling and adult eastern bluebirds had higher median Shannon diversity than those of tree swallows, (Table S1; Fig. 3A,B). Microbiotas of bluebirds and tree swallows were also significantly different in membership (PERMANOVA, $F_{2,134}$ = 4.65, p_{adj} = 0.016) and composition (PERMANOVA, $F_{2,134}$ = 8.59, p_{adj} = 0.016)(Fig. 3C,D). Host species identity explained more variation in membership (e.g. presence or absence of bacterial OTUs) than abundance-weighted composition (Table S2). Because host species identity had a strong effect on microbiotas, we analyzed the effects of heat treatment and parasitism on bacterial beta diversity separately for each species. We also limited these analyses to only the microbiota of nestlings since microbiota varied significantly according to host life stage (Table S2).

Effects of temperature and nest parasitism on nestling microbiota

For alpha diversity, we found that parasitized eastern bluebirds had lower bacterial richness than non-parasitized birds, regardless of heat treatment or life stage. In particular, heat treated and parasitized nestlings had the lowest bacterial richness of any treatment condition in this species (Table S1). In contrast, parasitized tree swallow nestlings had higher richness than non-parasitized nestlings, but only when heat was not applied. In the heat treated tree swallow nestlings, bacterial richness slightly decreased in the parasitized cohort (Table S1). There were too few observations of adult tree swallows to make such a comparison in this cohort, but there was a trend toward lower bacterial richness in parasitized adults in the non-heat treated group (Table S1). Heat and parasite treatments impacted the alpha diversity of bluebird and tree swallow microbiotas, but the significance of these effects varied between host species. For both species, we found that heat treatment alone did not significantly alter microbial richness, phylogenetic diversity, or community evenness (Fig. 4, Table S1). Overall, heat treatment in combination with parasitism significantly reduced all three alpha diversity estimates in bluebirds, while tree swallows were not affected (Fig. 4).

We tested the effects of heat and parasite treatments on each species at both the community level (i.e., beta diversity) and at the level of individual bacterial ASV. We used PERMANOVA to test the effects of these treatments on beta diversity of whole bacterial communities. For the eastern bluebirds, we found that parasite treatment and heat treatment significantly impacted microbial beta diversity in both membership and composition, but the interaction between these variables only significantly impacted composition (Table S3). In contrast, tree swallow microbiota membership and

composition were not significantly impacted by parasitism or heat treatment, nor their interaction (Table S3).

We next tested the effects of heat, parasite treatment, and their interaction on the individual abundances of 13 bacterial phyla. Models for several bacterial taxa failed to converge, likely due to a small number of observations. For the remaining taxa which achieved model convergence, we report parameter estimates and results of significance tests corrected for the false discovery rate (Table S4). The abundances of Fusobacteria, Actinobacteria, Chloroflexi, and Synergistes were negatively associated with the presence of nest parasites in eastern bluebirds, while only Chloroflexi abundance was impacted by heat treatment alone. For tree swallows, we did not detect an effect of either heat or parasitism on the abundance of any of the tested phyla. For both nestling species, the interaction between heat and parasitism was not determined to have a significant effect on the abundance of any one bacterial phylum.

DISCUSSION

This study showed that experimental heat treatment and parasite manipulation differentially impacts the microbiotas of two hosts, eastern bluebirds and tree swallows. Host species identity explained most of the variation in microbiotas among the two bird species (Fig. 2 & 3, Table S2), which is consistent with previous work showing strong patterns of bacterial host specificity in other wild vertebrates [33–36]. The bacterial communities of the two nestling species also showed idiosyncratic responses to both heat treatment and nest parasite treatment. Eastern bluebird nestling microbiotas showed a 14% decrease in mean richness in the presence of nest parasites when heat

was not applied (Table S1). When temperature was experimentally elevated, parasitized bluebird nestlings showed a 48% decrease in richness compared to the non-parasitized birds. On the other hand, tree swallow nestlings had similar microbiota alpha diversity in the presence or absence of nest parasites. When heat was applied, alpha diversity decreased by 22% in the parasitized nestlings compared to those in parasite-free nests, though this reduction was not statistically significant. Heat application alone did not significantly reduce alpha diversity in eastern bluebirds when parasites were absent, though there was a trend toward lower richness as well (Table S3).

The results of the heat treatment experiment are broadly concordant with previous studies examining the effects of heat on vertebrate gut bacterial communities. In cattle and tadpoles, heat alone appears to have no effect on microbiome alpha diversity [37, 38]. In other cases, higher ambient temperatures were found to be associated with lower alpha diversity in the gut microbiota of lizards [39], laying chickens [40], and salamanders [41]. Because endothermic animals such as birds are able to maintain thermal homeostasis, it is not likely that ambient temperature directly affects their gut microbiotas. Rather, the shifts we observed likely result from changes in host physiology spurred by the higher temperatures (e.g., [42, 43]). Accumulating evidence suggests that passerine nestlings respond physiologically to changes in nest temperature; elevated temperatures are associated with decreased body mass and wing length and higher corticosterone levels [30, 44, 45]. The exact mechanisms by which these physiological changes might alter gut microbiota composition remain largely unknown. One hypothesis is that corticosterone alters the structure of the gut mucosal barrier, which exerts an effect on the microbiota. For example, elevated cortisol

levels lead to reduced mucin production in urban squirrels [46]. Mucus may regulate the composition of the microbiota, and it is thought that perturbations to the mucus layer (e.g., by changes in host physiology in response to stress) may compromise the stability of these communities and give rise to dysbiosis [47]. Future studies could examine links between mucosal thickness, corticosterone levels, and microbiota attributes in birds experimentally exposed to heat to further develop our understanding of these interactions.

The two host species' microbiotas also responded differently to the treatments in terms of beta diversity. Both experimental variables impacted the membership and composition of eastern bluebird nestling microbiotas, including the interaction of the heat and parasitism treatments (Table S4). However, tree swallow beta diversity was robust to both experimental treatments, with neither heat, parasitism, nor their interaction, having a significant influence on either community membership or structure. These results are consistent with previous work on this system, which showed that while both species are tolerant to the presence of parasitic nest flies in terms of nestling mortality, tree swallows are both more tolerant *and* more resistant to the sublethal effects of the parasites than bluebirds [28, 48]. However, given that heat application still resulted in a trend toward lower alpha diversity in tree swallow nestlings, our results suggest that heat stress may interfere with the ability of both species to maintain stable associations with their microbiotas in the presence of nest parasites.

Interactions between temperature and parasitism have been reported in other systems, though few studies explicitly incorporate effects on host gut microbiota. In three-spined sticklebacks, rates of tapeworm infection were modulated by ambient

213 temperature such that parasites were more easily able to exploit hosts in warmer
214 experimental conditions [49]. *P. sialia* is an ectoparasite that feeds non-subcutaneously
215 on nestlings, and thus may respond differently to heat than internal parasites, which are
216 buffered from changes in the broader environment due to living within their hosts.
217 Evidence suggests that elevated temperatures result in higher parasite burdens in
218 eastern bluebird nests, while the opposite effect is observed for tree swallows [30].
219 Eastern bluebirds therefore appear to be more susceptible to the interaction of heat
220 stress and parasitism than tree swallows, and our results suggest that these effects in
221 combination have the power to alter their gut bacterial communities. Shifts in microbial
222 composition may signal destabilization of the microbiota in ways that are maladaptive
223 for their hosts. For example, urbanization-driven changes in the microbiotas of
224 American white ibises were significantly associated with pathogen prevalence,
225 suggesting that wild hosts may suffer decreased fitness as a result of microbiome
226 perturbation [50]. However, not all microbiome shifts are necessarily maladaptive.
227 Future work using metagenomic or metatranscriptomic techniques could assess the
228 functional implications of these shifts for hosts and determine the fitness consequences
229 of altered microbial communities.

230 Heat and parasitism did not significantly impact the abundance of any bacterial
231 phylum in tree swallow nestlings (Table S4). In eastern bluebirds, we found that parasite
232 presence caused a decrease in the abundance of Chloroflexi, Fusobacteria,
233 Synergistes, and Actinobacteria, while heat treatment only had a significant effect on
234 Chloroflexi abundance (Table S4). The reduced abundance of these bacterial phyla has
235 been linked to parasitism and disease outcomes in other study systems. One study

found that treatment-induced reduction of Fusobacteria in tadpole microbiotas was linked to higher parasite susceptibility in adult frogs [31]. Similarly, IgY and IgG antibody production has been strongly correlated with Chloroflexi and Fusobacteria abundance in wild Galapagos mockingbirds [27] and a mouse model [51], respectively, suggesting that these bacterial taxa may be instrumental for priming the immune system in early life. Phylum Synergistes is a recently described bacterial phylum that is common in animal guts but about which relatively little is known. However, at least one study comparing captive and wild wood grouse (*Tetrao urogallus)* found that Synergistes was completely lost and Actinobacteria significantly reduced in captive birds and suggested that these changes may compromise the performance of the cecum in birds released into the wild [52].

Taken together, these results suggest that parasitized eastern bluebird nestlings are sensitive to exposure to heat and parasitism. Parasitism may reduce the abundance of key microbiota members that are known to prime immune function, and as a result, the birds may be more susceptible to heat stress and other environmental stressors throughout their lives [30]. The interaction of temperature and parasitism on host-associated microbiotas may be of particular relevance in the context of climate change. Mean global surface air temperature is projected to increase by 1.4° to 5.8°C by 2100 relative to 1990 [53], which falls within the range of our experimentally elevated nest temperatures relative to normal [54]. Given our results, we might expect eastern bluebirds to fare worse under climate change than tree swallows. An interesting mechanistic hypothesis for this prediction is that the tree swallow microbiotas may be mediating their susceptibility to both heat and parasitism. Previous studies have

demonstrated that tree swallows mount immune responses to *P. sialia* that reduce parasite burdens [48], and given the links between the microbiota and immune function, we speculate that the ability of tree swallows to maintain stable associations with their microbes may partially be explained by their immune performance. In general, internal microbiota diversity is correlated with immune complexity across all organisms, suggesting a role for the immune system in promoting stable associations with microbes [55]. Eastern bluebirds, on the other hand, do not produce elevated immune responses to parasites and sustain twice the parasite burden per gram of nestling compared with tree swallows [48]. In contrast with tree swallows, eastern bluebird microbiotas shifted in response to heat and parasitism, suggesting that their immune systems and microbiotas may be less coupled. Future studies elucidating these mechanisms would add depth to our understanding of the importance of the microbiome in mediating host immune performance.

Given that both parasitism and heat reduced the abundance of key microbiota members associated with immunity, we predict that the net effects of a warming world and natural nest parasitism may threaten eastern bluebird survival in the future. This prediction is consistent with other studies raising concerns about the impacts of climate change on bluebird reproduction and survival (e.g., [56, 57]). In contrast, a long-term study on breeding performance found that between the periods 1962-1972 and 2006-2016, tree swallow reproductive performance increased as a result of earlier breeding induced by warmer winter temperatures [58]. Thus, the combined effects of a warming climate in concert with variation in physiological responses to heat and nest parasites are likely to impact eastern bluebirds and tree swallows differently. Future work should

explicitly test the survival and lifetime fitness costs of nestlings exposed to heat in early life and incorporate metatranscriptomic assays or metabolomics to assess the functional contributions of gut bacteria in determining host immune outcomes.

CONCLUSIONS

In conclusion, this study demonstrated that two species of free-ranging avian hosts respond differently to heat and parasitism at the level of the microbiota, and that specific bacterial phyla change in relative abundance in response to the treatments. Our work joins a growing body of literature suggesting that the gut microbiota may have the power to mediate external stressors such as heat and ectoparasite presence in wild hosts. This experimental study and future work will be instrumental in predicting how natural host-parasite-microbe systems respond to a warming world.

METHODS

Study Site

The experiment was conducted at the University of Minnesota Itasca Biological Station and Laboratories (47°13′33″N, −95°11′42″W), Minnesota, USA from May to August 2018. In 2018, approximately 170 nest boxes were established on private properties and near Itasca State Park. Tree swallows and eastern bluebirds are abundant in the area and nest readily in artificial cavities. At this site, *Protocalliphora sialia* is the main species of blowfly that parasitizes both bluebird and tree swallow nests [48]. Tree swallow clutch size ranges from one to nine eggs incubated for 13-14 days. Nestlings spend an average of 20 days in the nest [48]. Bluebird clutch size ranges from

one to seven eggs, which are incubated for 13-14 days. Nestlings spend an average of 18.8 days in the nest [59].

Experimental Manipulation of Nest Parasites and Temperature

We checked nest boxes once per week for evidence of nest construction. Once eggs were found, we recorded lay date and checked nests every other day until the eggs hatched. During the nestling stage, we conducted a fully factorial experiment by manipulating parasite presence (parasites vs. no parasites) and nest temperature (heat treatment vs. no heat) for both eastern bluebird and tree swallow nests (Fig. 1). At hatching, the nestlings and the top liner of the nests were removed for parasite manipulation. For the control treatment, we treated nests with sterile water to allow for natural parasitism (parasitized). For the experimental treatment, we treated nests with a 1% permethrin solution to remove all parasites (non-parasitized; Permectrin II) [27, 28]. We initially determined parasite treatment for each species by a coin flip, and alternately assigned all subsequent nests to a treatment.

For the heat treatment, we used a metal spatula to lift nest material from the bottom of the box and placed a fresh UniHeat 72+ Hour heat pack (heat-treated) or an exhausted heat pack (non-heat treated) in the open space. The packs contain a mixture of charcoal, iron powder, vermiculite, salt, sawdust, and moisture, and produce elevated temperatures between 35 – 40°C for two days when exposed to the air [60]. Nest boxes were revisited every two days to replace active heat packs so that nest boxes were maintained at constant elevated temperature until nestlings were 10 days old. For control nests, nest material was lifted with a metal spatula to control for nest

disturbance. Heat packs were always checked for parasites before they were removed; any parasites that were on the heat pack were returned to the nest. Heat treatment for each species was initially determined by a coin flip and all subsequent nests were assigned to alternating treatments.

When nestlings were approximately 10 days old, we collected feces from them and ended the heat treatment. To collect feces, nestlings were removed from the nest and held over a sterile weigh-boat until they defecated. The fecal sample was then moved from the tray to a sterile tube with approximately 500µL of DNA/RNA Shield (Zymo Research, Inc.), placed on ice in the field for up to 6 hours, and then stored in a -80°C freezer until the bacterial DNA was extracted. The samples were then transported to the University of Connecticut and stored in a -80°C freezer for downstream 16S sequencing. Although studies show that the bacterial community in avian feces does not always represent the entire digesta of the host (e.g., in the cecum; [61]), fecal samples are generally representative of the bacterial community in the large intestines [61, 62] and are used when hosts cannot be euthanized [63].

When nests were empty, they were carefully removed, along with the heat packs, from the nest box and stored in a gallon-sized, labeled plastic bag. Nest material was then dissected over trays lined with a white piece of paper. All *P. sialia* larvae (1^{st}, 2^{nd}, and 3^{rd} instars), pupae, and pupal cases were counted to determine total parasite abundance for each nest for Albert et al. (2021) [30].

DNA Isolation and 16S rRNA Gene Sequencing

Before starting the extraction, samples were centrifuged for 10 minutes at 10,000 rpm and 4°C and the supernatant (i.e. DNA/RNA Shield) was then removed. Total DNA was extracted from nestling feces using a Qiagen PowerFecal DNA Isolation Kit (Qiagen, Inc.). DNA extractions were then sent to the University of Connecticut Microbial Analysis, Resources and Services for sequencing with an Illumina MiSeq platform and v2 2x250 base pair kit (Illumina, Inc.). We also sequenced a laboratory blank to control for kit contamination and found no detectable sequences. Bacterial inventories were conducted by amplifying the V4 region of the 16S rRNA gene using primers 515F and 806R [64] and with Illumina adapters and dual indices [65].

Bioinformatic and Statistical Analyses

A total of 138 fecal samples were successfully sequenced. Raw forward and reverse reads for each sample were imported into QIIME2 v. 2020.8 [66]. Initial quality control was performed manually by summarizing demultiplexed sequences and visualizing per-base quality scores in QIIME2. Next, we used the DADA2 algorithm to trim low-quality base calls (as identified in the previous summary step), join forward and reverse reads, and identify and remove chimeric sequences [67]. The resulting feature table of amplicon sequence variants (ASVs) was then used to construct a bacterial 16S phylogeny, using the MAFFT alignment algorithm (q2-alignment) and the FastTree maximum likelihood estimation (q2-phylogeny) plugin [68, 69]. Bacterial taxonomy was assigned using the q2-feature-classifier [70] naive Bayesian classifier against the Greengenes 13_8_99% OTUs reference database [71]. After visual inspection of the resulting taxonomic bar plots, we filtered out mitochondrial and chloroplast sequences

373 from the ASV feature table and exported the data for further analysis in the R statistical
374 environment [72].
375 Basic data preprocessing was performed using packages *phyloseq* v. 1.32.0 [73]
376 and *microbiome* v. 2.1.25 [74]. First, we filtered the feature table to retain only those
377 samples with at least 1000 total reads, which reduced the dataset to 135 samples.
378 Because microbiome samples are prone to ambient laboratory contamination [75, 76],
379 we also used *decontam* v. 1.8.0 [77] to filter out potential contaminant ASVs from the
380 samples based on the prevalence of bacteria in the extraction negative control. We
381 performed alpha diversity calculations on the contaminant-filtered dataset, computing
382 Shannon diversity for each sample. Prior to beta diversity analyses, we performed a
383 Hellinger transformation (i.e., square root of the relative abundance given at the scale
384 [0,1]) on the ASV table to account for differences in library size [78].
385 Because our dataset contained relatively few adult mothers (n = 29), we decided
386 to focus on only nestlings for the beta diversity analyses (n = *107*). We used R package
387 *vegan* [79] to compute alpha (Shannon, Faith's PD, Evenness) and beta (Bray-Curtis,
388 Unifrac) diversity metrics for all samples [80]. To test for differences in alpha diversity
389 among experimental treatments, we compared treatments within species using the
390 Wilcoxon test and applying a Bonferroni correction to all significance values. To
391 compare microbiota among experimental conditions, we conducted PERMANOVA tests
392 on both unweighted (composition) and weighted (structure) Unifrac distances. Distance
393 matrices were estimated separately for each bird species. The full model call was
394 (dist.matrix ~ Parasite + Heat + Parasite * Heat). An important assumption of
395 PERMANOVA is homogeneity of dispersion among groups, so we also performed a

beta dispersion test for each distance matrix using the command “betadisper” in *vegan*. Because we tested the same hypotheses separately on each bird species, we controlled for multiple testing by adjusting all *p*-values using a Bejamini-Hochberg correction.

To specifically test for the effects of nest temperature treatment and parasite treatment on detection of gut microbial taxa, we used general linear models with mixed effects (GLMMs). To reduce the overall number of ASVs tested, we pruned the dataset to remove those ASVs that were not detected in at least 10 individual birds as well as those that had fewer than 100 total read counts. This data stringency left us with a total of 16 phyla of 112 bacterial families available for testing. Using package *glmmTMB* [81], we constructed models testing the effects of parasite treatment, heat treatment, and the interaction between parasitism and heat on each bacterial phylum. We fit models using a type-2 negative binomial distribution with a log link and modeled nest ID as a random effect to account for brood sampled from the same nests. Because microbiome features tables include many zeroes, we additionally included a zero-inflation component modelled on a single intercept. All *p*-values were adjusted for false discovery rate with a Benjamini-Hochberg correction.

All visualizations were created using *phyloseq* [73] and *ggplot2 [82].* Bar plots were created using *fantaxtic* (https://github.com/gmteunisse/Fantaxtic) and all color palettes are adapted from *wesanderson* (https://github.com/karthik/wesanderson).

DECLARATIONS

Ethics approval and consent to participate: All applicable institutional guidelines for the care and use of animals were followed (University of Connecticut IACUC protocol #A18-005).

Consent for publication: Not applicable.

Availability of data and material: All statistical analyses and code can be accessed publicly at https://github.com/MelissaIngala/ItascaBirdMicrobiota. Raw 16S rRNA data is publicly archived on the NCBI SRA under BioProject # PRJNA733473.

Competing interests: The authors declare no competing interests

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Authors' contributions: LA and SAK designed the study, LA and SAK collected the data, AA and MW conducted the molecular work. MRI analyzed the data and wrote the manuscript. All authors participated in improving the manuscript. Bird illustrations by MRI.

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occupy the land in which our research was conducted. The University of Minnesota Itasca Biological Station and Laboratories is located on the land ceded by the Mississippi and Pillager Bands of Ojibwe in the Treaty of Washington, commonly known as the 1855 Treaty. This treaty affirms the reserved rights doctrine and the inalienable rights of Ojibwe people to uphold their interminable relationship to the land. With affiliation to the aforementioned academic institutions, it is our responsibility to acknowledge Native rights and the institutions' history with them. We are committed to continue building relationships with the Ojibwe People through recognition, support, and to advocate for all Native American Nations. We strive to be good stewards of our place and privilege. *(This land acknowledgement was revised and written with support from Rebecca Dallinger and Joe Allen. It is a living document open to changes).*

REFERENCES

1. LeBlanc JG, Milani C, De Giori GS, Sesma F, Van Sinderen D, Ventura M. Bacteria as vitamin suppliers to their host: a gut microbiota perspective. *Current opinion in biotechnology*. 2013;24:160–8.

2. Sannino DR, Dobson AJ, Edwards K, Angert ER, Buchon N. The *Drosophila melanogaster* Gut Microbiota Provisions Thiamine to Its Host. *mBio*. 2018;9.

3. Thaiss CA, Zmora N, Levy M, Elinav E. The microbiome and innate immunity. *Nature*. 2016;535.

4. Ximenez C, Torres J. Development of microbiota in infants and its role in maturation of gut mucosa and immune system. *Archives of medical research*. 2017;48:666–80.

5. Zhu L, Yang Z, Yao R, Xu L, Chen H, Gu X, et al. Potential Mechanism of Detoxification of Cyanide Compounds by Gut Microbiomes of Bamboo-Eating Pandas. *mSphere*. 2018;3. doi:10.1128/mSphere.00229-18.

6. Turner LA, Bucking C. The role of intestinal bacteria in the ammonia detoxification ability of teleost fish. *Journal of Experimental Biology*. 2019;222.

7. Kohl KD, Oakeson KF, Orr TJ, Miller AW, Forbey JS, Phillips CD, et al. Metagenomic sequencing provides insights into microbial detoxification in the guts of small mammalian herbivores (*Neotoma* spp.). *FEMS Microbiology Ecology*. 2018.

8. Colston TJ, Jackson CR. Microbiome evolution along divergent branches of the vertebrate tree of life: what is known and unknown. *Molecular Ecology*. 2016;25.

9. Ley R, Hamady M, Lozupone C. Evolution of mammals and their gut microbes. *Science*. 2008;320:1647–51.

10. Youngblut ND, Reischer GH, Walters W, Schuster N, Walzer C, Stalder G, et al. Host diet and evolutionary history explain different aspects of gut microbiome diversity among vertebrate clades. *Nature Communications*. 2019;10:1–15.

11. Kartzinel TR, Hsing JC, Musili PM, Brown BR, Pringle RM. Covariation of diet and gut microbiome in African megafauna. *Proceedings of the National Academy of Sciences*. 2019;116:23588–93.

12. Liu X, Zeng B, Zhang J, Li W, Mou F, Wang H, et al. Role of the gut microbiome in modulating arthritis progression in mice. *Scientific reports*. 2016;6:1–11.

13. Bahrndorff S, Alemu T, Alemneh T, Lund Nielsen J. The Microbiome of Animals: Implications for Conservation Biology. *International Journal of Genomics*. 2016;2016.

14. Trevelline BK, Fontaine SS, Hartup BK, Kohl KD. Conservation biology needs a microbial renaissance : a call for the consideration of host-associated microbiota in wildlife management practices. *Proc R Soc B*. 2019;286.

15. West AG, Waite DW, Deines P, Bourne DG, Digby A, McKenzie VJ, et al. The microbiome in threatened species conservation. *Biological Conservation*. 2019;229 July 2018:85–98.

16. Kannan R, James DA. Effects of climate change on global biodiversity: a review of key literature. *Tropical Ecology*. 2009;50:31.

17. Rinawati F, Stein K, Lindner A. Climate change impacts on biodiversity—the setting of a lingering global crisis. *Diversity*. 2013;5:114–23.

18. Smale DA, Wernberg T, Oliver EC, Thomsen M, Harvey BP, Straub SC, et al. Marine heatwaves threaten global biodiversity and the provision of ecosystem services. *Nature Climate Change*. 2019;9:306–12.

19. Lokmer A, Wegner KM. Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. *The ISME journal*. 2015;9:670–82.

20. Thapa S, Zhang Y, Allen MS. Effects of temperature on bacterial microbiome composition in *Ixodes scapularis* ticks. *MicrobiologyOpen*. 2019;8:e00719.

503 21. Lima LFO, Weissman M, Reed M, Papudeshi B, Alker AT, Morris MM, et al.
504 Modeling of the Coral Microbiome: the Influence of Temperature and Microbial Network.
505 *mBio*. 2020;11. doi:10.1128/mBio.02691-19.

506 22. Zhang X-Y, Sukhchuluun G, Bo T-B, Chi Q-S, Yang J-J, Chen B, et al. Huddling
507 remodels gut microbiota to reduce energy requirements in a small mammal species
508 during cold exposure. *Microbiome*. 2018;6:1–14.

509 23. Campbell TP, Sun X, Patel VH, Sanz C, Morgan D, Dantas G. The microbiome and
510 resistome of chimpanzees, gorillas, and humans across host lifestyle and geography.
511 *The ISME journal*. 2020;14:1584–99.

512 24. Gaona O, Cerqueda-García D, Moya A, Neri-Barrios X, Falcón LI. Geographical
513 separation and physiology drive differentiation of microbial communities of two discrete
514 populations of the bat *Leptonycteris yerbabuenae*. *MicrobiologyOpen*. 2020;9:1113–27.

515 25. Dheilly NM, Bolnick D, Bordenstein S, Brindley PJ, Figuères C, Holmes EC, et al.
516 Parasite Microbiome Project: Systematic Investigation of Microbiome Dynamics within
517 and across Parasite-Host Interactions. *mSystems*. 2017;2:e00050-17.

518 26. Boyd EM. The external parasites of birds: a review. The Wilson Bulletin.
519 1951;63:363–9.

520 27. Knutie SA, Owen JP, McNew SM, Bartlow AW, Arriero E, Herman JM, et al.
521 Galápagos mockingbirds tolerate introduced parasites that affect Darwin's finches.
522 *Ecology*. 2016;97:940–50.

523 28. DeSimone JG, Clotfelter ED, Black EC, Knutie SA. Avoidance, tolerance, and
524 resistance to ectoparasites in nestling and adult tree swallows. *Journal of Avian Biology*.
525 2018;49:jav-01641.

526 29. Bulgarella M, Knutie SA, Voss MA, Cunninghame F, Florence-Bennett BJ, Robson
527 G, et al. Sub-lethal effects of permethrin exposure on a passerine: implications for
528 managing ectoparasites in wild bird nests. *Conservation physiology*. 2020;8:coaa076.

529 30. Albert L, Rumschlag S, Parker A, Vaziri G, Knutie SA. Elevated nest temperature
530 has opposing effects on host species infested with parasitic nest flies. *bioRxiv*. 2021.

531 31. Knutie SA, Wilkinson CL, Kohl KD, Rohr JR. Early-life disruption of amphibian
532 microbiota decreases later-life resistance to parasites. *Nature Communications*.
533 2017;8:1–8.

534 32. Knutie SA, Gabor CR, Kohl KD, Rohr JR. Do host-associated gut microbiota
535 mediate the effect of an herbicide on disease risk in frogs? *Journal of Animal Ecology*.
536 2018;87:489–99.

33. Lutz HL, Jackson EW, Webala PW, Babyesiza WS, Kerbis Peterhans JC, Demos TC, et al. Ecology and Host Identity Outweigh Evolutionary History in Shaping the Bat Microbiome. *mSystems*. 2019;4:e00511-19.

34. Ingala MR, Simmons NB, Wultsch C, Krampis K. Comparing microbiome sampling methods in a wild mammal : fecal and intestinal samples record different signals of host ecology , evolution. *Frontiers in Microbiology*. 2018;9 May:1–13.

35. Capunitan DC, Johnson O, Terrill RS, Hird SM. Evolutionary signal in the gut microbiomes of 74 bird species from Equatorial Guinea. *Molecular Ecology*. 2020;29:829–47.

36. Smith SN, Colston TJ, Siler CD, Finley SJ. Venomous Snakes Reveal Ecological and Phylogenetic Factors Influencing Variation in Gut and Oral Microbiomes. *Frontiers in Microbiology*. 2021;12 March:1–12.

37. Tajima K, Nonaka I, Higuchi K, Takusari N, Kurihara M, Takenaka A, et al. Influence of high temperature and humidity on rumen bacterial diversity in Holstein heifers. *Anaerobe*. 2007;13:57–64.

38. Kohl KD, Yahn J. Effects of environmental temperature on the gut microbial communities of tadpoles. *Environmental Microbiology*. 2016;18:1561–5.

39. Bestion E, Jacob S, Zinger L, Di Gesu L, Richard M, White J, et al. Climate warming reduces gut microbiota diversity in a vertebrate ectotherm. *Nature ecology & evolution*. 2017;1:1–3.

40. Zhu L, Liao R, Wu N, Zhu G, Yang C. Heat stress mediates changes in fecal microbiome and functional pathways of laying hens. *Applied microbiology and biotechnology*. 2019;103:461–72.

41. Fontaine SS, Novarro AJ, Kohl KD. Environmental temperature alters the digestive performance and gut microbiota of a terrestrial amphibian. *Journal of Experimental Biology*. 2018;221.

42. Sepulveda J, Moeller AH. The Effects of Temperature on Animal Gut Microbiomes. *Frontiers in Microbiology*. 2020;11 March:1–9.

43. Moeller AH, Ivey K, Cornwall MB, Herr K, Rede J, Taylor EN, et al. The Lizard Gut Microbiome Changes with Temperature and Is Associated with Heat Tolerance. *Appl Environ Microbiol*. 2020;86. doi:10.1128/AEM.01181-20.

44. Crino OL, Driscoll SC, Brandl HB, Buchanan KL, Griffith SC. Under the weather: corticosterone levels in wild nestlings are associated with ambient temperature and wind. *General and comparative endocrinology*. 2020;285:113247.

571 45. Salaberria C, Celis P, López-Rull I, Gil D. Effects of temperature and nest heat
572 exposure on nestling growth, dehydration and survival in a Mediterranean hole-nesting
573 passerine. *Ibis*. 2014;156:265–75.

574 46. Stothart MR, Palme R, Newman AE. It's what's on the inside that counts: stress
575 physiology and the bacterial microbiome of a wild urban mammal. *Proceedings of the*
576 *Royal Society B*. 2019;286:20192111.

577 47. Wang BX, Wu CM, Ribbeck K. Home, sweet home: how mucus accommodates our
578 microbiota. *FEBS Journal*. 2020;:1–11.

579 48. Grab KM, Hiller BJ, Hurlbert JH, Ingram ME, Parker AB, Pokutnaya DY, et al. Host
580 tolerance and resistance to parasitic nest flies differs between two wild bird species.
581 *Ecology and evolution*. 2019;9:12144–55.

582 49. Franke F, Armitage SA, Kutzer MA, Kurtz J, Scharsack JP. Environmental
583 temperature variation influences fitness trade-offs and tolerance in a fish-tapeworm
584 association. *Parasites & vectors*. 2017;10:1–11.

585 50. Murray MH, Lankau EW, Kidd AD, Welch CN, Ellison T, Adams HC, et al. Gut
586 microbiome shifts with urbanization and potentially facilitates a zoonotic pathogen in a
587 wading bird. *PloS one*. 2020;15:e0220926.

588 51. Slack E, Hapfelmeier S, Stecher B, Velykoredko Y, Stoel M, Lawson MA, et al.
589 Innate and adaptive immunity cooperate flexibly to maintain host-microbiota mutualism.
590 *Science*. 2009;325:617–20.

591 52. Wienemann T, Schmitt-Wagner D, Meuser K, Segelbacher G, Schink B, Brune A, et
592 al. The bacterial microbiota in the ceca of Capercaillie (*Tetrao urogallus*) differs between
593 wild and captive birds. *Systematic and Applied Microbiology*. 2011;34:542–51.

594 53. Harvell CD, Mitchell CE, Ward JR, Altizer S, Dobson AP, Ostfeld RS, et al. Climate
595 warming and disease risks for terrestrial and marine biota. *Science*. 2002;296:2158–62.

596 54. Cooper CB, Hochachka WM, Phillips TB, Dhondt AA. Geographical and seasonal
597 gradients in hatching failure in Eastern Bluebirds (*Sialia sialis*) reinforce clutch size
598 trends. *Ibis*. 2006;148:221–30.

599 55. Woodhams DC, Bletz MC, Becker CG, Bender HA, Buitrago-Rosas D, Diebboll H, et
600 al. Host-associated microbiomes are predicted by immune system complexity and
601 climate. *Genome biology*. 2020;21:23.

602 56. Dunn PO, Winkler DW. Effects of climate change on timing of breeding and
603 reproductive success in birds. Effects of climate change on birds. 2010;:113–28.

604 57. Harrod SE, Rolland V. Demographic responses of eastern bluebirds to climatic
605 variability in northeastern Arkansas. *Population Ecology*. 2020;62:317–31.

606 58. Imlay TL, Mills Flemming J, Saldanha S, Wheelwright NT, Leonard ML. Breeding
607 phenology and performance for four swallows over 57 years: Relationships with
608 temperature and precipitation. *Ecosphere*. 2018;9:e02166.

609 59. Gowaty PA, Plissner JH. Eastern bluebird (Sialia sialis), the birds of North America.
610 Ithaca: Cornell Lab of Ornithology. 2015.

611 60. Dawson RD, Hillen KK, Whitworth TL. Effects of experimental variation in
612 temperature on larval densities of parasitic *Protocalliphora* (Diptera: Calliphoridae) in
613 nests of tree swallows (Passeriformes: Hirundinidae). *Environmental Entomology*.
614 2005;34:563–8.

615 61. Wilkinson TJ, Cowan AA, Vallin HE, Onime LA, Oyama LB, Cameron SJ, et al.
616 Characterization of the microbiome along the gastrointestinal tract of growing turkeys.
617 *Frontiers in microbiology*. 2017;8:1089.

618 62. Videvall E, Strandh M, Engelbrecht A, Cloete S, Cornwallis CK. Measuring the gut
619 microbiome in birds: Comparison of faecal and cloacal sampling. *Molecular Ecology*
620 *Resources*. 2017; August:1–11.

621 63. Kohl KD. An introductory how to guide for incorporating microbiome research into
622 integrative and comparative biology. *Integrative and comparative biology*. 2017;:1–8.

623 64. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh
624 PJ, et al. Global patterns of 16S rRNA diversity at a depth of millions of sequences per
625 sample. *Proceedings of the National Academy of Sciences of the United States of*
626 *America*. 2011;108 Suppl Supplement_1:4516–22.

627 65. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a
628 dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence
629 data on the miseq illumina sequencing platform. *Applied and Environmental*
630 *Microbiology*. 2013;79.

631 66. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al.
632 Reproducible, interactive, scalable and extensible microbiome data science using
633 QIIME 2. *Nature biotechnology*. 2019;37:852–7.

634 67. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Amy Jo A Johnson, Holmes SP.
635 DADA2: High resolution sample inference from Illumina amplicon data. *Nature Methods*.
636 2016;13:48–56.

637 68. Katoh K, Misawa K, Kuma K, Miyata T. MAFFT: a novel method for rapid multiple
638 sequence alignment based on fast Fourier transform. *Nucleic acids research*.
639 2002;30:3059–66.

640 69. Price MN, Dehal PS, Arkin AP. FastTree 2 - Approximately maximum-likelihood
641 trees for large alignments. *PLoS ONE*. 2010;5.

70. Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, et al. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2 ' s q2-feature-classifier plugin. *Microbiome*. 2018;6:1–17.

71. McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, et al. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *The ISME journal*. 2012;6:610–8.

72. Team RDC, R Development Core Team R. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. 2016.

73. McMurdie PJ, Holmes S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE*. 2013;8.

74. Lahti L, Shetty S, Blake T. Tools for microbiome analysis in R. Microbiome Package Version 099. 2017.

75. Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF, et al. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biology*. 2014;12:87–87.

76. Glassing A, Dowd SE, Galandiuk S, Davis B, Chiodini RJ. Inherent bacterial DNA contamination of extraction and sequencing reagents may affect interpretation of microbiota in low bacterial biomass samples. *Gut Pathogens*. 2016;8:24–24.

77. Davis NM, Proctor D, Holmes SP, Relman DA, Callahan BJ. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome*. 2018;6:221499–221499.

78. Legendre P, Gallagher ED. Ecologically meaningful transformations for ordination of species data. *Oecologia*. 2001.

79. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, et al. vegan: Community Ecology Package. 2017;:v. 2.4-5.

80. Lozupone C, Hamady M, Knight R. UniFrac-an online tool for comparing microbial community diversity in a phylogenetic context. *BMC bioinformatics*. 2006;7:371–371.

81. Brooks ME, Kristensen K, Van Benthem KJ, Magnusson A, Berg CW, Nielsen A, et al. glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *The R journal*. 2017;9:378–400.

82. Wickham H, Chang W. ggplot2. Computer software] Retrieved from http://ggplot2 org. 2012.

FIGURES & TABLES

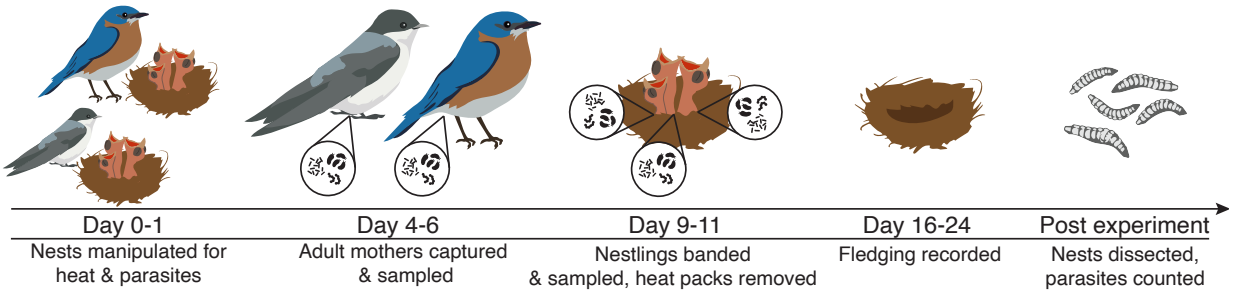
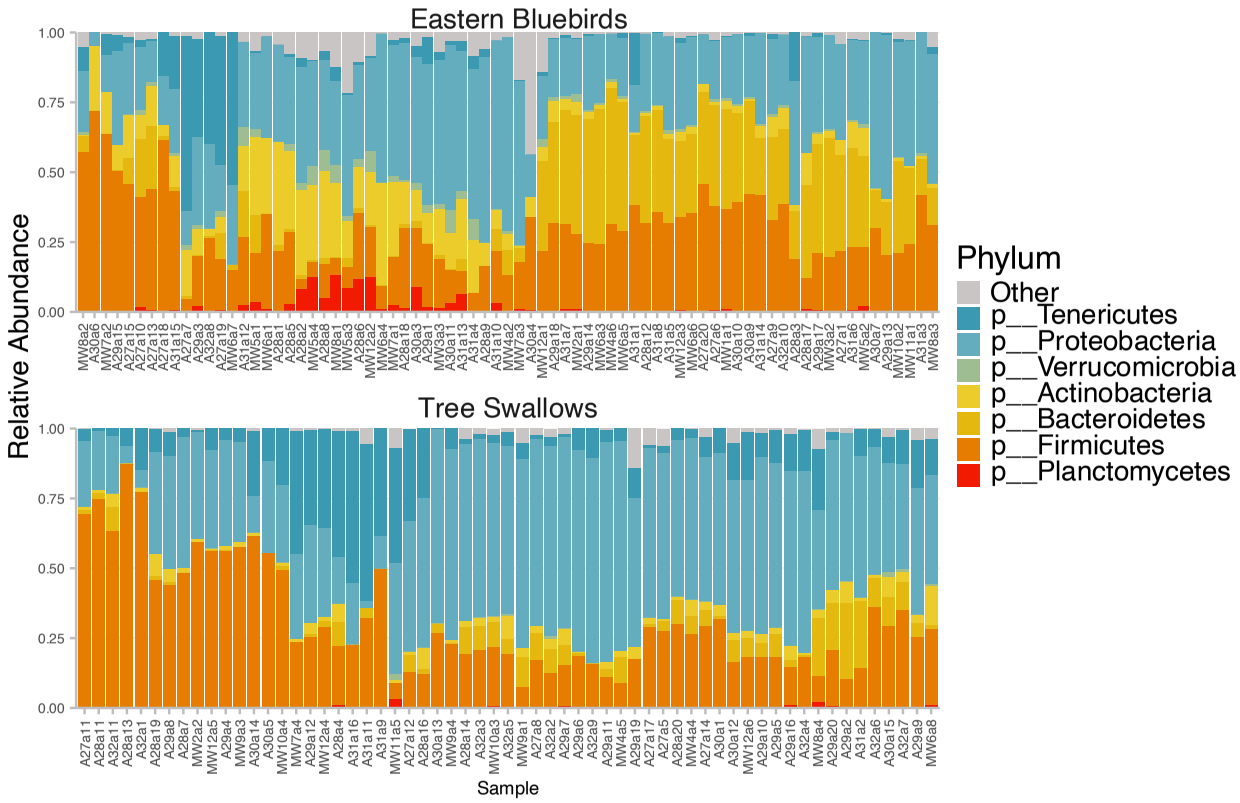


Figure 1. Eastern bluebird and tree swallow nests were manipulated to test the effects of heat treatment and parasitism on microbiota. Days represent nestling age from hatch date.



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689 **Figure 2.** Proportional abundance of bacterial phyla identified from eastern bluebird

690 (top) and tree swallow (bottom) microbiota. Each bar represents a sample from an

691 individual bird.

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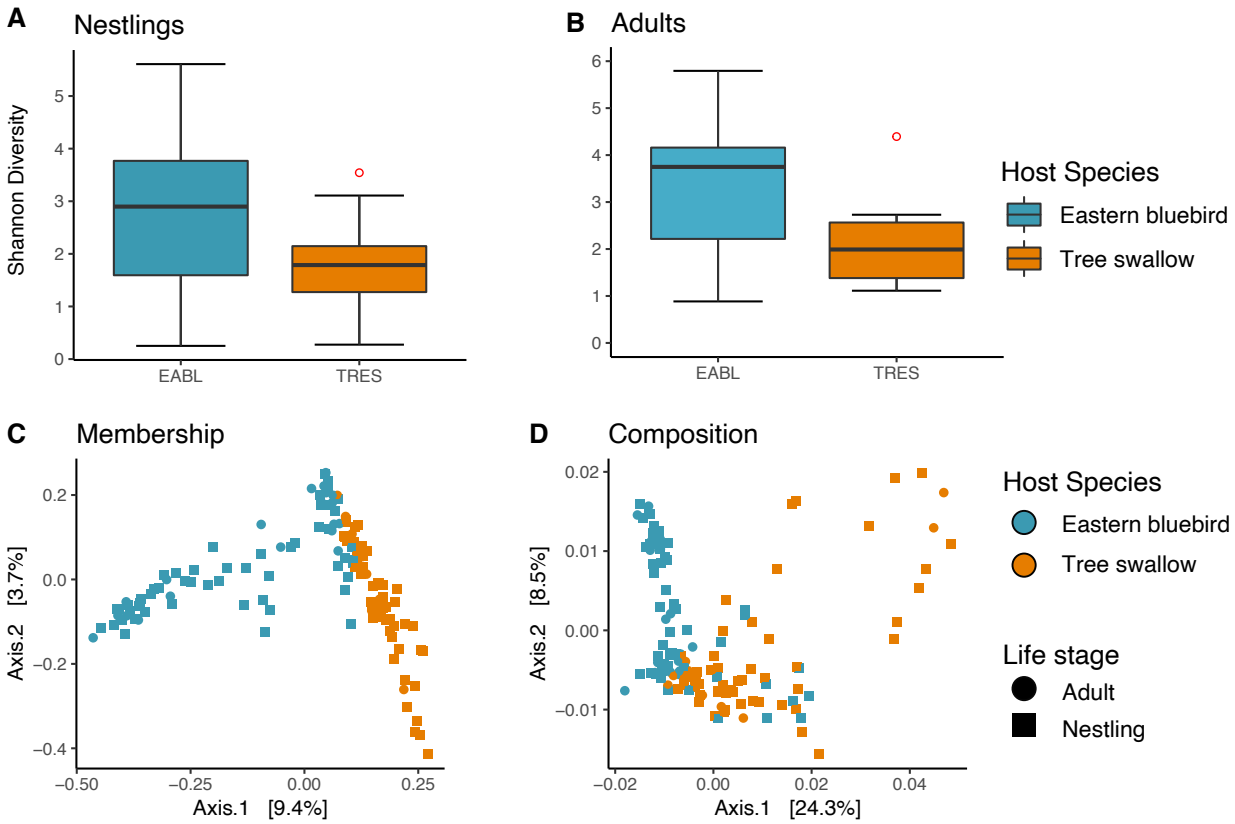

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695 **Figure 3.** Shannon diversity boxplots for nestlings (A) and adult birds (B) of both

696 species. Midline represents the median Shannon diversity value. Red points indicate

697 outliers. Principal coordinates ordination of (C) unweighted (membership) and (D)

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699 individual sample colored according to host species. Point shape indicates host life

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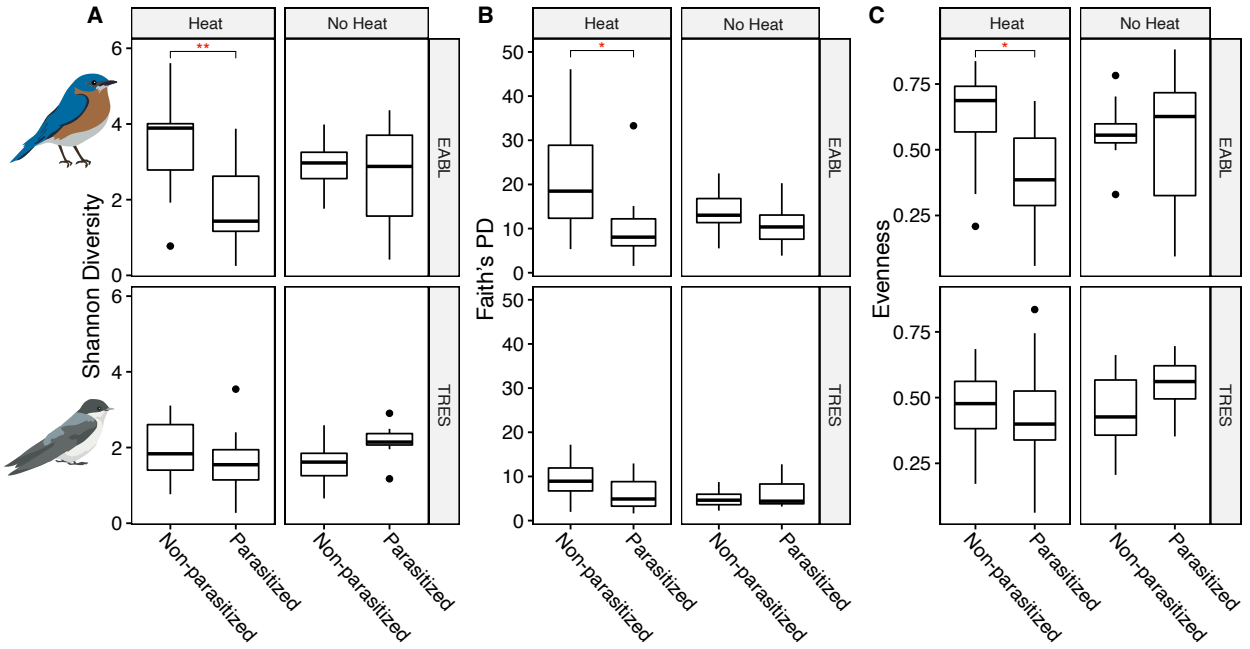

Figure 4. Effect of heat and parasite treatments on alpha diversity of eastern bluebird and tree swallow nestling microbiotas. Panels depict A) Shannon diversity, B) Faith's phylogenetic diversity, and C) Evenness. Horizontal bars represent median values while solid points indicate outliers. Significance codes: ** = $p_{adj} < 0.001$, * = $p_{adj} < 0.05$.

Supplemental Information

Supplemental Table S1. Effect of experimental heat and parasite treatment on alpha diversity of bird microbiotas. Mean Shannon diversity is reported ± standard error. Numbers in parentheses indicate sample size for each condition.

	No heat		Heat	
	Non-parasitized	**Parasitized**	**Non-parasitized**	**Parasitized**
Eastern bluebirds				
Nestlings	2.90 ± 0.2 (10)	2.55 ± 0.33 (16)	3.49 ± 0.33 (14)	1.82 ± 0.28 (15)
Adults	3.71 ± 0.33 (6)	2.88 ± 0.46 (6)	4.99 ± 0.81 (2)	2.89 ± 0.56 (6)
Tree swallows				
Nestlings	1.58 ± 0.15 (13)	2.16 ± 0.14 (10)	1.99 ± 0.28 (9)	1.56 ± 0.16 (20)
Adults	3.56 ± 0.83 (2)	1.96 ± 0.60 (2)	1.38 (1)	1.76 ± 0.23 (4)

Supplemental Table S2. PERMANOVA results for Bray-Curtis distance matrix and both membership (unweighted Unifrac) and composition (weighted Unifrac) for all birds.

	Bray-Curtis		**Unweighted Unifrac**		**Weighted Unifrac**	
	r^2	P_{adj}	r^2	P_{adj}	r^2	P_{adj}
Host species	0.11142	0.012*	0.11144	0.012*	0.06445	0.016*
Parasite Treatment	0.00859	0.012*	0.00876	0.012*	0.00850	0.016*
Heat Treatment	0.01851	0.012*	0.02080	0.012*	0.01067	0.016*
Life Stage	0.01154	0.012*	0.00973	0.012*	0.00901	0.016*

*Significant at ($P_{adj} < 0.05$)

Table S3. Effect of parasite treatment, heat treatment, and their interaction on beta diversity of eastern bluebird and tree swallow microbiotas. Values labeled * are significant at $P_{adj} \leq 0.05$.

	Unweighted Unifrac (Membership)		Weighted Unifrac (Composition)	
	r^2	p_{adj}	r^2	p_{adj}
Eastern Bluebirds				
Parasite Treatment	0.024	0.047 *	0.029	0.047 *
Heat Treatment	0.027	0.047 *	0.041	0.018 *
Parasite*Heat	0.018	0.4370	0.029	0.047 *
Tree Swallows				
Parasite Treatment	0.019	0.80	0.019	0.80
Heat Treatment	0.023	0.46	0.036	0.33
Parasite*Heat	0.025	0.276	0.025	0.71

Table S4. Mixed effects model results (coefficient and standard deviation) for the impacts of heat and parasite manipulation on 16 bacterial phyla, split amongst eastern bluebirds and tree swallows nestlings. Values shown in bold are significant at $p_{adj} < 0.05$ after Benjamini-Hochberg correction. "NA" values indicate that the model failed to converge for the specified bacterial taxon. W = parasitized condition, NH = no heat applied

Host Species	Bacterial Phylum	Intercept	Parasitism (Parasitized)	Heat (No Heat)	Parasitism*Heat
Eastern Bluebirds	Tenericutes	**6.51 (± 1.75)**	3.35 (± 2.23)	-1.23 (± 2.57)	-5.94 (± 3.4)
	Proteobacteria	**9.88 (± 0.34)**	-0.75 (± 0.48)	0.70 (± 0.53)	0.79 (± 0.70)
	Fusobacteria	**6.32 (± 1.53)**	**-4.99 (± 2.13)**	-32.6 (± 158411)	31.1(± 158411)
	Deferribacteres	**3.19 (± 0.89)**	-0.62 (± 1.24)	0.80 (± 1.38)	-0.25 (± 1.83)

	Crenarchaeota	**3.88 (± 0.89)**	-0.26 (± 0.0002)	-1.08 (± 1.38)	-5.56 (± 0.00004)
	Verrucomicrobia	**5.67 (± 0.59)**	-0.81 (± 0.82)	-0.61 (± 0.91)	1.27 (± 1.21)
	Actinobacteria	**6.53 (± 0.48)**	**-1.38 (± 0.63)**	-0.0009 (± 0.72)	0.33 (± 0.92)
	Chlamydiae	3.92 (± 2.23)	-0.63 (± 3.10)	-4.27 (± 3.48)	8.33 (± 4.60)
	Acidobacteria	-5.26 (± 4.72)	-2.52 (± 4.26)	-20.0 (± 15342)	19.40 (± 15342)
	Chloroflexi	**4.93 (± 1.0)**	**-5.24 ± (1.43)**	**-3.21 ± (1.56)**	2.54 (± 2.12)
	p__Unknown	NA	NA	NA	NA
	Synergistetes	**4.91 (± 1.33)**	**-5.13 (± 1.87)**	0.42 (± 2.05)	1.37 (± 2.74)
	Bacteroidetes	**9.14 (± 1.28)**	-1.53 (± 1.17)	0.24 (± 1.44)	1.25 (± 1.65)
	Firmicutes	**9.24 (±0.50)**	-0.37 (± 0.68)	0.23 (± 0.78)	0.018 (±1.01)
	Cyanobacteria	NA	NA	NA	NA
	Planctomycetes	**4.16 (± 0.95)**	-2.68 (± 1.33)	-1.78 (± 1.48)	2.34 (± 1.96)
Tree Swallows	Tenericutes	**8.87 (±0.55)**	0.31 (±0.67)	-0.42 (±0.72)	-1.13 (±0.97)
	Proteobacteria	**9.65 (± 0.62)**	-0.46 (±0.82)	-0.63 (±0.82)	1.13 (±1.16)
	Fusobacteria	NA	NA	NA	NA
	Deferribacteres	-24.2 (± 39533)	-0.93 (± 60975)	-0.97 (± 69930)	3.87 (± 84132)
	Crenarchaeota	NA	NA	NA	NA

	Verrucomicrobia	NA	NA	NA	NA
	Actinobacteria	**5.46 (± 0.66)**	-0.70 (± 0.80)	-1.53 (± 0.86)	0.94 (± 1.16)
	Chlamydiae	NA	NA	NA	NA
	Acidobacteria	-24.16 (± 39533)	-0.93 (± 60975)	-0.97 (± 69930)	3.87 (± 84132)
	Chloroflexi	24.2 (± 39533)	-0.93 (± 60975)	-0.97 (± 69930)	3.87 (± 84132)
	p__Unknown	-24.16 (± 39533)	-0.93 (± 60975)	-0.97 (± 69930)	3.87 (± 84132)
	Synergistetes	NA	NA	NA	NA
	Bacteroidetes	**7.55 (± 0.96)**	-0.38 (± 1.15)	0.99 (± 1.25)	-1.85 (±1.67)
	Firmicutes	**9.62 (± 0.41)**	-0.35 (± 0.50)	-0.30 (± 0.54)	0.15 (± 0.72)
	Cyanobacteria	2.87 (± 2.54)	-1.12 (± 3.06)	-0.62 (± 3.30)	0.072 (± 4.44)
	Planctomycetes	-24.16 (± 39533)	-0.93 (± 60975)	-0.97 (± 69930)	3.87 (± 84132)

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Figures

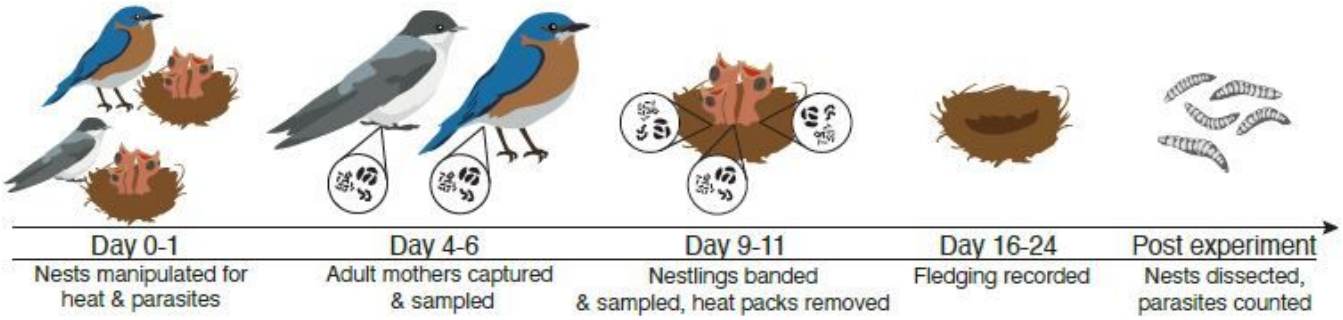

Figure 1

Eastern bluebird and tree swallow nests were manipulated to test the effects of heat treatment and parasitism on microbiota. Days represent nestling age from hatch date.

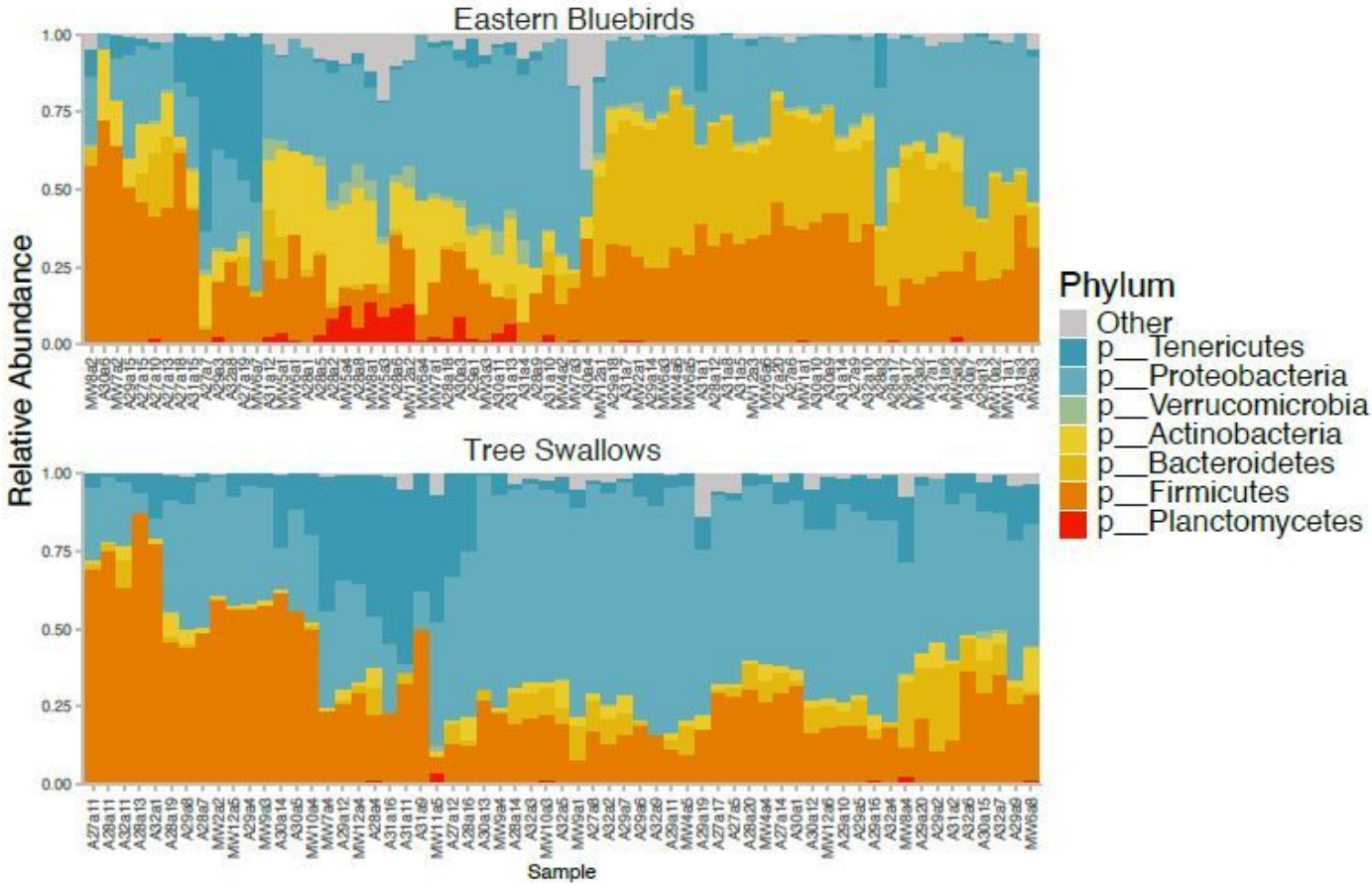

Figure 2

Proportional abundance of bacterial phyla identified from eastern bluebird (top) and tree swallow (bottom) microbiota. Each bar represents a sample from an individual bird.

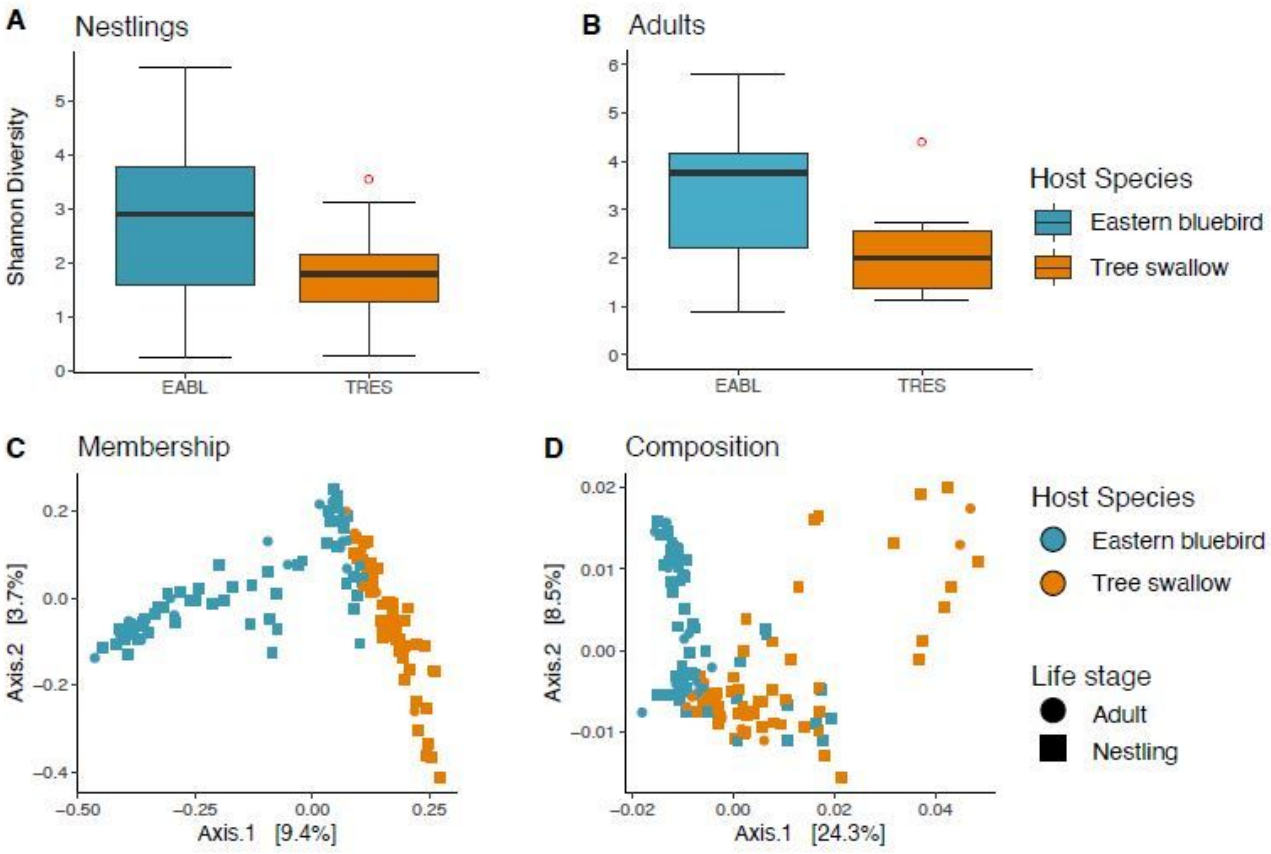

Figure 3

Shannon diversity boxplots for nestlings (A) and adult birds (B) of both species. Midline represents the median Shannon diversity value. Red points indicate outliers. Principal coordinates ordination of (C) unweighted (membership) and (D) weighted (composition) Unifrac distances for all birds. Each point represents an individual sample colored according to host species. Point shape indicates host life stage.

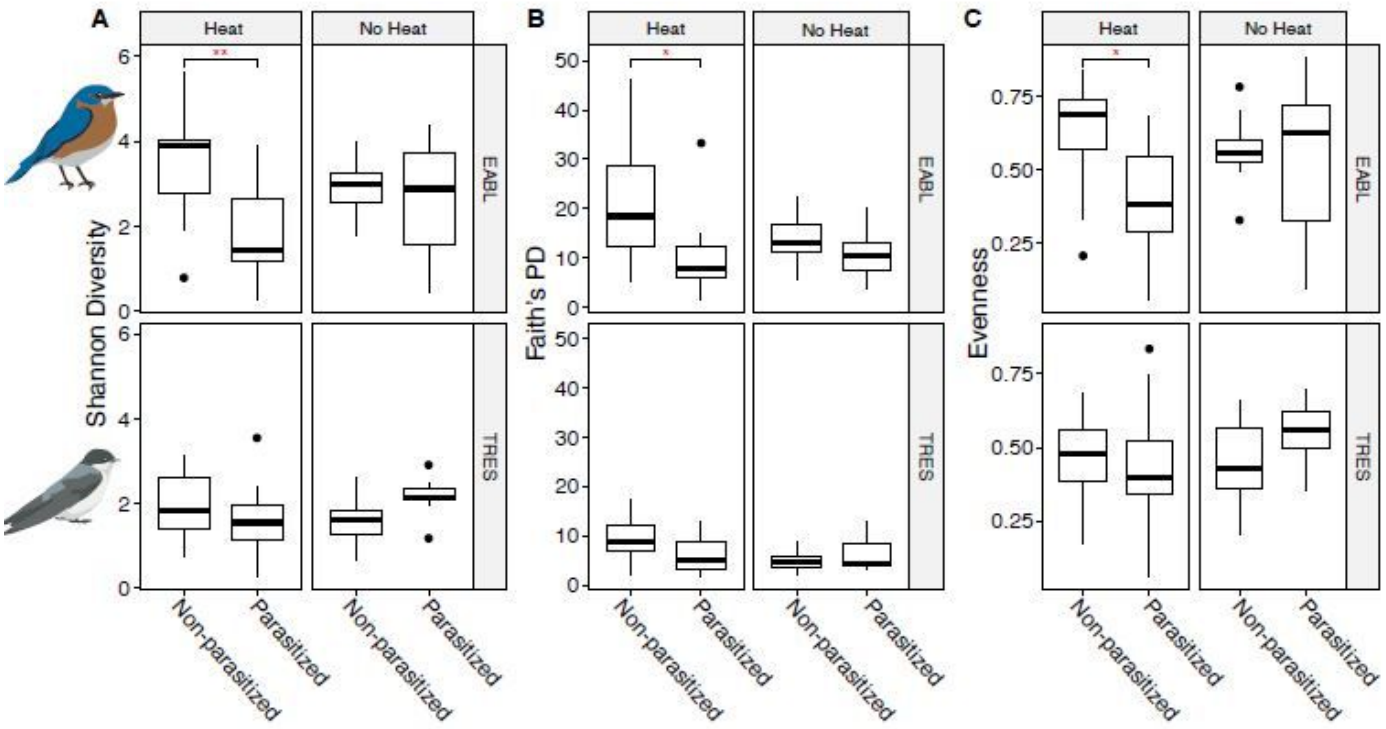

Figure 4

Effect of heat and parasite treatments on alpha diversity of eastern bluebird and tree swallow nestling microbiotas. Panels depict A) Shannon diversity, B) Faith's phylogenetic diversity, and C) Evenness. Horizontal bars represent median values while solid points indicate outliers. Significance codes: ** = padj < 0.001, * = padj < 0.05.