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Research article

Microbiome composition of Anna's hummingbirds differs among regions of the gastrointestinal tract

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The gastrointestinal (GI) microbiome is an important mediator and indicator of host physiology, health and fitness in many vertebrate systems, but sources of variation in microbiome composition within species are poorly understood, particularly in free-ranging birds. Hummingbirds have GI anatomy and physiology similar to other avian species, but they have a relatively rapid gastrointestinal transit rate, which could affect microbiome structure. To date, it has not been elucidated if microbiomes isolated from feces and samples from sections of the GI tract differ in an avian organism that has a rapid gastrointestinal time. Here, we test the hypothesis that the microbiome in free-ranging and wildlife rehabilitation-sourced Anna's hummingbirds *Calypte anna* differ between fecal and samples from three sections of the GI tract and between birds that differ in sex, age and poxvirus infection status. We characterized bacterial composition in fecal and GI tract samples from Anna's hummingbirds by amplicon sequencing of the 16S rRNA gene. We found strong evidence of differentiation in bacterial composition among GI tract regions and compared it to fecal samples. Actinobacteria, primarily genus *Corynebacterium*, Firmicutes and Fusobacteria were abundant in bird samples from regions of the GI tract. In contrast, fecal bacterial communities were more diverse and variable compared to GI tract samples. Bacterial community composition differed between male and female hummingbirds and with bird age (hatch year vs after-hatch year). Finally, birds with symptoms of avian poxvirus infection had a higher relative abundance of *Staphylococcus* spp. than birds with no symptoms of pox. Our results suggest that Anna's hummingbirds host differentiated microbiome among GI tract regions that is consistent among individual birds. The GI bacterial community also contained taxa not represented in fecal samples. This provides evidence for the possibility of a residential gut microbiome in Anna's hummingbirds, although functional significance of the bacterial microbiome remains unknown.

Keywords: avian poxvirus, gastrointestinal, gut microbiome, hummingbird, microbiome, pollinator

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Introduction

The vast majority of vertebrates possess a residential community of host-associated microbes located in the gastrointestinal tract (Ley et al. 2008, Amato 2013, Hird 2017) which are intimately tied to the health, physiological composition and fitness of the animal (Hooper et al. 2012, Sommer and Bäckhed 2013, Mosca et al. 2016). For birds, the functional significance of the microbiome is poorly understood. Studies have linked several diseases with abnormal microbial colonization in the gastrointestinal tract in bird species (Ganz et al. 2017, Murray et al. 2020) but functional importance remains elusive. Examining potential sources of variation in microbiome composition may inform drivers of its structure and function. A comprehensive meta-analysis of the avian GI microbiome (Waite and Taylor 2014) showed significant variation between host species, which corresponded to differing diets and geographic distribution. Adaptations that arose from flight such as the length of the intestinal tract and gut retention time also have been shown to affect microbiome composition, where small birds with faster gut turnover have higher intraspecific variation in community composition of the GI tract when compared to larger flighted and flightless birds (Song et al. 2020, Bodawatta et al. 2021). Within a bird species, microbial community composition often changes with age of the individual, often increasing in diversity with age (van Dongen et al. 2013, Awad et al. 2016, Kohl et al. 2019). Moreover, variation in microbial community composition can be explained by geographic location and environmental factors (Green and Bohannen 2006, Anderson et al. 2012, Colman et al. 2012, Hird et al. 2015, Grond et al. 2019). However, there exists a significant gap in knowledge of the drivers of variation in microbiome composition of wild bird species. This lack of understanding limits our ability to interpret variation in microbiome composition and assess its potential impact on the health of free-ranging animals.

Hummingbirds (family Trochilidae) are of conservation interest due to their importance as pollinators and indicators of environmental change and have been extensively studied for their metabolic rates, flight and cognitive abilities (Guevara et al. 2017, Maeda et al. 2017). However, their GI microbiome remains poorly understood (Preest et al 2003, Lee et al. 2019, Herder et al. 2021). Hummingbirds have a unique feeding style, with all hummingbird species typically spending 85–90% of their foraging time on nectar, and the rest of their time focused on hunting arthropods, with breeding females spending only 65–70% of their foraging time on nectar given their heightened protein requirements (Montgomerie and Gass 1981, Abrahamczyk and Kessler 2015). Additionally, hummingbirds are unique for their physiologies, including rapid gastrointestinal transit time (Karasov et al. 1986, McWhorter and del Rio 2000, Price et al. 2015) which is predicted to reduce associations with microbial communities in the gut (Kohl 2012). In particular, a study by Preest and colleagues (2003) implicated the contribution of GI bacteria to decomposition of nitrogenous compounds in the GI tract, but no further studies examine the presence or function of GI microbes on hummingbird health or physiology. In addition, hummingbirds can also use torpor when resources are limited and when they are energetically challenged. Torpor reduces metabolic rate, respiration, body temperature and potentially gastrointestinal activity (McWhorter and del Rio 2000, Ruf and Geiser 2015, Shankar et al. 2020). The basis for mortality of free-ranging hummingbirds is poorly understood, but disease syndromes and exposure to environmental contaminants could be fully or partial causes (Godoy et al. 2014, Mikoni et al. 2017, Bishop et al. 2018, Filigenzi et al. 2019, Graves et al. 2019, Magagna et al. 2019, Baek et al. 2020). Links between disease and microbial composition may lead to a better understanding of hummingbird health.

Here, we test the hypothesis that the hummingbird GI tract hosts a bacterial community that is distinct among regions of the GI tract. We compared bacterial composition between three regions of the GI tract: the combined proventriculus and ventriculus and two segments of the intestine. These regions of the GI tract were chosen because they differ in function and environmental conditions for microbes, with the proventriculus/ventriculus representing acidic and mechanical digestion, the upper intestine (the segment from the exit of the ventriculus to the jejunum) indicating the location where chemical digestion and food absorption takes place, and the lower intestine (jejunum to the cloaca) illustrating where absorption of fluid and electrolytes takes place. We also examined fecal samples to determine if fecal/cloacal samples are a good representation of GI tract microbiome composition (Momozawa et al. 2011, Berlow et al. 2020), as feces can be readily sampled from free-ranging birds. In addition, we examined if bacterial composition varied with bird age or sex, as has been demonstrated in other bird species (Kohl et al. 2019, Liu et al. 2020), and if it closely resembles food items. Finally, we examined if disease symptoms were associated with microbiome composition, with a focus on presence of the avian poxvirus (pox), which is a virus that commonly infects Anna's hummingbirds (Godoy et al. 2013, Baek et al. 2020).

Methods

Sample collection

To examine if the bacterial microbiome composition differs with bird age or sex, fecal samples from 48 Anna's hummingbirds *Calypte anna* were obtained from three locations in northern California: two sites at private residences in Winters, CA, and one site at a commercial business in Acampo, CA. Although 48 birds were sampled, only a subset was successfully sequenced (below). All three sites were chosen based on observed pre-existing populations of Anna's hummingbirds and collected between 7:00 and 12:00 h during mild weather conditions of partly cloudy and 21–26°C throughout June 2018. Hall feeder traps were used to capture the hummingbirds, and fecal samples were collected directly from the cloaca

into a hematocrit tube. All sampled birds were aged, sexed, weighed, banded and checked for presence of suspected pox viral lesions before release. Birds were evaluated for the presence of suspected pox lesions given that poxviridae infections have been described in hummingbirds (Godoy et al. 2013, Baek et al. 2020), and it is unknown if this disease process has impacts on the gastrointestinal microbiome. All birds with pox lesions used in the study were confirmed positive by a method utilizing a real-time quantitative PCR assay developed for detecting the hummingbird-specific *Avipox* 4b core protein gene, as described by Baek et al. (2020). No recaptured birds were used for this study. All live birds sampled for fecal collection (n = 48) were observed using hummingbird feeders. In addition, sugar–water solution samples from the feeders were collected on the same day as fecal samples were collected for additional comparison and potential food items (pooled insect samples) were also collected. The feeders were filled with a 1:4 sugar–water solution made with granulated pure cane white sugar and residential tap water. Permission to obtain the hummingbird carcasses for scientific study was approved by the United States Fish and Wildlife Service (permit: MB55944B-2) and the California Department of Fish and Wildlife (permit: SC-013066).

To sample bacteria directly from the GI tract, 20 deceased hummingbird carcasses, with no evidence of disease, were obtained from a wildlife rehabilitation center (Lindsay Wildlife Experience, Walnut Creek, CA). Birds were brought in from local good Samaritans, but data regarding the exact locations were limited. As with fecal samples, some samples from regions of the GI tract failed to yield sufficient sequence for analysis (described below). These carcasses had no external signs of illness or injury, did not receive medication while in care and all died within 24 h of arrival to the center. In addition, body conditions were aligned with healthy birds with high metabolic rates and living on an energetic edge, and there were no abnormalities on gross dissection. However, it could not be definitively confirmed that birds were completely devoid of any health, nutrition or toxicological conditions without running extensive diagnostic tests, which are limited for hummingbirds. As a comparison to birds that did not have any evidence of disease, seven hummingbird carcasses with obvious abnormalities on physical examination were evaluated. All the seven hummingbirds had visual evidence of disease in the form of external pox-like lesions on either the bill, wing joints, legs or a combination of all. All seven birds were confirmed to be positive for poxvirus using a previously published PCR method (Baek et al. 2020). Immediately after euthanasia or death, carcasses were placed in a −20°C freezer. The species, age and sex of the hummingbirds were determined as previously described (Pyle 1997). Three hummingbirds with no external signs of disease received minimal amounts of commercial food), and therefore dry samples of the food powder were additionally obtained for DNA extraction. Once transferred to the research laboratory, carcasses were stored at −70°C until dissection of digestive organs. Carcass freezing has been a technique employed by other microbiome studies (Capunitan et al. 2020, Song et al. 2020) and, given their small body size, hummingbird carcasses freeze rapidly, minimizing the time for change in bacterial community composition.

For each bird, organs were removed for DNA extraction using an aseptic technique. The acidic proventriculus and ventriculus ('proventriculus/ventriculus' hereafter; King and McLelland 1984) were sampled by cutting the esophageal–proventricular and ventricular–duodenal junctions. The small intestine sample was obtained by cutting the ventricular–duodenal junction and extending distally into the small intestine by approximately 0.5 cm. Similarly, the lower intestine sample was obtained by starting with the colonic–cloacal junction and cutting approximately 0.5 cm proximally into the large intestine.

Dissected organs were removed and collected in sterile microcentrifuge tubes and flushed using a syringe of sterile PCR-grade water, ensuring it passed through the entirety of the interior of the intestinal tract, in order to remove any potential dietary products. Following the flush, the intestinal tract was divided into upper and lower segments, which were sampled separately.

Microbiome characterization

To characterize the bacterial communities in the fecal and GI tract samples and in potential food items, we used metabarcoding of the 16S rRNA. Briefly, DNA was extracted from samples from regions of the GI tract using the DNeasy PowerSoil Kit and following manufacturer's instructions, modified by the addition of initial bead-beating and overnight lysis (Rubin et al. 2014). We also sequenced a sample of the commercial food, as some birds from Lindsay Wildlife Experience received minimal amounts of powder prior to being euthanized, and two blank control samples to ensure the quality of the sequencing data. From each extraction, DNA was sent to the Dalhousie IMR facility and sequenced using the Illumina MiSeq platform. Bacteria were characterized using the V6–V8 region of the 16S rRNA gene using 926F–1392R primers to avoid contamination by bird host tissues (Tremblay et al. 2015). The raw sequence data were submitted to the Sequence Read Archive (SRA) at the NCBI and can be located under access code PRJNA646258.

Reads were error-corrected and assembled into amplicon sequence variants (ASVs) using the R package DADA2 ver. 1.10.1 (Callahan et al. 2016) to represent the microbial taxa in and across all samples using recommended parameters. We chose to use ASVs rather than OTUs because of their greater precision and ability to compare across studies (Callahan et al. 2016). Briefly, primers were removed and reads filtered and trimmed using settings maxEE = 2,2 and length (forward = 280, reverse = 190). Reads were error-corrected and merged and chimeras detected and removed using the 'consensus' method. Bacteria were assigned taxonomy using SILVA training set v.132 (Quast et al. 2013). We restricted our analysis to bacteria by removing from the dataset all ASVs assigned to Archaea, Chlorophyta, Cyanobacteria, Arthropoda, Chloroflexi, class Chloroplast,

all mitochondrial sequences and all sequences without a taxonomic assignment. We recovered 291 468 reads after removing non-target sequences, resulting in an average of 28 183 sequences/sample and a minimum of 1264 sequences/sample. ASVs present in the extraction control samples were removed from the analysis as recommended (Davis et al. 2018). After removal of ASVs found in the control samples, followed by removal of low-abundance samples (<1000 sequences/sample), we were left with usable sequence from 20 (out of 20 submitted) proventriculus/ventriculus, 13/20 upper intestine, 11/20 lower intestine, 13/48 fecal samples, 3/5 insect samples, 2/2 feeder samples and 2/5 nectar powder samples submitted. We were able to determine sex, age and disease status out of the combined 68 birds used for the study, and we had useable sequences from 57/68 for sex, 57/68 for age and 57/68 for disease status. ASV abundance was normalized within individual samples by dividing the abundance of each ASV by the total sequence sum within a sample (McMurdie and Holmes 2014).

Statistical analyses

All statistical analyses focused on bird fecal and internal GI tract samples because too few samples were recovered from food items, precluding a formal analysis, but food item data are presented in Supporting information (described below). For bird samples, alpha diversity was calculated using Shannon's H-index, with all samples rarefied to have an equal sequencing depth using the function rarefy_evendepth in phyloseq (v. 1.26.1) (McMurdie and Holmes 2013). We used linear regression and ANOVA to assess if alpha diversity varied among regions of the GI tract, between bird ages or sexes and with pox infection status (yes/no) using linear models. To compare microbial community similarity across sample types, we visualized sample similarity using non-metric multidimensional scaling (NMDS) using Bray Curtis dissimilarity (Bray and Curtis 1957) using the 'ordinate' function in phyloseq (v. 1.26.1) (McMurdie and Holmes 2013) in R. We compared if microbiome composition was explained by GI tract sample type (fecal, proventriculus/ventriculus, upper intestine, lower intestine) using permutational multivariate analysis of variance (PERMANOVA) with the adonis2 function with 1000 permutations in the vegan (v. 2.5-5) package in R (Oksanen et al. 2019). We also examined if microbiome composition was associated with bird age (hatch year, after hatch year) or sex (male, female). To assess if health status (external visual evidence of pox-like lesions present, not present) varied with microbiome composition, we performed PERMANOVA but only used internal GI tract samples because only one free-ranging bird with a fecal sample presented with pox-like lesions. To observe the statistical differences between regions of the GI tract and fecal samples, we performed a post hoc test using the pairwise Adonis package (Martinez Arbizu 2017). In order to quantify the dispersion among samples within age groups, sexes, sample types and health status, we used the multivariate homogeneity of group dispersions test via the betadisper function in the vegan package in R (Oksanen et al. 2019) and Tukey's honest significant difference to compare among groups. All samples were plotted with ggplot2 (v. 3.2.1) (Wickham 2016).

To determine which ASVs were overrepresented within each bird GI tract sample type, age and sex, sequence counts were modeled with a local dispersion model and the reads per sample were normalized using the geometric mean using the DESeq2 (v. 1.24.0) package in R (Love et al. 2014). ASVs were considered differentially abundant with a false discovery rate (FDR) < 0.05. This analysis was repeated for health status (pox lesions/no pox lesions) on only regions of GI tract samples, subsampled to obtain an equal number of birds/treatment. In order to assess which taxa best discriminated the regions of the GI tract, we also performed a random forest analysis (machine learning method for classification) followed by an analysis of the taxa that contributed most to the decrease in Gini (greater decrease indicates an important variable for classification) using the 'randomForest' package (Liaw and Wiener 2002). All statistical analyses were performed in R (v. 3.5.2) (<www.r-project.org>) and RStudio (RStudio Team 2015).

Results

Alpha diversity

Shannon diversity was greatest in fecal samples (Fig. 1) compared to any GI tract types ($F_{3,53}=6.72$, $p < 0.001$), whereas GI tract regions did not differ significantly in diversity ($F_{2,41}=0.45$, $p=0.64$). Bacterial diversity did not differ significantly between bird sexes ($F_{1,53}=0.17$, $p=0.68$) nor ages ($F_{1,53}=0.50$, $p=0.48$; Supporting information). No interaction between GI tract sample type, bird age or bird sex was significant for alpha diversity ($p > 0.25$ for all). Bacterial diversity in GI tract samples was not significantly associated with pox status (pox $F_{2,38}=0.33$, $p=0.71$).

Community composition

Comparing fecal and internal GI tract samples, bacterial community composition differed among sample types (Fig. 2, 3, PERMANOVA: $R^2 = 0.12$; $p=0.001$), between sexes (PERMANOVA: $R^2 = 0.02$, $p=0.03$) and between hatch years and after hatch years (PERMANOVA $R^2 = 0.02$, $p=0.03$; Supporting information). Notably, bird fecal samples were the most variable in composition (Fig. 2; betadisper TukeyHSD $p < 0.05$) whereas the proventriculus samples were the least variable of all regions of the GI tract sampled (Fig. 2; betadisper TukeyHSD $p < 0.05$). Among GI tract samples, lower and upper intestine samples were more variable than the proventriculus/ventriculus (betadisper TukeyHSD $p=0.01$), while the upper and lower intestine samples did not differ significantly from each other in dispersion (betadisper TukeyHSD $p=0.99$). Post hoc pairwise tests showed significant differences between fecal samples and proventriculus (pairwise $p=0.006$), fecal samples and

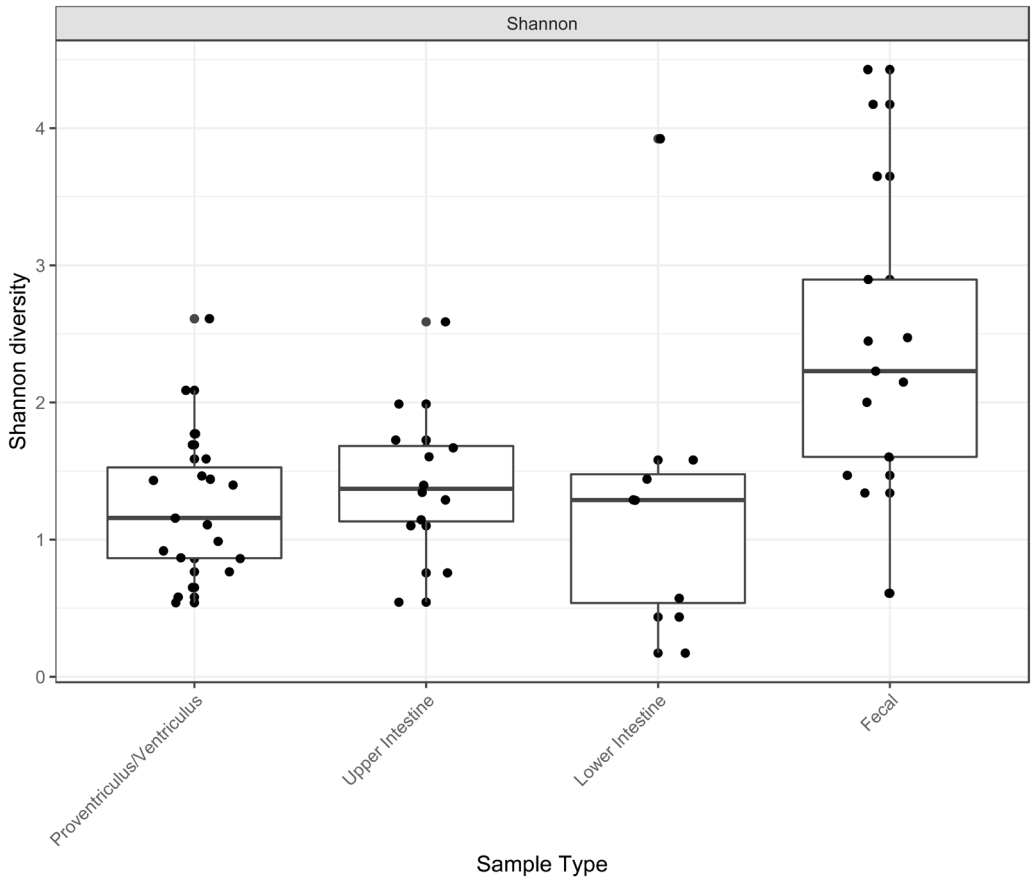

Figure 1. Shannon diversity of bacterial communities showing differences among fecal and GI tract sample types. The intestinal tract was divided into its upper and lower segments, the upper being the section contiguous with the ventriculus and the lower being the section closest to the cloaca. Fecal samples were collected from free-ranging birds while regions of the GI tract (proventriculus/ventriculus and upper and lower intestinal samples) were sampled from free-ranging birds that were brought to the Lindsay Wildlife Experience. Center line in each box indicates median, outer lines indicate the first and third quartiles, and whiskers extend to include 1.5 times the interquartile range.

the upper intestine (pairwise $p = 0.03$), the proventriculus and lower intestine (pairwise $p = 0.01$) and the proventriculus and the upper intestine (pairwise $p = 0.01$). Variation in microbiome composition (dispersion) differed neither between hatch year (HY) birds and AHYs (betadisper Age TukeyHSD $p = 0.41$, Supporting Information) nor between male and female birds (betadisper Sex TukeyHSD $p = 0.42$, Supporting Information). Within bird GI tract samples, microbial composition differed between birds that had visual lesions consistent with pox infections and those that did not (PERMANOVA: $R^2 = 0.05$; $p = 0.002$), but these groups did not differ in dispersion (betadisper TukeyHSD $p = 0.70$).

We also sampled feeder sugar water, insect and Nektar powder bacterial communities. Because we only sampled a low number of dietary items, we could not statistically compare their composition to that of bird GI tract samples but found that each contained distinct microbial communities that were contained within NMDS 95% confidence interval for fecal samples (Supporting information) and contained some taxa also represented within fecal samples (Supporting information).

Bacterial composition of GI regions

Hummingbird GI tract types were distinguished by particular taxa. The proventriculus/ventriculus contained 50% Fusobacteria, 25% Firmicutes, 10% Bacteroidetes and 10% Actinobacteria (Fig. 3A). The upper intestine contained 50% Firmicutes, 20% Fusobacteria and 20% Actinobacteria (Fig. 3A). Over 75% of both the lower intestine and fecal samples was characterized by Proteobacteria, along with <10% Bacteroidetes and Actinobacteria (Fig. 3A). *Oceanivirga* (Fusobacteria) and *Riemerella* (Bacteroidetes) were more abundant in the proventriculus/ventriculus than the intestine or fecal samples (Fig. 4; DESeq $p < 0.05$) and were important in distinguishing among sample types (Supporting information). Fecal samples were categorized by the presence of *Lawsonella* (Actinobacteria), a genus of the suborder Corynebacterineae and *Endozoicomonas* (Proteobacteria) (Fig. 4, Supporting information). The random forest algorithm correctly classified 11 of 13 fecal samples and 18 of 20 proventriculus samples despite a relatively poor overall classification success (38% out-of-box error

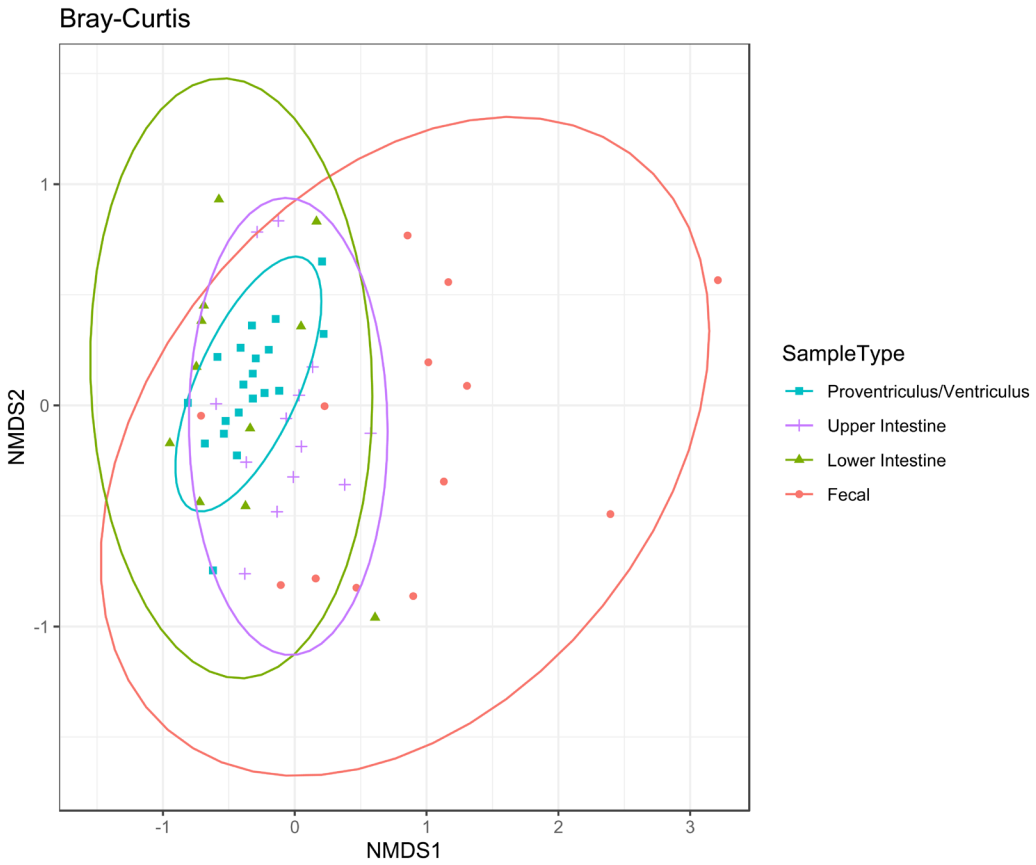

Figure 2. Nonmetric multidimensional scaling (NMDS) plot of Bray–Curtis dissimilarities between bacterial amplicon samples taken from regions of the GI tract or fecal material from Anna's hummingbirds (*Calpyte anna*; n = 57). Fecal samples were collected from free-ranging birds while internal samples from regions of the GI tract were from originally free-ranging birds that were brought to Lindsay Wildlife Experience and combined for sample size. Points represent individual samples and are colored by sample type. The intestinal tract was divided into its upper and lower segments, the upper being the section contiguous with the ventriculus and the lower being the section closest to the cloaca. Stress value = 0.22.

rate). Intestinal samples were more difficult to classify correctly and to distinguish from fecal samples (61% and 85% error in classifying these sample types using random forest). Birds with symptoms of pox viral infections had a higher relative abundance of *Staphylococcus* (Firmicutes) (Supporting information, DESeq $p < 0.05$), which were only detected in bird GI tract samples.

Discussion

Here, we report that the hummingbird GI tract hosts distinct and consistent bacterial communities among bird individuals, in contrast to fecal samples which were more diverse and variable in composition. Moreover, GI tract samples were characterized by distinct bacterial taxa (e.g. *Oceanivirga*, *Riemerella* and *Corynebacterium*), especially within the proventriculus/ventriculus. A graphical comparison of bacterial composition of potential dietary items to the hummingbird samples found minimal overlap between internal regions of the GI tract and diet items but some similarity between diet items and fecal samples (Supporting information). Previous studies have reported that fecal samples reflect the microbiome of dietary items rather than the host GI tract (Sekelja et al. 2012, Ingala et al. 2018, Videvall et al. 2018, Bodawatta et al. 2021). Overlap in the presence of some taxa between fecal and diet samples (Supporting information) and increased variability of fecal compared to internal GI tract bacterial composition (Fig. 1) suggest this may be the case for *C. anna*. However, an important caveat for this hypothesis is that in addition to our small sample size for dietary items, bird populations sampled for fecal and GI samples in our study differed. It is possible that the free-ranging birds sampled for fecal material could have experienced more variation in the environment than those brought to Lindsay Wildlife Experience (although these were also from free-ranging birds), resulting in the greater variation in fecal samples. However, all birds were from northern California, and rehabilitation birds chosen for inclusion in our study received no mediation and died soon after arrival at the center; moreover, we did not detect overlap between bacterial taxa in the diet fed at Lindsay Wildlife Experience and those in the GI tract (Supporting information). Paired sampling of fecal samples

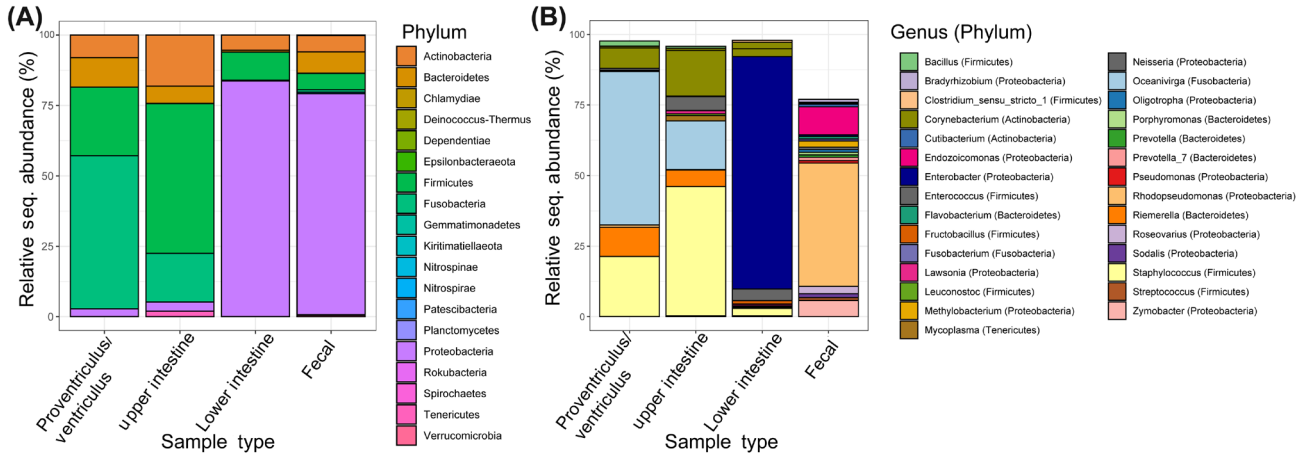

Figure 3. Relative sequence abundance (% of total) of bacterial taxa associated with fecal or samples from regions of the GI tract from Anna's hummingbirds (*Calpyte anna*; n = 57), averaged within each sample type. In (A), colors indicate bacterial phyla, and in (B), colors represent bacterial genera. The intestinal tract was divided into its upper and lower segments, the upper being the section contiguous with the ventriculus and the lower being the section closest to the cloaca. Fecal samples were collected from free-ranging birds while internal GI tract samples were from originally free-ranging birds that were brought to Lindsay Wildlife Experience. Only the most abundant 30 taxa are shown in the genus-level plot to enhance clarity; the rare taxa that comprise the remainder of the total for each sample type are not shown in this figure.

and internal samples from the same birds could resolve this question directly.

Focusing on the internal bacterial composition of the GI tract characterized here, the bacterial taxa of *C. anna* documented here (Fig. 3) differ in taxonomic composition from that of previously characterized pollinators, including social bees (Engel et al. 2016), nectivorous passerines (Bodawatta et al. 2018) and nectivorous bats (Carrillo-Araujo et al. 2015), suggesting a limited role of diet in shaping GI bacterial taxonomic composition in pollinators. These results are shared by a recent study from Bodawatta et al. (2021), which showed bacterial community composition being species-specific rather than resulting from phylosymbiosis. Comparing to other nectar feeding birds, the excreta of sunbird *Cinnyris osea* contained bacteria from the phyla Proteobacteria, Firmicutes and Actinobacteria (Gunasekaran et al. 2020), similar to the fecal samples in our study and *C. anna* in Herder et al. (2021). Nectivorous bats contained primarily Proteobacteria while having minimal amounts of Firmicutes (Carrillo-Araujo et al. 2015). Like *C. anna*, the nectar-feeding passerine *Toxorhamphus poliopterus* was not only characterized by a high relative abundance of Firmicutes, Actinobacteria and Proteobacteria but also contained Tenericutes which were detected at low abundance in *C. anna* (Bodawatta et al. 2018). Moreover, the dominant taxa between these taxa differed substantially at the genus level (Bodawatta et al. 2018). Future work could compare the internal GI tract bacterial composition in more nectar-feeding species to examine if phylogeny or diet is important in shaping microbiome structure.

As predicted, we found evidence for GI tract region-specific microbial communities, consistent with previous work on bird microbiomes (Waite and Taylor 2014, Grond et al. 2018, 2020, Videvall et al. 2018, Bodawatta et al. 2020). It is likely that physiochemical conditions in the GI tract (pH, morphology, etc.) could shape microbial composition among regions of the GI tract. The similarities in bacterial community composition of the proventriculus and upper intestine are not surprising, as normal movement of ingesta is circular, moving through the proventriculus, ventriculus and upper intestine before passing through the lower intestine. However, the proventriculus is often more acidic than the upper and lower portions of the intestinal tract (King and McLelland 1984, Grond et al. 2018), and our study hosted the most distinct bacterial composition, characterized by *Oceanivirga* (Fusobacteria) and *Riemerella* (Bacteriodetes). *Oceanivirga*, in the family Leptotrichiaceae, was abundant in the proventriculus. Although this species has not previously been detected in birds, the Leptotrichiaceae are anaerobic colonists of mucus membranes that are capable of fermentation and may contribute to host nitrogen availability in insect hosts (Lory 2014). Also abundant, *Riemerella* can be pathogenic to some bird species (Emerson et al. 1983, Hays 2006, Li et al. 2020), there was no evidence of disease in birds sampled for this study. Species within the genus *Riemerella* can exhibit trypsin, chymotrypsin and acid phosphatase activity (Hinz et al. 1998) which suggests possible contribution to host nutrition via protein lysis. Fecal samples were characterized by a high abundance of *Lawsonella* (Corynebacterineae) and *Endozoicomonas* (Hahellaceae), neither of which were present in the GI tract (Fig. 4, Supporting information). We detected *Corynebacterium* in abundance across many regions of the GI tract and in fecal samples, as was previously reported for *C. anna* (Lee et al. 2019, Herder et al. 2021). The genus *Corynebacterium* contains some pathogenic species (Katsukawa et al. 2016, Bratcher 2018), but members of this genus are frequently isolated from the cloaca of healthy birds, including *Calidris* shorebird species (Risely et al.

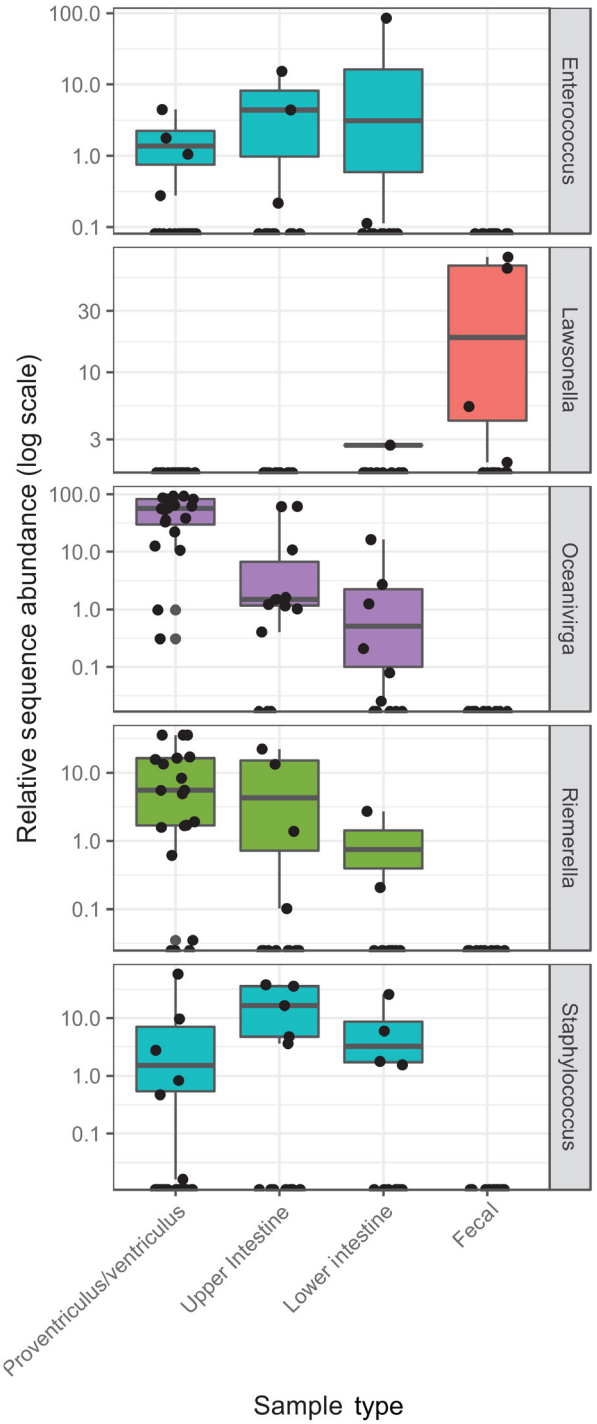

Figure 4. Relative sequence abundance (% of total) of bacterial genera found to be differentially abundant between fecal and sample types from regions of the GI tract from Anna's hummingbirds (*Calpyte anna*; n = 57) using DESeq2 with false discovery rate less than 0.05. Center line in each box indicates median, outer lines indicate the first and third quartiles, and whiskers extend to include 1.5 times the interquartile range. Note that the y-axis is on a log10 scale so that low relative abundance samples are still visible. Fecal samples were collected from free-ranging birds while regions of the GI tract were sampled from free-ranging birds taken to the Lindsay Wildlife Experience. Panels are labeled with bacterial genus.

2018), penguins (Goyache et al. 2003), eagles (Fernández-Garayzábal et al. 2003) and psittacines (Bangert et al. 1988), and their effects on host physiology in most systems remain unknown. Though *Corynebacterium* has previously been reported in Anna's hummingbirds and other bird gut microbiota, Hammer et al. (2015) reported that members of this genera, along with *Staphylococcus*, may be present in high abundance due to skin contamination as they are commonly present on human hands. Our approach removes all ASVs detected in the negative controls, so we believe that although the possibility of skin contamination is present, *Corynebacterium* and *Staphylococcus* found in our samples are likely to be associated with the birds.

The lower intestine was characterized by *Enterobacter*, *Enterococcus*, *Leuconostoc* and *Streptococcus* (Fig. 4, Supporting information), taxa which are frequently described as mucus-associated colonists of the GI tract of many organisms (Dieterich et al. 2018). Previous studies on *C. anna* have described *Enterococcus* and *Leuconostoc* (Herder et al. 2021) and *Enterobacter* (Lee et al. 2019) in fecal samples. Members of the family Enterobacteriaceae have previously been found populating bird intestinal tracts (García-Amado et al. 2018), and *Enterobacter* is a putative pathogen and that commonly resides in the cloaca in other bird species (Zeng et al. 2017) and easily adapts to gut conditions. Its presence in the lower intestine of our samples (Supporting information) may indicate further evidence of a residential gut microbiome.

Our data support predictions that the bacterial composition of young birds is distinct from older birds (Supporting information) and weakly support a difference in bacterial composition between sexes (Supporting information). In comparison to previous studies that have noted substantial turnover in bacterial composition in the GI tract of house sparrows with bird age (Kohl et al. 2019), in our study, bird age only explained about 2% of variance in community composition. It may be that microbial turnover and assembly is detectable only in younger birds, but here, we were only able to sample HYs that were actively foraging. Similarly, differences between male and female microbiomes in our study were significant but explained little variation in bacterial species composition. It may be that differences between sexes vary seasonally and be more pronounced during breeding season or when diet or habitat ranges diverge between sexes (Escallón et al. 2019).

Microbiome composition also varied with the presence of avipox lesions (Supporting information), suggesting a link between disease and dysbiosis in hummingbirds. While our methods were unable to determine the species of bacteria and are thus unable to claim if it is a pathogen for the birds, a higher abundance of *Staphylococcus* in birds presenting with avipox lesions suggests that viral disease processes are associated with variation in the microbiome and might have the potential to shift the relative abundance of particular microorganisms. Avian pox viral infections are typically not a primary cause of death in hummingbirds but can range in disease severity and compromise their overall nutritional status and immune function. In a previous study, mallard

ducks positive for influenza A had a lower bacterial species diversity, reduced species richness and evenness than those ducks that were reported negative for the virus (Ganz et al. 2017). In contrast with predictions that diseased organisms should show reduced variability than healthy organisms, we found no difference between birds with and without avipox symptoms in dispersion (variability in microbiome composition) but we note our limited sample size for this comparison. Mechanisms underlying such links will be essential for interpreting shifts in microbiome composition or potentially using microbiome data for health monitoring of free-ranging bird populations.

Our results have implications for methods of microbiome monitoring of free-ranging hummingbirds. The microbial communities of free-ranging animals are often limited to fecal or cloacal sampling methods (Allegretti et al. 2014, Videvall et al. 2018, Herder et al. 2021), largely due to the lethality of sampling other regions of the GI tract. The differences between fecal and GI tract microbes suggest that fecal sampling does not necessarily correspond with microbial composition found in regions from the hummingbird GI tract, so more invasive methods or repeat sampling of the same bird over a period of time might be required to more accurately characterize taxa within GI tract samples.

In conclusion, our results suggest that a consistent bacterial community exists within portions of the hummingbird GI tract. Observed variation in microbial community composition is driven primarily by the region of the GI tract, which varied significantly between sample type and region. Additionally, health status is also associated with GI tract microbiota composition. Although some taxa that we identified may contribute to previous reports of microbial involvement in nitrogen recycling (Preest et al. 2003), further investigation will be required. Metagenomic, transcript-based, functional or other types of inquiry will be required to examine the functional role of GI microbes for hummingbirds metabolic or digestive processes. The unique nature of the GI tract of *C. anna* warrants further study, as their nectivorous and insectivorous diet and rapid digestive transit time can help provide insight into the differences in community composition found in smaller birds, as previously shown in Bodawatta et al. (2021). Our study suggests that GI tract-associated microbiota in hummingbirds exists and that additional research into its function is warranted.

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Raw sequence data are available at NCBI SRA under project no. PRJNA646258. Metadata and code are available at Dryad <https://doi.org/10.25338/B81D0X>.

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