INTRODUCTION

A healthy gut microbiome is thought to be both diverse and relatively stable (García-García *et al*. 2019; Rinninella *et al*. 2019) but may be heavily affected by a variety of extrinsic and intrinsic factors, including host genetics, habitat, and diet (Hird *et al*. 2015; Rothschild *et al*. 2018). The composition and stability of a healthy microbiome may change as animals undergo recurrent physiological stressors, such as migration or changing climates across seasons (Sommer *et al*. 2016; Carey and Assadi-Porter 2017). Increased understanding of both variation and stability of gut microbial ecologies related to recurrent physiological stressors can further elucidate host adaptation to repetitive stress. Here, we ask what changes and what remains consistent within the gut microbiome of a migratory bird species across multiple time points and locations within the annual cycle.

Species experiencing seasonal variation in habitat, diet, or physiological stressors often exhibit correlated changes in their microbiome (Maurice *et al*. 2015; Sommer *et al*. 2016; Ren *et al*. 2017; Smits *et al*. 2017; Drovetski *et al*. 2019). Migratory animals may undergo seasonal fluctuations in metabolic needs that, in combination with changing habitats and diets, result in variable microbiota composition across their annual cycles, but the extent to which this occurs remains unclear (Jenni and Jenni-Eiermann 1998; Grond *et al*. 2018).

Gut microbiota of some migratory bird species have been characterized at discrete portions of the annual cycle, revealing substantial bacterial diversity (Lewis *et al*. 2016; Risely *et al*. 2017; Wu *et al*. 2018; Cao *et al*. 2020). Different environments, such as breeding grounds, wintering grounds, and stopover sites during migration, have been shown to impact the overall composition of gut microbiota, likely through exposure from local microbial communities or

food sources (Lewis *et al*. 2017; Wu *et al*. 2018). Additionally, physiological adaptations of migratory birds, such as intestinal atrophication during active migration, may further affect gut microbiota (Grond *et al*. 2018). Given the variability of gut microbiota and strong environmental effect, it may be difficult to directly correlate variation in gut microbiota to ongoing biological processes, specific host traits, or environmental factors without temporal sampling across different time points of the annual cycle. (Hird *et al*. 2014; Capunitan *et al*. 2020; Song *et al*. 2020). Here we recaptured individuals multiple times on their tropical wintering and temperate breeding grounds to better understand local and temporal variability in gut microbiota thus reducing sources of variability known to be associated with sampling different individuals and different populations (Flores *et al*. 2014; Hird *et al*. 2014; Baxter *et al*. 2015).

Until now, no migratory songbird has been sampled at multiple time points and locations across their annual cycles. Migratory birds have complicated annual cycles that involve twice-annual movements often spanning thousands of kilometers between stationary breeding and wintering periods. Once captured, researchers typically have no way to relocate or recapture the same individuals outside of the original capture site, especially for species with expansive wintering and breeding ranges and with populations that may number in the millions. This inhibits sampling from the same population, let alone the same individual, at multiple points in the annual cycle. As a result, one must attempt to measure and control for confounding factors, such as between population differences, and account for high inter-individual variability (Flores *et al*. 2014; Hird *et al*. 2014; Baxter *et al*. 2015). Thus, our inability to study the same individuals across the annual cycle has impeded identification and understanding of variation within birds associated with seasonal movement.

The Kirtland's Warbler (*Setophaga kirtlandii)* provides an unusual opportunity for studying changes across the annual cycle in a migratory species. Their small population size as well as restricted breeding and wintering ranges (Cooper *et al.* 2019) make it feasible to relocate individuals across seasons (Cooper *et al.* 2018; Cooper and Marra 2020). Following substantial population declines, only 167 singing males were recorded in 1974 and again in 1987, based on breeding surveys (Kepler *et al.* 1996). Through extensive conservation management efforts, the population has increased to approximately 2,300 singing males of which 97% breed across a relatively small area in Michigan's Lower Peninsula. This species winters primarily in the scrub forests of The Bahamas (Cooper *et al.* 2019), more than 2,000 km from the breeding grounds. For this study, we radio-tagged individuals on the wintering grounds and then relocated and recaptured the same birds on the breeding grounds in Michigan through the use of automated telemetry towers. We used 16S rRNA next generation sequencing technologies to catalogue the bacterial communities of individuals. Our goals were to: (1) characterize the bacterial diversity of Kirtland's Warblers at three unique periods of the annual cycle at the population and individual level; (2) evaluate host sex, age, period of annual cycle, and location effect on abundance and diversity of gut microbiota; and (3) determine if a core bacterial profile for Kirtland's Warblers exists and if so, establish a species-specific pattern.

MATERIALS AND METHODS

Initial sample collection in The Bahamas

We captured Kirtland's Warblers on Cat Island, The Bahamas, in March and April of 2017 and 2018 using vocalization playback and mist nets. We classified individuals into two age

categories (SY = second calendar year or ASY = after second year), sexed individuals following Pyle (1997), and attached a USGS metal band and three plastic colored bands. We then fitted a coded radio-tag (0.35g, Model = NTQBW-2, Lotek Wireless, Inc.) using a modified leg-loop harness (Rappole and Tipton 1991). Tags emitted coded pulses at regular intervals (29.3 s), which allowed for individual identification through handheld or automated telemetry receivers (Taylor *et al.* 2017). After attaching the radio tags, we collected fecal samples by placing birds in a wax paper bag for up to ten minutes. We transferred fecal materials from the bag to Whatman FTA Cards (Whatman, Florham Park, NJ) using Whatman sterile swabs (Whatman, Florham Park, NJ).

Relocation and recapture in Michigan

We erected 11 automated telemetry towers in Michigan which allowed us to detect tagged individuals as they arrived at the majority of breeding sites. Birds arrived between May 9 and June 3. We downloaded tower data daily and used handheld telemetry to search the few areas not well covered by towers at least every three days. We used these data to determine arrival dates in Michigan. Following initial detection, we used handheld telemetry to locate each individual's territory and target them for recapture. Birds were captured an average of 7.7 (SD ±8.1). days after their first detection in Michigan. We also attempted to recapture birds towards the end of the breeding season in early July. In May of 2018, we also captured and sampled non-tagged Kirtland's Warblers to compare microbial variation in individuals known to be from Cat Island with birds that may have wintered on other islands. Regardless of timing, we used identical capture and sampling protocols as those used in The Bahamas (see above).

Molecular Methods

We isolated DNA from fecal samples stored on Whatman FTA Cards using the Qiagen DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) following the manufacturer's extraction protocol. We included six blank negative controls to account for possible contamination during extraction and polymerase chain reaction (PCR). Following standardized procedures (Caporaso *et al.* 2012), we used PCR to amplify the V4 region of the 16S microbial small subunit ribosomal RNA (rRNA) gene using the Earth Microbiome Project universal primers 515f/816r. We then used the Illumina MiSeq sequencing platform to obtain paired-end 150 base pair reads. DNA extractions took place at the Field Museum of Natural History. All subsequent molecular work was conducted at the IGM Genomics Center of the University of California, San Diego.

Sequence Processing

We processed raw sequence data with the Quantitative Insights Into Microbial Ecology (QIIME2 version 2019.1) pipeline (Caporaso *et al.* 2010; Bolyen *et al.* 2019). In QIIME2, following standard demultiplexing and quality filtering, we generated amplicon sequence variants (ASVs) using the Divisive Amplicon Denoising Algorithm (DADA2) (Callahan *et al.* 2016). DADA2 statistically infers sample sequences and implements quality control elements including exclusion of singletons, chimera removal, and sequence error elimination. We trimmed all sequences outside base pair positions 13 and 145 base pairs to remove the primers. We classified ASV taxonomies using the Silva reference database (Quast *et al.* 2012, version 132). We identified bacterial contaminants using a frequency-based algorithm in the R package

Decontam (Davis *et al.* 2018). We removed contaminants and negative controls from subsequent analyses.

Rarefaction

Rarefaction of microbial data to normalize for varying library size can lead to data loss and may be detrimental to interpretation of results (McMurdie and Holmes 2014). To ensure that patterns observed in non-rarefied data are not due to bias in library size, we rarefied all libraries to 7,000 reads. We then conducted alpha and beta diversity analyses, described below. Results from rarefied tests did not qualitatively differ from the non-rarefied data (Figure S1). In the main text, we present and discuss the results of non-rarefied data.

Statistical Analysis

We analyzed community alpha diversity using log (observed ASV richness) and the Shannon Diversity Index. For modeling diversity, we used a linear mixed model as implemented in the R package *lme4* (Bates *et al.* 2007) and evaluated the importance of different variables, taking into account the repeated sampling of some birds. We included host age (SY or ASY), sex (male or female), year (2017 or 2018) and sampling period (The Bahamas, first recapture in Michigan, and second recapture in Michigan) as fixed effects and individual host as a random effect. Using *lmerTest* (Kuznetsova *et al.* 2015), we generated an ANOVA table from the linear model analysis, and subsequently conducted *a posteriori* pairwise tests to compare the three sampling periods. Additionally, we conducted a pairwise t-test to assess differences between tagged and randomly caught birds within the first recapture period of 2018. We tested for the influence of

outliers, which appeared to cause a deviation from normality in ASV richness (Shapiro-Wilks test), by repeating the analyses with outliers omitted and obtained very similar results. Finally, we tested for the effect of individual-level random effects with a likelihood ratio test comparing the model with and without individual ID as the random effect term, and we found individuals did not consistently differ from each other.

To examine community differences in the microbiome (beta diversity), we applied permutational multivariate analysis of variance (PERMANOVA) of Bray-Curtis dissimilarity and unweighted UniFrac distances, calculated among individual samples (Anderson 2014). For variables that showed significant differences in the PERMANOVA analyses, we conducted an *a posteriori* test to assess differences in dispersion or centroids using PERMDISP. We visualized beta diversity of significant variables using non-metric multidimensional scaling (nMDS) ordination of the Bray-Curtis measurements. Diversity calculations were implemented using the R packages *vegan* and *phyloseq* (Oksanen *et al.* 2007; McMurdie and Holmes 2013). Finally, to ask which taxa differ in abundance across sampling periods, we implemented analysis of composition of microbes (ANCOM) in QIIME2 (Mandal *et al.* 2105). ANCOM utilizes the underlying structure of the microbiome data to identify differentially abundant taxa between categories.

Core Microbiome

We defined the community core microbiome as ASVs present in at least 50% of all individuals in each of the three sampling periods (Astudillo-García *et al.* 2017, Grond *et al.* 2017). We studied the community core at multiple taxonomic levels using *Phylocore* (Ren and Wu 2016). We also

identified a temporal core in birds sampled in triplicate, defined as ASVs found at all three sampling periods within the same bird (Shade *et al.* 2012). We calculated the proportion of temporal core ASVs to those that are transient and not found at all three sampling periods to identify the average proportion of ASVs that are retained over time.

RESULTS

We collected 176 fecal samples from 116 Kirtland's Warblers at locations throughout Cat Island, The Bahamas, where we collected 93 samples, and Michigan's Lower Peninsula, with 63 samples collected in the first recapture period and 20 samples collected at second recapture. Thirty-four birds were sampled twice, once during initial capture in The Bahamas and a second time during first recapture in Michigan. Of those birds, 10 individuals were sampled a third time during a second recapture period in Michigan (Table S1). Additionally, 13 non-tagged Kirtland's Warblers were sampled in May 2018 in Michigan. Quality control measures resulted in the removal of 10 libraries for poor DNA or PCR yield and 52 contaminant ASVs from the overall dataset. Our final dataset is composed of 166 sequenced libraries (Table 1) totaling 5,007,844 reads, with an average 30,168 reads per library (range: 7,022 – 100,856). We detected 7,426 unique ASVs across all sampled with a mean of 107.3 ± 96.7(standard deviation [SD]) per library.

Bacterial community composition and diversity

Across all samples, bacteria from 37 phyla were detected. Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria composed 91.13% of the total reads; 5.8% of the reads

belonged to the 33 remaining phyla and 3.07% of reads did not align to any known bacterial phyla (Figure 1A). *Clostridia* (Phylum Firmicutes), *Gammaproteobacteria* (Phylum Proteobacteria), and *Bacteroidia* (Phylum Bacteroidetes) were the most abundant classes, representing 70.16% of all reads. The mean abundance of most phyla and classes differed between initial sampling in The Bahamas and subsequent samplings in Michigan (Figure 1B, Table S2). The birds shifted from a Firmicutes dominated microbiome in The Bahamas (mean abundance per individual 39.82% [SD, ±13.97%]) and Michigan following arrival (38.12% [SD, ±16.41%]) to Proteobacteria as the most abundant phylum in the second Michigan recapture period (47.07% [SD, ±27.90%]). Bacteroidetes and Actinobacteria were also proportionally more abundant in The Bahamas than in the second Michigan recapture period. Notably, Cyanobacteria represented 1.91% (SD, ±5.93%) of the total microbiota in The Bahamas, but decreased to 0.05% (SD, ±0.23%) by the second recapture period in Michigan.

Alpha diversity was not significantly affected by year, host age or host sex (Table S3). However, the three sampling periods significantly differed (Type III ANOVA with Satterthwaite's method; Observed richness: $F_{2,116.34} = 14.76$, $P < 0.0001$; Shannon Diversity: $F_{2,126.91} = 29.22$, $P < 0.0001$). All Bonferroni corrected pairwise comparisons on the fitted values from the linear model were significantly different from each other (Observed: Bahamas vs. each recapture period both $P < 0.0001$, first vs. second recapture period, $P = 0.002$; Shannon Diversity: all comparisons: $P < 0.0001$). Birds in The Bahamas showed increased bacterial diversity compared to either recapture period in Michigan, demonstrated through a comparison of all samples (Figure 2A) as well as with paired sampling of the same individuals (Figure 2B). In the birds sampled in triplicate, alpha diversity varied between first and second Michigan recaptures

(Figure 2C). A comparison of tagged and randomly captured birds in the first Michigan sampling period of 2018 revealed no significant differences in alpha diversity (pairwise t-test; Observed: $p = 0.13$, Shannon Diversity: $p = 0.22$). We observed decreased alpha diversity through the first three days after arrival in Michigan followed by a slight increase over the following six days (Figure 3).

Our results indicate that beta diversity was not significantly affected by age or sex of the birds within the full dataset or individual sampling periods (Table 2), with the exception of age in the second Michigan resampling period (unweighted UniFrac: PERMANOVA $p = 0.0128$, PERMDISP $p = 0.2213$). Community composition of the microbiota significantly differed by year in the full dataset and at each sampling period (Table 2, Figure 4A). Additionally, our PERMANOVA results suggest that sampling period exerted a significant effect on the microbiota composition (Bray-Curtis: $p = 0.0002$, unweighted UniFrac: $p = 0.0001$), though the significant unweighted UniFrac result can be explained through variation in spread of the sample composition, rather than with significantly different centroids such as with the Bray-Curtis dissimilarity matrix (PERMDISP; Bray-Curtis $p = 0.7104$, unweighted UniFrac $p = 3.71e\text{-}6$). This indicates that although the abundances of microbiota are significantly different during sampling periods, the taxonomic variation of bacterial lineages present are not. The effect of sampling period on the gut microbiota explained 2.5% and 2.4% of the variation in microbiota composition for Bray-Curtis and unweighted UniFrac respectively. Taken together, all variables tested (Sampling period, Year, Sex, Age) explained less than 5% of the total variation in the microbiome (Bray-Curtis: 4.91%, unweighted UniFrac: 4.6%). No consistent changes were observed in the beta diversity of the birds sampled in triplicate (Figure 4B, Figure S3).

Across the three sampling periods, six ASVs and six genera were identified by ANCOM as differentially abundant. Genera *Solirubrobacter*, *Nocardioides*, and *Rubrobacter* were significantly associated with The Bahamas, *Endobacter* and *Candidatus Hamiltonella* associated with first recapture period in Michigan, and *Serratia* with second recapture period. ASVs within families *Beijernickiaceae* and *Pseudomonadaceae* were significantly more abundant during first recapture in Michigan and families *Enterobacteriaceae*, *Beijernickiaceae*, and *Synergistaceae* contained ASVs associated with the second recapture in Michigan. No ASVs were statistically associated with The Bahamas.

Core Microbiome

We identified Class *Bacilli*, Order *Pseudomonadales*, Family *Beijernickiaceae*, Genera *Sutterella* and *Eubacterium elegans* group, and 27 ASVs as representing the community core microbiota of Kirtland's Warbler within the full dataset, overlapping at all time periods (Table S4). Thirteen of the 27 core ASVs are members of class *Clostridia* and nine ASVs are found within genus *Bacteroides*. The remaining core ASVs belong to Phyla Actinobacteria, Proteobacteria, and additional classes within Firmicutes. One ASV is unclassified after Kingdom Bacteria. We also identified a temporal core in the birds sampled at all three sampling points. Individuals retained 18-26 ASVs, present at each sampling period, which represented an average of 25.06% (range: 8.58%-50.00%) of ASVs detected per individual per time point.

DISCUSSION

Gut microbiota of Kirtland's Warblers change as individuals and populations migrate from the wintering grounds in The Bahamas to breeding territories in Michigan. Repeated sampling at multiple points across the annual cycle was only possible because we were able to capture, sample, and radio-tag individuals on the wintering grounds and then use automated telemetry to relocate the same individuals thousands of kilometers away on the breeding grounds (Cooper and Marra 2020). Through the resampling of individuals we remove potential biases associated with sampling multiple populations. Therefore, the effects observed can be attributed to true changes within individuals and our study population. We found that period within the annual cycle exerts a notable effect on the overall diversity of the microbiome and birds on their wintering grounds have a significantly different and more diverse community of gut microbiota than those on their breeding grounds. We also identified a common, core microbial profile of Kirtland's Warbler that persisted throughout multiple portions of the annual cycle.

Community Composition

The overarching composition of Kirtland's Warbler microbiota is consistent with that of most wild bird surveys to date, with members of Phyla Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria comprising the majority of all bacteria detected (Dewar et al. 2014; Lewis et al. 2016; Grond *et al.* 2018). However, the relative abundances of all phyla changed, sometimes dramatically, as the birds migrated from The Bahamas to Michigan and over time in Michigan. Variation across the annual cycle may reflect difference in presence or abundance of environmental bacteria, responses to altered diets that in turn favor some bacteria over others

264 or vary with host characteristics and requirements. Below, we consider plausible examples of
265 each.
266
267 *Environmental effect*
268 The avian gut microbiome frequently reflects the local environment (Hird et al. *2014;* Hird *et al.*
269 2018; Cao *et al.* 2020). Cyanobacteria, found in marine and brackish waters (Sivonen 1996), was
270 common in birds in The Bahamas but nearly absent from most individuals in Michigan.
271 Cyanobacteria has previously been found in the gut microbiota of island birds (García-Amado *et*
272 *al.* 2018) and is known to be acquired through food (Birrenkott *et al.* 2004). Kirtland's Warblers
273 may acquire environmentally derived Cyanobacteria in The Bahamas via food consumption, as
274 most birds were captured within 2km of the ocean and much of the groundwater on the island
275 is brackish. We detected two common environmental bacterial genera, *Solirubrobacter* and
276 *Nocardioides*, as more abundant with birds in The Bahamas (Janssen 2006; Topp *et al.* 2000).
277 While local habitats exert a notable influence on the gut microbiota of birds, it is
278 unknown if microbial diversity increases or decreases during active migration or timing of gut
279 microbiota to acclimate to new habitats. During migration birds are exposed to varying
280 environments at stopover sites where they could acquire novel microbes (Lewis et al. 2017),
281 resulting in temporarily inflated diversity. In contrast, possible adaptations to long distance
282 flight, such as relatively shorter intestinal length and atrophication of intestines during active
283 migration, might result in decreased microbial diversity (McWilliams and Karasov 2005;
284 Caviedes-Viral *et al.* 2007). Using the ability to determine what day individuals arrive in
285 Michigan following migration, we observed a decrease in microbial diversity the first three days

before slowly increasing over days four through nine. Birds may be spending the first few days at their breeding grounds shedding transient microbes acquired at stopover sites. This suggests that during spring migration microbial diversity increases due to exposure at stopover sites rather than decreases as an adaptation to long-distance flight. However, sample size per day is small and additional research with larger sample size is needed to further support these results.

Gut microbiota are dynamic, displaying influence of novel microbial pools within 24-48 hours of exposure (Lewis *et al*. 2017; Grond *et al*. 2019; Capunitan *et al*. 2020). Two of our findings further support rapid acclimation to local microbiota. First, we observed no significant variation in gut microbial diversity of the 12 birds we sampled early in Michigan that were not part of the individuals we sampled in The Bahamas, implying rapid turnover of microbiota sourced from the local Michigan habitat. Second, we observed significant variation in beta diversity between 2017 and 2018, as well as within each sampling period. Environmental microbes often exhibit high turnover over time (Faust *et al*. 2015). As such, our observations further support significant influence of local environment on the gut microbiome. This highlights the continued need for long term monitoring of microbiomes as community-wide differences between years are demonstrable within the same geographic regions.

Diet

Dietary shifts throughout the annual cycle correspond to changes in gut microbiota (Ren *et al*. 2017; Smits *et al*. 2017; Drovetski *et al*. 2019). Kirtland's Warblers shift from a fruit-rich diet in The Bahamas to a diet composed primarily of insects in Michigan (Deloria-Sheffield *et al*. 2001; Wunderle *et al*. 2010; Wunderle *et al*. 2014). Firmicutes and Actinobacteria, which are often

associated with frugivorous diets and known to aid in digestion through cellulose and carbohydrate degradation, were more abundant in The Bahamas where the Kirtland's diet is rich in fruit (Anand and Kandula 2012; Segawa *et al*. 2019). The abundance of Cyanobacteria throughout the sampling periods similarly shifts with decreased frugivory exhibited by the Kirtland's from The Bahamas to Michigan. Cyanobacteria may be acquired as environmental byproduct; it may also be represented by ingested chloroplasts (Brice *et al*. 2019). Though Cyanobacteria is often removed from gut microbial studies (Knight *et al*. 2018), the proportional variation between sampling periods further illustrates environmental and diet related differences throughout the annual cycle.

Proteobacteria, often abundant in insectivorous species (Edenborough *et al*. 2020) more than doubled in relative abundance from The Bahamas to the second Michigan recapture period. This may be in response to the shift in diet between locations. Additionally, specific lineages of this phylum, such as genus *Serratia*, are known to produce chitinase which facilitates the degradation of insects' exoskeletons (Rathore and Gupta 2015). We identified *Serratia* as significantly abundant in the second Michigan sampling period, corresponding to the insect-rich diet of that time.

Host

Bacterial taxa presence and abundance may fluctuate in response to host requirements. Phylum Firmicutes has been linked to weight gain, increased nutrient uptake, and metabolic efficiency in birds (Angelakis and Raoult 2010; Teyssier *et al*. 2018). The abundance of this phylum was lower in the second recapture period in Michigan than in the first recapture period

or The Bahamas. Initial capture in The Bahamas occurred within the two months prior to the start of spring migration. During this time birds accumulate fat deposits to sustain them throughout long-distance migration (Fox and Walsh 2012). At the first recapture in Michigan, individuals are actively seeking and defending breeding territories. Both activities are energetically expensive and associated with increased metabolism, potentially associated with higher abundance of Firmicutes in gut microbiota. It is also possible that the bacteria in early Michigan are residual from The Bahamas and stopover sites (Lewis et al. 2017). Further research is needed to better identify bacterial lineages associated with specific metabolic demands of birds throughout the annual cycle.

Sex specific conditions, such as hormones, behaviors, and reproductive physiology may influence or be influenced by the microbiome (Pearce *et al*. 2017; Escallón *et al*. 2019). In the breeding season, close proximity of male and female birds can lead to convergence of microbial composition resulting in reduced variation between males and females (*White* et al. 2010). We found no significant variation in overall beta diversity between sexes, although female showed slightly higher alpha diversity than males. In Rufous-collared Sparrows (*Zonotrichia capensis*), cloacal microbiome diversity increased as males transitioned from non-breeding to breeding condition (Escallón *et al*. 2019), which is the opposite of what we observed in the fecal microbiome of Kirtland's Warblers, which showed a decrease in diversity. These sparrows are non-migratory and do not experience the same extreme habitat change that the Kirtland's do, which could potentially explain the alpha diversity differences between species.

We generally found no significant compositional differences between SY and ASY age groups in the full dataset implying that adult age does not influence the microbiome of these birds.

However, we did see a difference in beta diversity between SY and ASY in the first recapture period in Michigan. Second year males often do not successfully establish and defend breeding territories against older males which in turn results in these individuals moving at larger spatial scales than territorial adults (Cooper and Marra 2020). The lack of an established breeding territory and subsequent floating behavior could result in those individuals being exposed to a different suite of environmental bacteria.

Core Microbiome

Identification of microbes that persist within the gut over time will help identify those that are inherently tied to biological processes, termed the community core microbiome. Our analyses identified a group of microbial lineages, including several that likely play a role in digestion and nutrient uptake, as the species-specific community core of Kirtland's Warblers. Eight ASVs in genus *Bacteroides* (Phyla Bacteroidetes*)* were identified as core. *Bacteroides* are common gut microbes in humans that are frequently associated with food materials breakdown and production of nutrients and energy (Wexler 2007). Though common in birds, the exact function of *Bacteroides* is unknown; however, it is thought they play a similar role in food digestion to that in humans (Bennett *et al*. 2013; Waite and Taylor 2015; Grond *et al*. 2018). Family *Ruminococcaceae* (Phyla Firmicutes*)*, contains numerous bacterial species that degrade cellulose (Duncan *et al*. 2007). Our sampling of Kirtland's Warblers identified three ASVs from this family that are common throughout the population. Similarly, the Greater Sage-Grouse (*Centrocercus urophasianus)* has a rich diversity of *Ruminococcaceae* associated with seasonal variation in foliage consumption (Drovetski *et al*. 2019). These bacteria may aid in the digestion

374 of the various fruits and berries ingested throughout the year, but which become a primary part
375 of the diet on the wintering grounds.
376 Defining the core microbiome is a critical step in understanding the consistent
377 components of often dynamic and complex microbial assemblages. These stable components
378 are commonly tied to biological processes within the host and their identification lead to an
379 increased understanding of host-microorganism interactions and dependencies (Tschöp *et al.*
380 2009). Identifying core microbes can be confounded by environmental inocula which could
381 inflate the number of ASVs identified as essential core. By resampling the same population we
382 establish a core microbiome that is persistent across multiple environments and time periods.
383 These bacterial lineages will likely play an important role in facilitating biological processes
384 within the birds.
385 Additionally, through repeated sampling of the same birds at three separate time
386 periods, we have documented the proportion of ASVs that individuals retain over time.
387 Although several previous studies have described the proportion of core ASVs to total ASVs
388 detected within their study, interpretations may vary depending on the number of birds
389 sampled and may therefore not represent the number of core ASVs in each individual (Lewis *et*
390 *al.* 2016; Grond *et al.* 2017). We show that individuals sampled in triplicate retain 18-26 ASVs
391 over time. This represents an average of 25.1% of all lineages detected per individual per
392 sampling point, and we argue it best reflects the proportion of stable, persistent bacteria within
393 an individual. Documenting the species-specific core microbiome of Kirtland's Warblers as well
394 as persistent lineages across seasons and changing environments provides model data from
395 which we can begin to understand the extent to which birds depend on their gut microbiota.

CONCLUSION

The ability to study the same individuals and populations throughout the annual cycle greatly enhances our understanding of the consequences of changing environments and seasonal physiological stressors on gut microbiota. We demonstrate that a significant compositional shift occurs in the community structure of gut bacteria as Kirtland's Warblers migrate from The Bahamas to Michigan. Additionally, we describe a species-specific core microbiome and the proportion of bacterial lineages retained across three periods of the annual cycle within individuals. Though Kirtland's Warblers were recently removed from the endangered species list, continued management and research is needed for this species to survive. Healthy gut microbiota should be included in the maintenance of threatened and endangered species (Allan *et al.* 2018; Roth *et al.* 2019; DeCandia *et al.* 2020) and this study provides model data as to how species with small population sizes and extreme habitat specialization react to changing environments.

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Figure Legends

Figure 1 Relative abundance of bacterial phyla. (A) Stacked barplots showing the relative abundance of each phylum with each column representing one individual sample, ordered by day of capture and separated by sampling period. Phyla with total abundance less than 1% and unclassified phyla are represented by gray. (B) Relative abundance boxplots of the five most common phyla per individual by sampling period representing the change in relative abundance from Cat Island, The Bahamas (CIB) to the first Michigan recapture period (MI1) and the second Michigan recapture period (MI2). Individual points represent the relative abundance of each phyla per individual per sampling period. Significance levels are pairwise comparisons between sampling periods are shown (ns: $p>0.05$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$).

Figure 2 Alpha diversity measurements of amplicon sequence variants (ASVs) including Observed ASV richness (log transformed, top row) and Shannon Diversity index (bottom row) Boxplots of alpha diversity at each sampling period (Column A). Individual points represent the alpha diversity measure of the individual at that period. Significance levels are pairwise comparisons between sampling periods are shown (ns: $p>0.05$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$). Alpha diversity change over time in the individuals sampled two (Column B) or three times (Column C). Each line connects the measurements of the same individual between the respective sampling periods. Continuous lines represent a negative change in alpha diversity and dotted lines represent a positive change.

Figure 3 Association between gut microbiome alpha diversity and length of time birds have been in Michigan following end of Spring migration. Each point shows alpha diversity of an individual bird. The blue line represents the moving average between days

Figure 4

(A) Non-metric multidimensional scaling (nMDS) ordination of Kirtland's Warbler gut microbiome community by sampling period, compared using Bray-Curtis distances. Ellipses show 95% confidence intervals around the centroid of each sampling period. Three outliers were removed from ordination plot for visualization purposes, plot including outliers is shown in Supplemental Figure 2. (B) Ordination of individual birds sampled in triplicate placed within the nMDS space of all samples, highlighting intra-individual change over time.

Figure S1 Alpha and beta diversity plots of rarefied data. All libraries were rarefied to a depth of 7,000 sequences. Rarefaction resulted in the loss of 319 ASVs (4.3% of total ASVs). Alpha and beta diversity analyses were performed. All results were qualitatively similar to non-rarefied data; no variable or category gained or lost statistical significance compared to non-rarefied data. (A) Boxplots illustrating the relative abundance of the top five most common phyla per individual by sampling period representing the change in relative abundance from Cat Island, The Bahamas (CIB) to the first Michigan recapture period (MI1) and the second Michigan recapture period (MI2). (B) Changes in alpha diversity across the sampling periods. For plots A and B significance levels are pairwise comparisons between sampling periods are shown (ns: $p>0.05$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$). (C) Non-metric multidimensional

scaling (nMDS) ordination of Kirtland's Warbler gut microbiome community by sampling period, compared using Bray-Curtis distances. Ellipses show 95% confidence intervals around each sampling period.

Figure S2 – Non-metric multidimensional scaling (nMDS) ordination of Kirtland's Warbler gut microbiome community by sampling period, compared using Bray-Curtis distances and including outliers omitted in Figure 4. Ellipses show 95% confidence intervals around the centroid of each sampling period.

Figure S3 Comparison of beta diversity measures for individuals captured at all three sampling periods. Each line represents one individual connecting the Bray-Curtis measurements between the first and second sampling period to the measurement between the second and third sampling period.

Table Legends

Table 1 Individuals sampled per time period, including age (SY = second calendar year, ASY = after second calendar year) and sex (M = male, F = female) breakdown. Numbers reflect libraries included in analyses and do not include those removed for poor sequencing or PCR yield.

Sampling Period	Date	Samples Collected	Age			Sex		
			SY	ASY	Unk.	M	F	Unk.
Initial Capture (CIB)	March 29 - April 16, 2017	41	18	22	1	38	3	0
	March 23 - April 24, 2018	51	37	14	0	36	15	0
First Recapture (MI1)	May 20 - June 6, 2017	19	10	9	0	19	0	0
	May 13 - June 26, 2018	24	18	6	0	23	1	0
Non-tagged Birds (MI1)	May 13 - 20, 2018	13	6	6	1	12	0	1
Second Recapture (MI2)	July 2 - 10, 2017	8	4	4	0	8	0	0
	July 1 - 11, 2018	10	7	3	0	8	2	0
	Total	**166**	**100**	**64**	**2**	**144**	**21**	**1**

Table 2 Results of permutational multivariate analysis of variance (PERMANOVA) tests indicating if ASV beta diversity measures are significantly different for the tested variable based on Bray-Curtis and unweighted UniFrac distance metrics. Results reported for full dataset and within sampling periods for variables year, sex, and age. Asterisks denote statistically significant results of PERMANOVA with Bonferroni correction, $p < 0.05$. PERMDISP analysis results reported when PERMANOVA results significant. All tests conducted with 999 permutations.

	Bray-Curtis				
	PERMANOVA			PERMDISP	
Variable	*Pseudo-F*	R^2	*Pr(>F)*	*f-value*	*P-value*
Sampling Period	2.058	0.025	<0.001*	0.343	0.710
Year (full dataset)	1.900	0.011	0.002*	1.659	0.200
Year (CIB Only)	1.485	0.016	0.019*	0.936	0.336
Year (MI1 Only)	2.474	0.044	<0.001*	0.304	0.583
Year (MI2 Only)	2.223	0.172	0.003*	0.000	0.984
Sex (full dataset)	1.203	0.007	0.137		
Sex (CIB Only)	1.035	0.011	0.345		
Sex (MI1 Only)	1.169	0.216	0.482		
Sex (MI2 Only)	1.425	0.082	0.082		
Age (full dataset)	0.929	0.006	0.595		
Age (CIB Only)	0.927	0.010	0.681		
Age (MI1 Only)	0.926	0.017	0.586		
Age (MI2 Only)	1.020	0.060	0.343		

	Unweighted UniFrac				
	PERMANOVA			PERMDISP	
Variable	*Pseudo-F*	R^2	*Pr(>F)*	*F*	*P-value*
Sampling Period	2.001	0.024	<0.001*	13.514	<0.001
Year (full dataset)	1.314	0.008	0.121		
Year (CIB Only)	2.027	0.022	0.003*	0.180	0.673
Year (MI1 Only)	1.295	0.025	0.003*	0.7258	0.398
Year (MI2 Only)	1.541	0.878	0.007*	0.001	0.974
Sex (full dataset)	1.159	0.007	0.074		
Sex (CIB Only)	0.939	0.010	0.737		
Sex (MI1 Only)	0.862	0.016	0.695		
Sex (MI2 Only)	0.862	0.016	0.695		
Age (full dataset)	1.131	0.007	0.099		
Age (CIB Only)	0.887	0.010	0.925		
Age (MI1 Only)	1.279	0.023	0.013*	1.532	0.221
Age (MI2 Only)	0.942	0.056	0.615		

Table S1 Host associated metadata including sampling date and location for each individual per sampling period, including age (SY = second year, ASY = after second year) and sex (M = male, F = female).

*Denotes non-tagged birds from the 2018 first recapture period.

**For samples included in the comparison of alpha diversity over time in the first recapture period in Michigan individual's date of arrival is included.

Table S2 Relative abundance of each bacterial phyla (highlighted in gray) and classes with the standard deviation listed in parentheses. Phyla and classes are listed in order of most abundant in the full dataset. Relative abundances were calculated for the full dataset and within individual sampling periods.

Table S3 Results of the linear mixed model analyses of alpha diversity values for Observed ASV richness and Shannon Diversity Index. Model factors include sex (male or female), age (second year or after second year), year (2017 or 2018), and sampling period (initial capture in The Bahamas, first recapture in Michigan, or second recapture in Michigan). Asterisks denote statistically significant results of model, $p < 0.05$.

Table S4 (A) Taxonomic classifications of ASVs identified as core throughout all sampling periods. (B) Core taxonomic groups as identified by the *Phylocore* program. Terminal taxonomic groups bolded.