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Research article

Disentangling relationships between physiology, morphology, diet, and gut microbial diversity in American kestrel nestlings

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Gut microbiota are increasingly recognized as important drivers of host health and fitness across vertebrate taxa. Given that gut microbial composition is directly influenced by the environment, gut microbiota may also serve as an eco-physiological mechanism connecting host ecology, such as diet, and physiology. Although gut microbiota have been well-studied in mammalian systems, little is known about how gut microbial diversity and composition impact morphological and physiological development in wild birds. Here, we characterized both diet and gut microbial diversity of free-living American kestrel *Falco sparverius* nestlings throughout development to test whether gut microbial diversity predicts host morphological and physiological traits in either contemporary or time-lagged manners. Gut microbial alpha diversity on day 21 of nestling development was positively correlated with diet alpha diversity representative of the majority of nestling development (days 5–20). Gut microbial alpha diversity early in development was negatively correlated with body mass in both contemporary and time-lagged manners. Gut microbial alpha diversity early in development was positively correlated with blood glucose later in development. As nestlings experience rapid growth demands in preparation to fledge, these time-lagged associations may indicate that gut microbial diversity at early critical developmental windows may determine the future trajectory of morphological and physiological traits underlying metabolism that ultimately impact fitness.

Keywords: development, diet diversity, *Falco sparverius*, gut microbiota, raptor

Introduction

Gut microbiota are the collection of bacteria, archaea, viruses, eukaryotic organisms (e.g. fungi), and other microorganisms residing in the host gastrointestinal tract (reviewed by Berg et al. 2020). These microorganisms can benefit the host through increased nutrient uptake and metabolism, development of intestinal morphology,

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detoxification, and immune system training (Kohl et al. 2012, Ballou et al. 2016, Grond et al. 2018, Videvall et al. 2019). Given the numerous microbiota-mediated functions within vertebrates, the diversity and composition of the gut microbial community and its functional capacity can reflect the health status of its animal host (Park 2018).

High gut microbial alpha diversity is typically indicative of greater vertebrate host health, with low alpha diversity common in multiple disease states (reviewed by Heiman and Greenway 2016). Mice experimentally fed antibiotics to reduce microbial alpha diversity display altered metabolism and body condition compared to controls (Cox et al. 2014). Other studies, however, suggest that gut microbial composition, rather than overall alpha diversity, has a greater impact on host health. For example, Worsley et al. (2021) found that gut microbial composition but not alpha diversity significantly differs between adults that survived to the next breeding season and those that did not. Investigations of microbiota-mediated effects on host health have historically been focused on mammalian hosts but, given their potentially significant link with fitness, they have recently gained traction in other vertebrate hosts, such as birds (reviewed by Grond et al. 2018 and in Bodawatta et al. 2021b). Microbiota-mediated impacts on host health may be further exaggerated in avian hosts, as free-living birds have diverse and temporally dependent diets that may impact gut microbial diversity and composition (Davidson et al. 2020).

The first step to understanding the importance of gut microbiota to avian health is to investigate the factors that drive its colonization from hatching and through development into adulthood. Mammalian microbiota colonization occurs during live birth, through the offspring's contact with microbiota in the birth canal, which is a unique colonization strategy that contrasts with many other taxa (Koenig et al. 2011, Perez-Muñoz et al. 2017). Oviparous vertebrates such as birds were presumed to hatch from internally sterile eggs (Grond et al. 2017); however, recent evidence discovered distinct in ovo bacterial communities in wild bird eggs, suggesting that oviparous vertebrates may acquire maternal microbiota through the inoculation of egg yolk prior to shelling (Trevelline et al. 2018). However, these in ovo microbial communities occur in low abundance (Lauder et al. 2016), and birds exhibit weak to no specificity between gut microbial community similarity and host phylogeny (Song et al. 2020). Thus, the majority of gut microbiota within developing birds must be obtained horizontally from the nest environment (van Veelen et al. 2017, Campos-Cerda and Bohannan 2020, van Veelen et al. 2020) or diet items either individually foraged or delivered via parents to the nest (Hird et al. 2015, Ballou et al. 2016, Youngblut et al. 2019, Dion-Phénix et al. 2021).

Diet plays a predominant role in shaping gut microbial communities even at an individual scale (Bolnick et al. 2014). Diet determines a high proportion of the variation in microbial community composition in avian systems with natural (Hird et al. 2015, Youngblut et al. 2019, Dion-Phénix et al. 2021, Bodawatta et al. 2022, Schmiedová et al. 2022) and experimentally manipulated diets (Davidson et al. 2020, Teyssier et al. 2020, Bodawatta et al. 2021a). For example, rural house sparrows *Passer domesticus* naturally host more diverse gut communities than urban conspecifics, and birds experimentally given a rural-like diet exhibit increased gut microbiota diversity compared to controls, likely driven by protein, fat, and fiber level differences between rural versus urban diets (Teyssier et al. 2020).

Because of their implications for host health and development, gut microbial composition and diversity may have profound impacts on the developmental trajectory of phenotypic traits related to individual fitness (Mitchell et al. 2013, Burton and Metcalfe 2014, Öst et al. 2020). Within birds, individual variation in nestling development has been well documented, and in many cases linked to variation in ecological conditions such as lay date (McKinnon et al. 2012, Samplonius et al. 2016, Cornell and Williams 2017), interannual variation (Kaliński et al. 2015, Markowski et al. 2015), weather and temperature patterns (Ardia 2013, Rodríguez and Barba 2016, de Zwaan et al. 2020), food availability, or parental provisioning rate (Merino and Potti 1998, Forero et al. 2002, Scheuerlein and Gwinner 2006). However, few studies have explored how natural gut microbial composition may be related to these ecological conditions or individual variation in development of key morphological and physiological traits related to nestling fitness.

Physiology underlying aerobic capacity, such as hematocrit (i.e. volume percentage of red blood cells in blood), may be a particularly important aspect of development at the critical life history transition of fledging, as birds go from a sedentary to a highly active, aerobic lifestyle necessary to sustain flight, and experience high post-fledging mortality rates (reviewed by Naef-Daenzer and Grüebler 2016, Newton et al. 2016). Hematocrit has been linked to fledgling flight ability (Cornell et al. 2017), body condition (Lill et al. 2013), and recruitment (Bowers et al. 2014). Glucose concentration, a physiological metric related to metabolism, is also vital to host health, as sustaining energetically expensive flight requires fast metabolism and efficient absorption of glucose (Hazelwood 2000). The mechanistic link connecting individual variation in nestling development with ecological variation could be the gut microbiota of the host, given that its composition may be directly determined by the environment, and its substantial role in host health (Vispo and Karasov 1997, Rowland et al. 2018).

Although studies focused on host health implications of avian gut microbiota are gaining popularity, much of the present literature focuses on poultry and the application of probiotics to enhance chicken growth (reviewed by Patterson and Burkholder 2003), leaving our understanding of the role of gut microbiota to the health and condition of wild hosts limited. Of the studies conducted on wild birds, many have investigated the role of gut microbiota in host weight gain (Potti et al. 2002, Kohl et al. 2018, Teyssier et al. 2018, Videvall et al. 2019). In wild great tits *Parus major*, gut microbial composition early in nestling development predicts body mass later in the nestling period (Davidson et al. 2021), and changes in gut microbial composition throughout the nestling period are

associated with mass gain (Teyssier et al. 2018). A negative relationship between gut microbial alpha diversity and future weight gain was found in juvenile ostriches (*Struthio camelus*; Videvall et al. 2019) and nestling great tits (Davidson et al. 2021), but a positive relationship was reported in a different population of great tit nestlings (Teyssier et al. 2018). Due to the intraspecific and interspecific variation in the timing and direction of the relationship between gut microbial diversity and host weight, we hypothesized that gut microbial diversity would be related to host body mass in either a contemporary or time-lagged manner, but with no a priori predictions about directionality of the relationship. No studies to our knowledge have examined how individual variation in development of both nestling morphology (e.g. body mass) and physiology underlying aerobic capacity (e.g. hematocrit) and metabolism (e.g. blood glucose concentration) change throughout the nestling period in relation to gut microbial diversity in wild birds. Owing to the lack of investigations between gut microbial diversity and host physiology such as hematocrit and glucose in wild birds, we explore both contemporary and time-lagged associations.

Here, we investigated how natural variation in diet (i.e. percentage of prey types, diet diversity) influences gut microbial diversity, and how gut microbial diversity predicts morphological (i.e. body mass) and physiological development (i.e. hematocrit and glucose) in American kestrel *Falco sparverius* nestlings throughout the nestling period over two years. The American kestrel is a common falcon species that has a diverse diet consisting of small mammals, songbirds, and invertebrates, potentially allowing for gut microbiota to be obtained from a variety of prey sources (Boal et al. 2021). In addition, some kestrel breeding pairs exhibit bias towards a single prey type (Melo, M., unpubl.), making them an ideal model species for correlating diet with gut microbial community diversity and composition. Correlations between ecological conditions and nestling development have been previously identified in this system (Cornell et al. 2021). In this paper, we test whether gut microbiota may link ecological conditions, via diet, to individual variation in nestling development. We predicted that 1) host intrinsic factors such as age and sex would be significantly related to gut microbiota across both sampling years of this study; 2) nestling diet diversity would positively predict gut microbial diversity; 3) gut microbial diversity would be related to host body mass either in a contemporary or time-lagged manner; and 4) gut microbial diversity would be related to nestling physiology either in a contemporary or time-lagged manner.

Material and methods

Study species

The American kestrel is a cavity-nesting rural falcon species that readily occupies artificial nest-boxes, allowing for ease of repeated measures in free-living individuals. American kestrels typically lay a clutch size of 4–5 eggs with an average incubation time of 26–32 days and have a fledge age of 28–31 days (Smallwood and Bird 2020). They hatch asynchronously within 1–2 days with incubation usually starting with the penultimate egg laid (Love et al. 2003). Kestrel nestlings follow mass overshoot recession growth profiles whereby nestling mass exceeds adult mass values prior to fledging and subsequently recedes post-fledging (Cornell et al. 2021). Kestrels also display reverse sexual dimorphism (i.e. female kestrels typically weigh about 10% more than male kestrels), with sex-related differences in mass being detected as early as 14 days post-hatch (Cornell et al. 2021). Development of tarsus length and wing length is unrelated to sex; however, female nestlings exhibit longer tarsi by 21 days post-hatch (Cornell et al. 2021). Individual variation is prevalent in both somatic and physiological trait development but correlations between the two on the individual level appear absent (Cornell et al. 2021), and there are presently no investigations relating development to post-fledge survival. Somatic traits and offspring survival have, however, been correlated with environmental conditions such as weather and prey abundance (Dawson and Bortolotti 2000). In our study, survival prior to fledging was 95.45%, with only two nestlings dying before day 21 (i.e. nestlings 633-1 and 630-1).

Sample collection

We monitored American kestrel nest-boxes in Berks, Lehigh, and Schuylkill Counties, Pennsylvania, USA, beginning in early May 2018 and 2019 to document occupancy, egg-laying date, and hatching as described in Cornell et al. (2021). In 2018, we sampled 12 nestlings from 4 nests, and in 2019 we sampled 32 nestlings from 10 nests (n = 44 nestlings total). On days 7, 14, and 21 after hatching (hatch day = day 0), we removed nestlings from the nest and collected morphometrics, blood samples, and fecal samples between 08:00 and 12:00 (following university IACUC and US Fish and Wildlife Service banding permits). Birds were sexed on day 21 from coloration of primary feathers: blue for males and brown for females (Smallwood and Bird 2020). We measured mass to the nearest 0.01 g using a digital scale. We banded nestlings with uniquely colored temporary leg bands on day 7 for identification within nests and banded with USGS aluminum bands on day 21. We obtained blood samples by piercing the brachial vein with a 26.5 gauge needle and collecting whole blood into heparinized tubes. We switched the sampled wing (left or right) between repeated sampling events to reduce soreness. We measured glucose in mg/dL with the first drop of blood to appear using a blood glucose meter. Hematocrit was measured as described in Cornell et al. (2021). To characterize gut microbial diversity, we collected fecal samples (n = 131 total) from nestlings by abdominal palpation in sterile collection containers. We swabbed fecal matter with sterile flocked swabs placed in autoclaved microcentrifuge tubes. We stored fecal samples on ice in the field and then at −80°C until DNA extractions. See Supporting information for all metadata associated with each sample.

In the second sampling year only (2019), we assessed nest level diet by monitoring the nests with cameras mounted to the outside of the nest-box for an average of 122 min (range 60–253 min) on days 5, 6, 12, 13, 19, and 20 of nestling development. Two nests had only five days of recording due to equipment malfunction; however, longer recordings on other days mitigated this difference such that there was no effect on overall time recorded (t-test, $t = -0.14$, $p = 0.91$). To avoid influencing natural behavior, we mounted a dummy camera to the box when not filming. We filmed nests between 07:00 and 14:00. We recorded weather during filming categorically (sunny, cloudy, light rain, steady rain), with over 90% of filming completed during no precipitation. There was no effect of weather (one-way ANOVA, $F_{3,421} = 1.5$, $p = 0.20$) or time of day (linear mixed effects model, nest as a random effect, $F_{1,47} < 0.1$, $p = 0.88$) on provisioning rate. We categorized prey items recorded on video into groups: mammal, passerine, arthropod, annelid, and other. The 'other' category included two novel prey items observed only once each: a green frog *Rana clamitans* and a northern fence lizard *Sceloporus undulatus*. We used mass values of each prey type from the literature (Supporting information) to determine the percentage of each prey type in the diet representative of the majority of the nestling period (days 5–20 of nestling development), by summing the total mass of prey delivered to the nest and dividing by the total mass of all prey and multiplying by 100. We calculated diet diversity (Shannon index) representative of the majority of the nestling period (days 5–20 of nestling development) using the *diversity* function in the 'vegan' package ver. 2.6-2 in R (www.r-project.org, Oksanen et al. 2019) using the percentage of prey types corrected for mass of the prey as described above.

DNA extractions, PCR, and sequencing

We extracted DNA from whole swabs using DNeasy PowerSoil Pro DNA Isolation Kits (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocol. We amplified the V4 region of the 16S rRNA gene (i.e. a universal marker gene for bacteria and archaea) using the primers 515F and 806R with Illumina adapters added. We followed the Earth Microbiome Project 16S Illumina Amplicon protocol (Caporaso et al. 2011, Caporaso et al. 2012) except for using 10 µl total reaction volumes instead of 25 µl. Each PCR reaction was run in triplicate and included 5 µl of 2× Platinum Hot Start Master Mix (Invitrogen, Waltham, MA, USA), 0.5 µl of 10 µM primers, 3 µl of nuclease free water, and 1 µl of template DNA. Cycling conditions were 3 min at 94°C followed by 35 cycles of 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s before a final extension at 72°C for 10 min.

We pooled the three replicate reactions for each sample and ran a 1% agarose gel to confirm that amplification was successful for the V4 region of the 16S rRNA gene (~ 350 bp). Each PCR run included negative controls (nuclease-free water in place of template DNA but were not sequenced). We also extracted, amplified, and sequenced 4 negative kit reagent controls. We submitted our final pooled PCR products to the Cornell Biotechnology Resource Center for quantification, normalization, library preparation, and sequencing in one Illumina MiSeq paired-end 2 × 250 bp run (n = 131 fecal samples, n = 4 negative controls).

Gut microbiota bioinformatics

We demultiplexed forward and reverse sequences and quality filtered using default settings in QIIME2 (ver. 2021.11) (Bolyen et al. 2019). After visually inspecting quality scores, we used the DADA2 plugin to remove primers, truncate reads at 180 bp, and generate amplicon sequence variants (ASVs). We assigned taxonomy to the ASVs by fitting a naive-Bayes classifier trained on the Silva 132 database using the sk-learn classifier (Quast et al. 2012, Yilmaz et al. 2014). The phylogeny plugin was applied to construct a rooted phylogenetic tree by employing FastTree and MAFFT. We removed singletons, archaea, eukaryotes, mitochondria, chloroplasts, and ASVs unassigned to a bacterial phylum in QIIME2 with the filter-table plugin before we combined the ASV table, sample metadata, taxonomy table, and phylogenetic tree using the 'phyloseq' package ver. 1.40.0 in R (www.r-project.org, McMurdie and Holmes 2013). We used the 'decontam' package ver. 1.16.0 in R (www.r-project.org) to identify and remove likely contaminants based on associations with sample biomass and comparisons with negative kit controls using the default settings (Davis et al. 2018). We identified 25 out of 7173 ASVs as contaminants and removed them from the ASV table.

After potential contaminants were removed, we imported all data objects back into QIIME2 to calculate alpha and beta diversity metrics and removed the negative control samples for all subsequent analyses. To limit bias due to differing read library sizes, we rarefied samples to 1640 reads to retain the majority of samples (see Supporting information for rarefaction curve), eliminating 13 samples, leaving us with a final sample size of 122 samples (n = 118 fecal samples, n = 4 negative controls). We calculated alpha diversity metrics including Shannon index (Shannon and Weaver 1949), Chao1 index (Chao 1984), and Faith's phylogenetic diversity (PD, Faith 1992) with the diversity plugin in QIIME2. The Shannon index incorporates both richness (the number of observed ASVs) and evenness, whereas the Chao1 index estimates the number of rare taxa missed from under-sampling. Faith's PD takes phylogenetic relatedness of microbial taxa into account by summing the branch lengths of a phylogenetic tree (Faith 1992). We calculated comparisons of beta diversity by computing Bray–Curtis dissimilarity distances (Sorenson 1948) which account for richness and abundance of the ASVs in the communities, and Jaccard similarity distances (Jaccard 1908), which only measure community richness, also using the diversity plugin in QIIME2. We exported raw alpha and beta diversity values from the QIIME2 artifacts with the export plugin for statistical analyses in R (www.r-project.org).

Statistical analyses

Alpha diversity: age, sex, and year

All statistical analyses were conducted in R ver. 4.2.1 (www.r-project.org). We checked for suitability of models by examining plots of residuals versus fits and qq normality to identify

outliers. We approximated normality of alpha diversity metrics with Shapiro Wilk tests ($W > 0.95$). We log transformed the Chao1 index and Faith's PD; the Shannon index was normal without transformation. We used linear mixed effects models (LMMs) run in the 'nlme' package ver. 3.1-157 (Pinheiro et al. 2020). We used separate models for each gut microbial alpha diversity metric: Shannon index, log Chao1 index, and log Faith's PD in relation to each fixed effect as described below. The purpose of the following analyses was exploratory in order to generate rather than confirm hypotheses (Bender and Lange 2001, Ranstam 2019); therefore, we did not correct p values for multiple comparisons as discussed in Davidson et al. (2021).

To test the relationship between host intrinsic factors including age and sex on gut microbial alpha diversity across sampling years, we ran separate LMMs with each gut microbial alpha diversity metric as a response variable and an age*sex interaction and year as fixed effects with nested random effects of nestling ID within nest (fixed = microbial alpha diversity ~ age*sex + year + random = ~ 1|nest/nestling ID). The age*sex interaction was not significant, so we removed the interaction term and ran age, sex, and sampling year as separate fixed effects in each model (fixed = microbial alpha diversity ~ age + sex + year + random = ~ 1|nest ID/nestling ID). Sampling year was significant for log Chao1 index so sampling year was controlled for in all appropriate Chao1 models. Sample sizes for each host intrinsic factor were as follows: age (day 7: n = 39, day 14: n = 41, day 21: n = 38), sex (female: n = 58, male: n = 60), and sampling year (2018: n = 34, 2019: n = 84).

Alpha diversity: diet diversity and prey percentage

We assessed nest level diet at the scale of the majority of the nestling period (average of 122 min total across days 5, 6, 12, 13, 19, and 20 of nestling development); therefore, we only compared diet diversity metrics with gut microbial diversity on nestling day 21 since it was the terminal sample at the end of the nestling period and the most likely timepoint to capture the scale of diet diversity measured. To test for relationships between diet alpha diversity and gut microbial alpha diversity, we ran separate LMMs with each gut microbial alpha diversity metric from day 21 samples only as a response variable and diet alpha diversity (Shannon index) as a fixed effect with nest as a random effect (fixed = microbial alpha diversity ~ diet alpha diversity + random = ~ 1|nest). Only nest was included as a random effect instead of nestling ID nested within nest because only one timepoint was used from each nestling (i.e. day 21). We also ran separate LMMs with each gut microbial alpha diversity metric from day 21 samples only as a response variable and percentage of each prey type as separate fixed effects with nest as a random effect (fixed = microbial alpha diversity ~ mammals + passerines + arthropods + annelids + random = ~ 1|nest). Sampling year was not included as a fixed effect for log Chao1 models because diet data were only collected in the second sampling year (2019). Sample size for all diet analyses includes day 21 samples in 2019 only (n = 27).

Alpha diversity: morphology and physiology

To test for relationships between gut microbial alpha diversity and nestling morphological and physiological traits, we examined whether gut microbial alpha diversity at the time of sampling was associated with host morphological or physiological trait values at the same developmental stage (i.e. contemporary relationship), or whether gut microbial alpha diversity at an early developmental stage predicted future host trait values (i.e. time-lagged relationship), while controlling for the starting trait value. Raw trait values were used as the response variable, instead of change in trait values, as this allowed us to control for the starting trait value as a covariate in time-lagged models. Sample sizes for all contemporary analyses were: day 7 (n = 39), day 14 (n = 41), and day 21 (n = 38). For time-lagged relationships, we only compared consecutive time points using day 7 microbial diversity with day 14 trait values (n = 39), and day 14 microbial diversity with day 21 trait values (n = 41). Sampling year was controlled for as a fixed effect in all models with log Chao1 microbial alpha diversity.

To test for an effect of gut microbial alpha diversity on host body mass in a contemporary manner, we ran separate LMMs for each age (days 7, 14, and 21) with host body mass as a response variable and microbial alpha diversity (Shannon index, log Chao1 index, or log Faith's PD) as a fixed effect with nested random effects of nestling ID within nest (fixed = day 7 mass ~ day 7 microbial alpha diversity + random = ~ 1|nest/nestling ID). We controlled for sex in mass models at days 14 and 21 when kestrels are sexually dimorphic for mass (fixed = day 14 mass ~ day 14 microbial alpha diversity + sex + random = ~ 1|nest/nestling ID).

To test for an effect of gut microbial alpha diversity on host body mass in a time-lagged manner, we ran separate LMMs for each age time-lagged comparison (day 7 microbial diversity and day 14 trait values; day 14 microbial diversity and day 21 trait values) with host body mass at the latter timepoint as the response variable and microbial alpha diversity at the previous timepoint (Shannon index, log Chao1 index, or log Faith's PD) and starting body mass and sex as fixed effects with nested random effects of nestling ID within nest (fixed = day 14 mass ~ day 7 microbial alpha diversity + day 7 mass + sex + random = ~ 1|nest/nestling ID).

To test for an effect of gut microbial alpha diversity on host physiological traits (i.e. glucose and hematocrit) in a contemporary manner, we ran separate LMMs for each age (days 7, 14, and 21) with either glucose or hematocrit as the response variable and microbial alpha diversity (Shannon index, log Chao1 index, or log Faith's PD) as a fixed effect with nested random effects of nestling ID within nest (fixed = day 7 glucose ~ day 7 microbial alpha diversity + random = ~ 1| nest/nestling ID).

To test for an effect of gut microbial alpha diversity on host physiological traits in a time-lagged manner, we ran separate LMMs for each age time-lagged comparison (day 7 microbial diversity and day 14 trait values; day 14 microbial diversity and day 21 trait values) with either glucose or hematocrit at

the latter timepoint as the response variable and microbial alpha diversity at the previous timepoint (Shannon index, log Chao1 index, or log Faith's PD) and starting trait value as fixed effects with nested random effects of nestling ID within nest (fixed = day 14 hematocrit ~ day 7 microbial alpha diversity + day 7 hematocrit + random = ~ 1| nest/nestling ID).

Beta diversity: age, sex, and year

Distance matrices for both Bray–Curtis and Jaccard dissimilarity were exported from QIIME2 with the export plugin for statistical analyses in R (www.r-project.org). To test for changes in microbial beta diversity, we conducted a separate permutational multivariate analysis of variation (PERMANOVA) with each distance matrix (Jaccard or Bray–Curtis) as the response variable to statistically partition the sources of variation in microbial community structure with the *adonis2* function in the 'vegan' package ver. 2.6-2, with the 'by' parameter set for 'margin' to account for marginal effects of the tested variables. Permutations (1000) were restricted within nested blocking factors of nestling ID within nest with the *how* function in the 'permute' package ver. 0.9-7 (Simpson et al. 2016) unless otherwise noted. An assumption of the adonis test is that groups have homogeneity of variance. The dispersion of the groups outlined in each section below was checked for homogeneity of variance using the *Betadisper* and *permutest* functions from the 'vegan' package. All model formulas and sample sizes for each beta diversity analysis were the same as their corresponding alpha diversity analysis (above).

To test the relationship between host intrinsic factors including age and sex on gut microbial beta diversity across sampling years, we ran separate PERMANOVAs with each microbial beta diversity distance matrix (Bray–Curtis or Jaccard) as the response variable and an age*sex interaction and sampling year as separate fixed effects with the nested random effects structure described above. The age*sex interaction was nonsignificant for both beta diversity metrics, so we removed the interaction term and ran age, sex, and sampling year as separate fixed effects in each model. Age was the only significant host intrinsic factor, so we subsequently conducted pairwise PERMANOVAs between consecutive time points (day 7 versus day 14 and day 14 versus day 21) with age as the only fixed effect. We conducted a similarity percentage (SIMPER) analysis to identify the average contribution of each bacterial genus to the Bray–Curtis dissimilarity of each significant pairwise age group comparison in PAST (ver. 4.11), with a 70% cutoff for low contributions to dissimilarity. Beta diversity ordinations were visualized with principal component analyses (PCoA) calculated with the 'phyloseq' package.

Beta diversity: diet diversity and prey percentage

We ran Mantel tests (1000 permutations) to test for relationships between gut microbial beta diversity (Jaccard and Bray–Curtis) and nest level diet beta diversity (Jaccard) in the 'vegan' package. To test for relationships between prey type and gut microbial beta diversity, we ran separate PERMANOVAs with each microbial beta diversity distance matrix (Bray–Curtis or Jaccard) as the response variable and percentage of each prey type (mammal mass, passerine mass, arthropod mass, and annelid mass) as separate fixed effects with nest as a random effect. To investigate whether specific diet prey types impact bacterial genera differently, we examined the correlations (Kendall's rank correlations) between relative abundances of the top 11 bacterial genera and the percentage of diet prey type (mammal, passerine, arthropod, and annelid) in individual diets using the *cor* and *cor.test* functions in base R (www.r-project.org).

Microbial composition: morphology and physiology

We conducted separate LMMs with body mass, glucose, or hematocrit as a response variable with either phylum-level relative abundances of Firmicutes or Proteobacteria as the main fixed effect (see 'Alpha diversity: morphology and physiology'). Firmicutes and Proteobacteria were selected for these analyses because they were the top two phyla, whereas Bacteroidota was only present in 7 samples. All visualizations were made with the 'ggplot2' package ver. 3.3.6 and edited in Biorender.

Results

Sequencing results

Before filtering, there were 4 461 203 total reads and 7198 unique ASVs. Mean reads per sample were 33 046 with samples ranging from 0 to 74 625 reads. After filtering, we retained a total of 122 samples (n = 118 fecal samples, n = 4 negative controls) resulting in 4 089 054 reads and 6447 unique ASVs that were used to calculate gut microbial alpha and beta diversity metrics. Mean reads per sample were 31 946 with samples ranging from 36 to 73 720 reads.

Nestling age, sex, and gut microbial diversity across sampling years

Across sampling years, gut microbial alpha diversity differed for log Chao1 ($F_{1,12} = 7.71$, $p < 0.02$) but not Shannon ($F_{1,12} = 0.44$, $p = 0.52$) or log Faith's PD ($F_{1,12} = 1.22$, $p = 0.29$) with higher log Chao1 diversity in 2019 than 2018 (Fig. 1a, Table 1). Gut microbial composition did not differ by sampling year for either metric (Bray–Curtis: pseudo-$F_{1,83} = 4.11$, $p = 0.47$; Jaccard: pseudo-$F_{1,83} = 5.01$, $p = 0.55$) (Fig. 1b, Supporting information).

No alpha diversity metric (Shannon index, log Chao1 index, or log Faith's PD) showed any significant relationship to age (Fig. 1c, Table 1), but microbial composition did differ by age (Supporting information). When comparing consecutive timepoints, nestling day 7 and day 14 differed in microbial composition for both beta diversity metrics (Bray–Curtis: pseudo-$F_{1,71} = 1.47$, $p = 0.05$; Jaccard: pseudo-$F_{1,71} = 1.47$, $p = 0.04$). Nestling day 14 also differed from day 21 for both beta diversity metrics (Bray–Curtis: pseudo-$F_{1,71} = 1.52$,

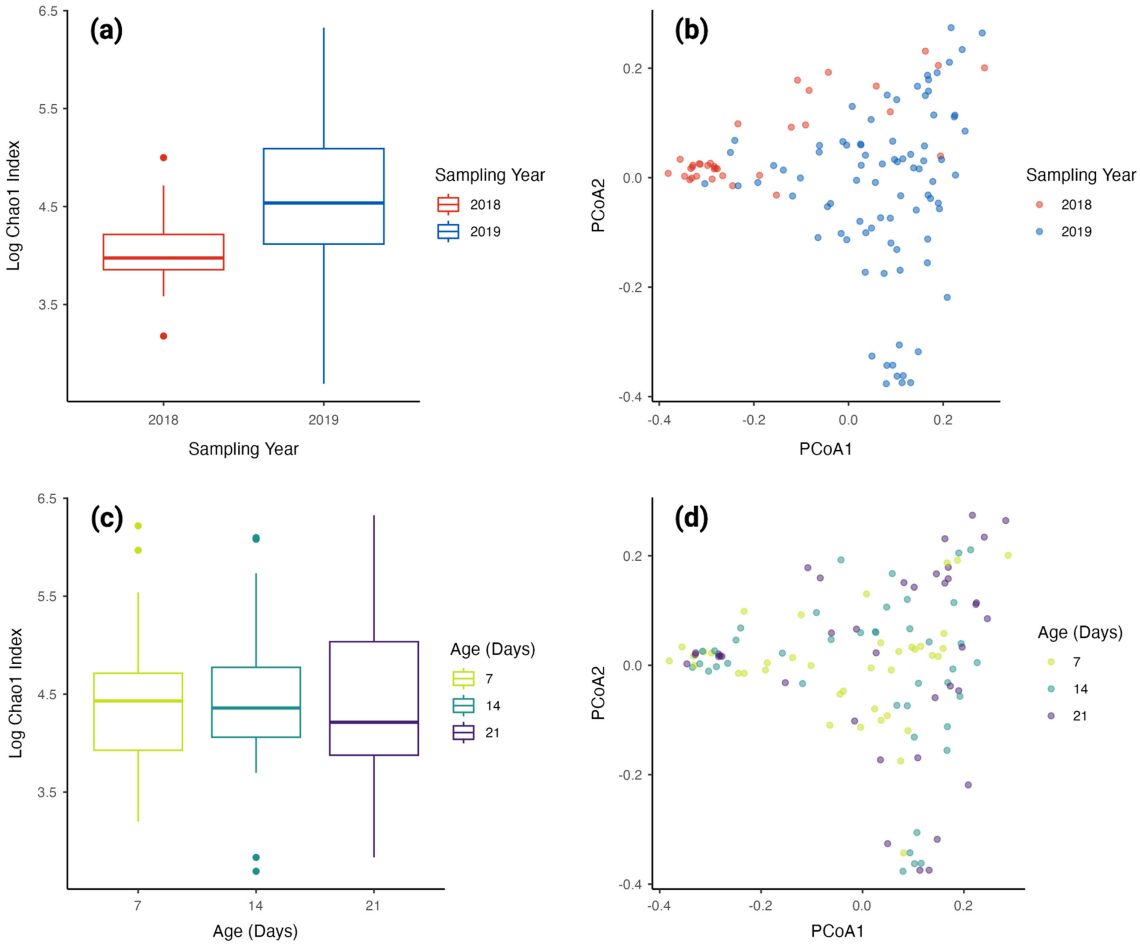

Figure 1. Relationships between nestling age, sampling year, and gut microbial alpha diversity of American kestrel *Falco sparverius* nestlings. (a) Gut microbial alpha diversity (log Chao1 index) differed between sampling years (2018: n = 34, 2019: n = 84). (b) Principal coordinates analysis (PCoA) ordination plot of beta diversity (Jaccard) differences between the two sampling years. (c) Gut microbial alpha diversity (log Chao1 index) did not change between nestling age (day 7: n = 39, day 14: n = 41, day 21 = 38). (d) PCoA ordination plot of beta diversity (Jaccard) differences between nestling age (days).

Table 1. Results of linear mixed effects models testing the effect of host intrinsic traits including age and sex across sampling years on gut microbial alpha diversity of American kestrel *Falco sparverius* nestlings (n = 44). Each model included (a) Shannon index, (b) log Chao1 index, or (c) log Faith's phylogenetic diversity (PD) as the response variable; and age, sex, and year as fixed effects with nested random effects of nestling ID within nest. An asterisk and bold denote a significant p value (α = 0.05).

Response variable	Fixed effects	Degrees of freedom	Estimate	F value	p value
(a) Shannon index	(Intercept)	1, 72	4.06	414.65	**< 0.0001***
	Age	2, 72	0.18	0.64	0.5295
	Sex	1, 29	0.22	0.83	0.3704
	Year	1, 12	−0.30	0.44	0.5205
(b) Chao1 index (log)	(Intercept)	1, 72	3.96	2200.57	**< 0.0001***
	Age	2, 72	0.07	0.08	0.9217
	Sex	1, 29	0.02	0.00	0.9511
	Year	1, 12	0.58	7.71	**0.0168***
(c) Faith's PD (log)	(Intercept)	1, 72	1.81	848.48	**< 0.0001***
	Age	2, 72	0.00	0.01	0.9898
	Sex	1, 29	0.03	0.05	0.8293
	Year	1, 12	0.16	1.22	0.291

p < 0.01; Jaccard: pseudo-$F_{1,71}$ = 1.52, p = 0.01, Fig. 1d, Supporting information).

At the phylum level, Firmicutes dominated across all ages (day 7 = 94.69%, day 14 = 94.03%, day 21 = 91.70%) followed by lower relative abundances of Proteobacteria (day 7 = 5.24%, day 14 = 5.97%, day 21 = 8.24%) and less than 1% relative abundances of Bacteroidota (Bacteria) (Supporting information). At the genus level, the top 10 genera, including an unassigned genus from class Bacilli, dominated across all ages (day 7 = 45.49%, day 14 = 48.13%, day 21 = 45.03%) followed by lower relative abundances of *Enterococcus* (day 7 = 21.13%, day 14 = 20.87%, day 21 = 16.86%), an unassigned genus from order Lactobacillales (day 7 = 13.18%, day 14 = 10.89%, day 21 = 12.83%), *Bacillus* (day 7 = 6.74%, day 14 = 7.56%, day 21 = 8.30%), *Carnobacterium* (day 7 = 4.41%, day 14 = 1.08%, day 21 = 4.28%), an unassigned genus in order Bacillales (day 7 = 1.64%, day 14 = 3.10%, day 21 = 2.00%), *Vibrio* (day 7 = 0.53%, day 14 = 1.92%, day 21 = 4.02%), an unassigned genus in class Gammaproteobacteria (day 7 = 1.40%, day 14 = 1.27%, day 21 = 1.74%), *Exiguobacterium* (day 7 = 1.20%, day 14 = 1.37%, day 21 = 1.58%), and an uncultured genus in family Comamonadaceae (day 7 = 1.34%, day 14 = 1.24%, day 21 = 1.01%) (Supporting information).

Next, we ran a SIMPER analysis to determine the average contribution of each bacterial genus to the average dissimilarity in microbial composition among consecutive age timepoints (Supporting information). Over 80% of the dissimilarity between nestling days 7 versus 14 and 14 versus 21 can be attributed to changes in the relative abundances of 5 bacterial genera. Increases in the relative abundances of an unassigned genus from class Bacilli, and the genus *Bacillus,* respectively, explained 26.77% and 10.34% of the average dissimilarity between nestling day 7 and day 14, whereas decreases in *Enterococcus*, an unassigned genus from order Lactobacillales, and *Carnobacterium* explained 26.01, 12.60, and 9.24% of the dissimilarity, respectively. When comparing nestling days 14 and 21, decreases in the relative abundances of *Enterococcus* and an unassigned genus from class Bacilli explained 25.38 and 23.98% of the average dissimilarity, respectively; whereas increases in an unassigned genus from order Lactobacillales, and in *Bacillus* and *Carnobacterium* explained 13.17, 11.08, and 9.18% of the dissimilarity, respectively.

We found no significant differences in gut microbial alpha diversity (Table 1) or composition according to the sex of the nestlings (Supporting information).

Diet and gut microbial diversity

In the second sampling year (i.e. 2019 when diet data were collected), the Shannon index for gut microbial diversity at day 21 was positively correlated with the Shannon index for diet diversity ($F_{1,7}$ = 7.05, p = 0.03, Fig. 2, Table 2) but log Chao1 index ($F_{1,7}$ = 3.97, p = 0.09) and log Faith's PD ($F_{1,7}$ = 4.05, p = 0.08) for gut microbial diversity were not related to diet alpha diversity (Table 2). Percentage of prey items including mammal, passerine, arthropod, and annelid were not related to gut microbial alpha diversity for either the Shannon index or log Chao1 index, or overall microbial composition for either Bray–Curtis or Jaccard (Supporting information). Diet beta diversity (Jaccard) did not correlate significantly with day 21 gut microbial beta diversity (Bray–Curtis: Mantel r = 0.04, p = 0.26; Jaccard: Mantel r = 0.02, p = 0.37). The relative abundance of *Lactobacillus* was negatively correlated with the percentage of arthropods in the diet ($\tau = -0.49$, p < 0.01). The relative abundance of *Enterococcus* was positively correlated with the percentage of passerines ($\tau = 0.42$, p < 0.01) but negatively correlated with the percentage of mammals in the diet ($\tau = -0.31$, p = 0.03, Supporting information).

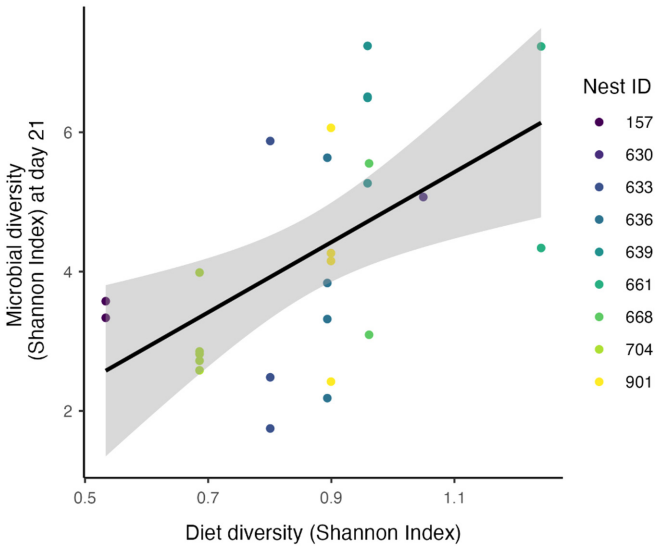

Figure 2. Nestling diet diversity (Shannon index) was positively correlated with gut microbial alpha diversity (Shannon index) on day 21 (n = 27) in American kestrel *Falco sparverius* nestlings in 2019. Diet diversity (Shannon index) is representative of the entire sampling period (days 5–20 of nestling development). Each datapoint denotes an individual nestling colored by nest ID, and gray shading denotes 95% confidence interval around the line of best fit.

Morphological and physiological development and gut microbial diversity

For contemporary or same day relationships, microbial diversity was negatively related to nestling body mass on day 14 for log Faith's PD ($F_{1,25}$ = 6.05, p = 0.02, Fig. 3a) but not for Shannon index ($F_{1,25}$ = 3.01, p = 0.09) or log Chao1 index ($F_{1,25}$ = 3.09, p = 0.09; Supporting information). In addition to the contemporary relationship found on day 14, early nestling gut microbial diversity was predictive of nestling body mass at a later stage of development. Microbial alpha diversity at day 7 negatively predicted nestling body mass at day 14 for log Faith's PD ($F_{1,21}$ = 11.21, p < 0.01, Fig. 3b) and log Chao1 ($F_{1,21}$ = 10.86, p < 0.01, Fig. 3c), but not for Shannon index ($F_{1,21}$ = 0.02, p = 0.88; Supporting information). Microbial alpha diversity (Shannon index)

Table 2. Results of linear mixed effects models testing the relationship between diet alpha diversity and gut microbial alpha diversity of American kestrel *Falco sparverius* nestlings. Each model included (a) Shannon index, (b) log Chao1 index, or (c) log Faith's phylogenetic diversity (PD) for day 21 microbiome samples only (n = 27) as the response variable and diet alpha diversity (Shannon index) as a fixed effect with nest as a random effect. Year was not included as a fixed effect in the Chao1 model because diet data were only collected in 2019. Diet alpha diversity is representative of the majority of nestling development (days 5–20). An asterisk and bold denote a significant p value (α = 0.05).

Response variable	Fixed effects	Degrees of freedom	Estimate	F value	p value
(a) Shannon index	(Intercept)	1, 18	0.14	183.18	**< 0.0001***
	Diet alpha diversity (Shannon index)	1, 7	4.75	7.05	0.0327
(b) Chao1 index (log)	(Intercept)	1, 18	2.74	601.49	**< 0.0001***
	Diet alpha diversity (Shannon index)	1, 7	2.18	3.97	0.0865
(c) Faith's PD (log)	(Intercept)	1, 18	0.7	238.10	**< 0.0001***
	Diet alpha diversity (Shannon index)	1, 7	1.48	4.05	0.0839

at day 14 negatively predicted nestling body mass at day 21 ($F_{1,24} = 6.87$, p = 0.02, Fig. 3d), but not for log Chao1 index ($F_{1,23} = 1.00$, p = 0.33) or log Faith's PD ($F_{1,24} = 2.39$, p = 0.14; Supporting information). There were no other significant contemporary or time-lagged relationships between gut microbial alpha diversity and nestling body mass for Shannon index, log Chao1 index, or log Faith's PD (Supporting information).

Nestling body mass at day 21 was negatively correlated with the day 21 relative abundance of Firmicutes ($F_{1,23} = 5.86$, p = 0.02) and positively correlated with the relative abundance of Proteobacteria ($F_{1,23} = 5.75$, p = 0.03; Supporting

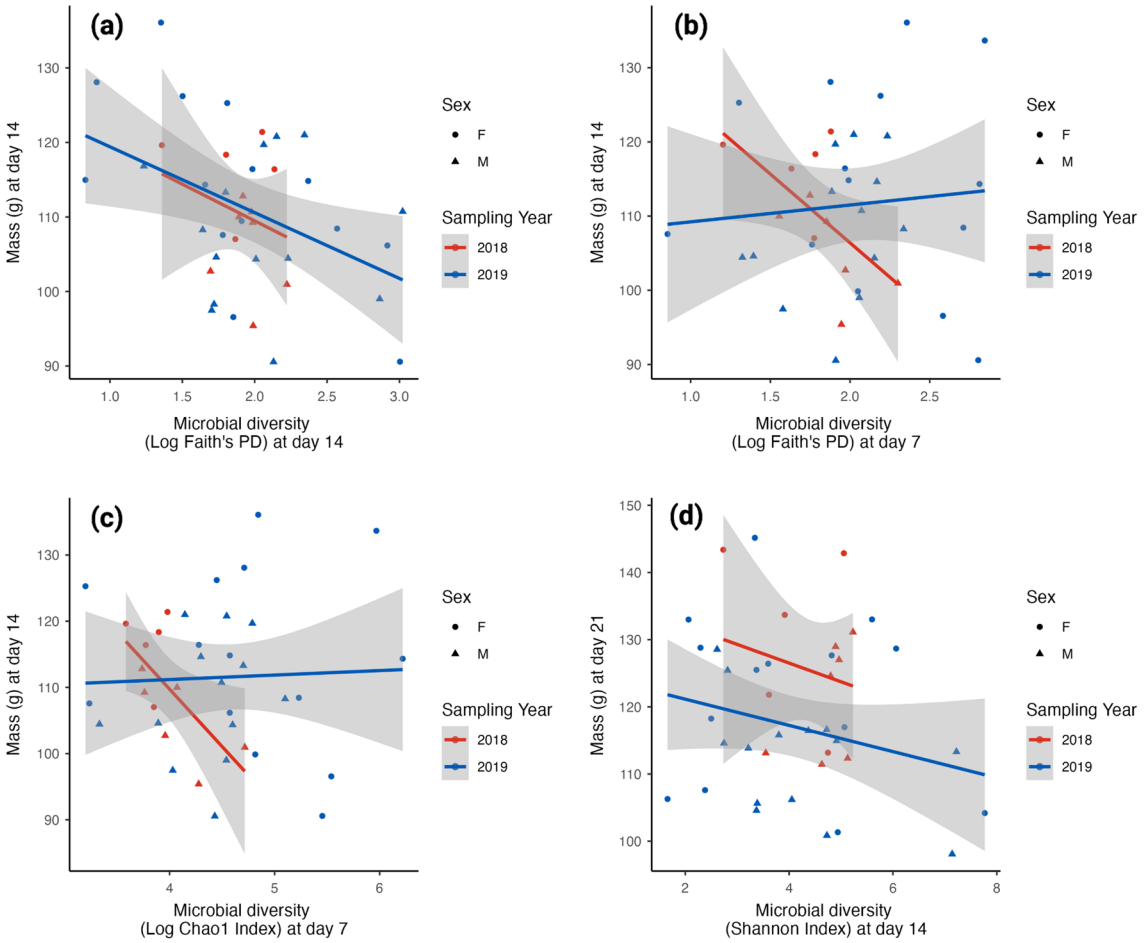

Figure 3. Contemporary and time-lagged relationships between nestling body mass and gut microbial alpha diversity of American kestrel *Falco sparverius* nestlings. (a) Nestling body mass (g) on day 14 was negatively correlated with gut microbial alpha diversity (log Faith's phylogenetic diversity (PD)) on day 14 (n = 39). (b–c) Nestling body mass (g) on day 14 was negatively correlated with gut microbial alpha diversity on day 7 (n = 39) for both (b) log Faith's PD and (c) log Chao1 index. (d) Nestling body mass (g) on day 21 was negatively correlated with gut microbial alpha diversity (Shannon index) on day 14 (n = 41). Each datapoint denotes an individual nestling colored by sampling year with shapes corresponding to nestling sex (F = female, M = male), and shading denotes 95% confidence interval around the line of best fit for each sampling year.

information). The relative abundance of Firmicutes at day 14 positively predicted nestling body mass at day 21 ($F_{1,24}=11.03$, $p < 0.01$), whereas the relative abundance of Proteobacteria at day 14 negatively predicted body mass at day 21 ($F_{1,24}=11.03$, $p < 0.01$). There were no other significant contemporary or time-lagged relationships between the relative abundances of Firmicutes and Proteobacteria and nestling body mass (Supporting information).

Early gut microbial diversity was related to physiology underlying metabolism (i.e. blood glucose) but not aerobic capacity (i.e. hematocrit) at a later stage of development. Gut microbial diversity on day 7 (Shannon index) was positively related to blood glucose concentrations on day 14 ($F_{1,22}=10.34$, $p < 0.01$; Fig. 4) but not for log Chao1 index ($F_{1,22}=3.66$, $p=0.07$) or log Faith's PD ($F_{1,22}=3.09$, $p=0.09$, Supporting information). There were no other significant contemporary or time-lagged relationships between gut microbial alpha diversity and blood glucose for Shannon index, log Chao1 index, or log Faith's PD (Supporting information). Gut microbial diversity was not related to hematocrit in a contemporary or time-lagged manner (Supporting information). The relative abundances of Firmicutes and Proteobacteria were not related to blood glucose or hematocrit for any contemporary or time-lagged analysis (Supporting information).

Discussion

Our results suggest that diet diversity may play a major role in gut microbial diversity, which in turn may impact morphological and physiological development in American kestrel nestlings. Gut microbial alpha diversity on nestling day 21 was positively correlated with diet alpha diversity representative of the majority of the nestling period (days 5–20), and the relative abundances of specific bacterial genera were correlated with the percentage of certain prey types. Increased gut microbial diversity was negatively correlated with body mass in both contemporary and time-lagged manners. Gut microbial alpha diversity was positively related to host physiology underlying metabolism (i.e. blood glucose), which may impact host fitness, given the importance of metabolism at the time of fledging. Contemporary and time-lagged associations between gut microbial diversity and host development further support the hypothesis that diversity of gut microbial communities may be a key component of determining individual nestling condition and could ultimately affect fitness.

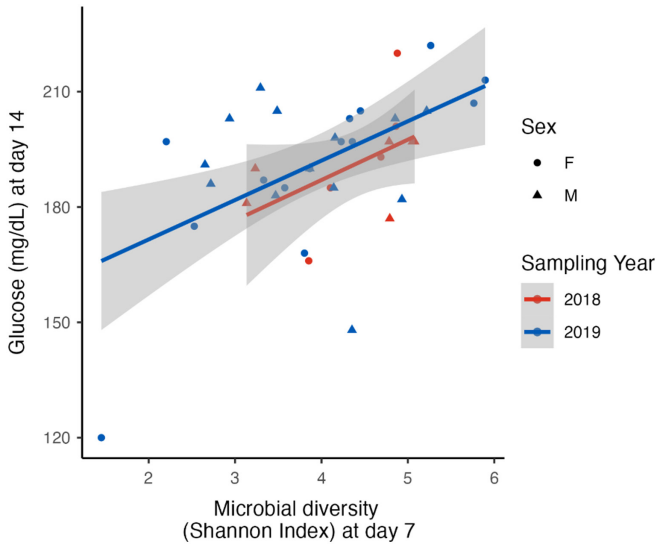

Figure 4. Nestling blood glucose (mg/dL) on day 14 was positively correlated with gut microbial alpha diversity (Shannon index) on day 7 (n = 39) in American kestrel *Falco sparverius* nestlings. Each datapoint denotes an individual nestling colored by sampling year with shapes corresponding to nestling sex (F = female, M = male), and shading denotes 95% confidence interval around the line of best fit for each sampling year.

Nestling age impacts gut microbial beta diversity but not alpha diversity

Gut microbial alpha diversity did not increase with age in American kestrel nestlings. This pattern parallels findings in Eurasian kestrel *Falco tinnunculus* nestlings, in which gut microbial alpha diversity did not vary significantly across different stages of nestling development (Zhou et al. 2020). However, we cannot rule out the possibility that gut microbial alpha diversity increased from hatching to seven days post-hatch. Our data suggest that nestlings can attain stable gut microbial alpha diversity levels by seven days post-hatch, which are maintained throughout development until fledging the nest, paralleling the results of Diez-Mendez et al. (2022). This early procurement of gut microbial diversity is consistent with Grond et al. (2017) in which alpha diversity starkly increased between hatching and two days post-hatch and then remained stable for the rest of the nestling period. Other avian host species display variation in gut microbial alpha diversity trajectory, with some species increasing in alpha diversity throughout development, including black-legged kittiwakes *Rissa tridactyla* (which increased in alpha diversity from 5 to 30 days post-hatch: van Dongen et al. 2013), pigeons *Columba livia* (which increased in community richness from 14 to 21 days post-hatch; Ji et al. 2020), chickens *Gallus gallus* (which increased from 1 to 28 days post-hatch; Awad et al. 2016), and ostriches (which increased over the first three months of life; Videvall et al. 2019). Great tits exhibited the opposite pattern of decreasing in gut microbial alpha diversity between two developmental time points (decreased between 8 and 15 days post-hatch; Teyssier et al. 2018).

The lack of an effect of age on gut microbial alpha diversity could also be attributed to the fact that avian gut microbial diversity and composition are more influenced by extrinsic factors such as environment and diet than by intrinsic factors such as host phylogeny (Hird et al. 2014, Song et al. 2020, Bodawatta et al. 2021c). Birds hatch with little to no gut microbial diversity (Grond et al. 2017, Trevelline et al. 2018) and then slowly inoculate their gut microbial communities

via horizontal transmission with the nest environment and prey items (Ballou et al. 2016, van Veelan et al. 2020). Even when nestling gut microbial communities are disrupted with antibiotics or probiotics, continuous recolonization from the nest environment and vertical transfer of microbes during feeding washes out any sign of disturbance to the nestling microbial communities (Diez-Méndez et al. 2022).

Although gut microbial alpha diversity did not differ among ages, beta diversity differences suggest that gut microbial composition may transition over time while maintaining similar alpha diversity levels. Kestrel gut microbial communities were dominated by Firmicutes across all ages, followed by a lower relative abundance of Proteobacteria, paralleling gut microbial community compositions of other wild bird species (reviewed by Waite and Taylor 2014, Grond et al. 2018, Bodawatta et al. 2021b). Other species of raptors including the Eurasian kestrel (Zhou et al. 2020), Cooper's hawk (*Accipiter cooperii*, Taylor et al. 2019), and red kite (*Milvus milvus*, Blanco 2014) harbored a higher relative abundance of Proteobacteria rather than Firmicutes in their gut microbiota. However, another study documented fluctuating dominance in the relative abundance of Proteobacteria versus Firmicutes across consecutive samples of an individual Eurasian kestrel depending on the host condition. For example, a high relative abundance of Proteobacteria was observed mainly in several samples that were collected during surgeries or drug treatments (Guan et al. 2020). Thus, dominance of specific microbial taxa in relative abundance of total gut microbial communities should be interpreted with caution as environmental factors, such as diet shifts, could result in variations in gut microbial relative abundance over time within a single individual.

Our data from the SIMPER analysis demonstrate that the majority of the differences in microbial composition between consecutive age timepoints can be attributed to changes in the relative abundances of 5 bacterial genera within the Firmicutes phylum. Of particular interest, decreases in *Enterococcus* explain more than 25% of these differences in microbial composition between each consecutive age comparison. Though the *Enterococcus* genus contains some beneficial species (Hanchi et al. 2018), potentially pathogenic species such as *E. faecalis* can lead to decreased hatchability in birds (Reynolds and Loy 2020). *Enterococcus* spp. produce antimicrobial compounds including bacteriocins (Hanchi et al. 2018), allowing them to competitively exclude other bacterial taxa. Semi-altricial species such as American kestrels are immunologically immature as hatchlings, but they acquire increased adaptive immunity as they age (Fairbrother et al. 2004). Increased adaptive immunity may explain the decrease in *Enterococcus* and increases in other taxa that could colonize the open niche space within the gut (Kreisinger et al. 2018). Diet may also influence gut microbial composition shifts throughout development if prey types are preferentially fed at different time points in development. Future studies capable of DNA metabarcoding fecal samples for diet composition or collecting more detailed provisioning data may seek to investigate this idea to fill this knowledge gap.

Nestling sex does not impact gut microbial alpha or beta diversity

Microbial alpha and beta diversity did not differ between the sexes in American kestrel nestlings, despite exhibiting sexual dimorphism in plumage characteristics and body mass by day 14 post-hatch (Anderson et al. 1997). Previous studies of sexually dimorphic avian species detected sex-related differences in gut microbial composition (Liu et al. 2020, Góngora et al. 2021). However, differences in microbial alpha and beta diversity based on sex in kestrels may not be apparent until adulthood, when intrinsic factors such as sex-related differences in immunosuppressant hormones are fully developed (Grond et al. 2018). Future studies should investigate the presence of sex-related gut microbial differences in adult American kestrels and how the gut microbial profiles shift across the breeding season as demands change (i.e. from incubation to nestling provisioning).

Sampling year impacts gut microbial alpha diversity but not beta diversity

In our study, microbial alpha diversity but not beta diversity differed between sampling years. Several environmental factors including ambient temperature (reviewed by Sepulveda and Moeller 2020), predation pressure (Zha et al. 2018), food availability (Knutie 2020), or nutritional value of prey items (Chapman et al. 2003) could explain the interannual differences in gut microbial alpha diversity. Intra-annual variation in microbial diversity has been attributed to seasonally driven dietary changes (Davenport et al. 2014, Wu et al. 2017, Hicks et al. 2018). In wild rhesus macaques *Macaca mulatta*, individuals vary significantly in gut microbial composition among all 4 seasons of the year based on total macronutrient intake (Cui et al. 2021). In our kestrel system, diet limitations due to annual variation in prey availability may have also influenced gut microbial alpha diversity between years. Diet data were not collected in 2018, leaving us unable to test this hypothesis. Alternatively, alpha diversity may have been higher in 2019 than in 2018 due to the larger sample size of nests monitored in 2019 (i.e. 2018: $n = 4$ nests versus 2019: $n = 10$ nests), elevating detection of uncommon species.

Diet diversity is positively correlated with gut microbial alpha diversity on nestling day 21

Diet is a major driver of gut microbial diversity and community composition (Muegge et al. 2011, Hird et al. 2015). Decreased diet variation can lead to reduced gut microbial alpha diversity (Wang et al. 2021). As gut microbiota in avian hosts are typically obtained horizontally from the environment and prey items, it is logical that a variety of prey types in the diet would introduce a greater diversity of gut microbiota to the host (Ballou et al. 2016). Our data support this idea as gut microbial alpha diversity on day 21 was positively correlated with diet alpha diversity representative of the majority of nestling development. This positive association between

diet alpha diversity and gut microbial alpha diversity is consistent with findings in other avian (Maul et al. 2005), reptilian (Wang et al. 2021), and mammalian systems (Heiman and Greenway 2016, Chi et al. 2019).

We did not find significant correlations between gut microbial beta diversity and diet beta diversity, suggesting that individuals with different diets did not carry consistently different gut microbial communities. Paralleling the results of Bodawatta et al. (2022), the lack of an association between microbial beta diversity and diet beta diversity could imply that individual variation in bird gut microbiomes leads to associations of different bacterial taxa with the same dietary item. As we broadly categorized prey items (songbird, mammal, annelid, etc.), it is possible that our diet beta diversity values do not account for variation in microbial contribution of specific prey species within these broader categories. For example, granivorous songbird prey may contribute different microbiota than omnivorous songbirds (García-Amado et al. 2018), where differences in gut microbial diversity or composition based on dietary guild would remain undetected by our methods. Indeed, Bodawatta et al. (2022) discovered differences in diet–microbiome associations depending on diet description method (e.g. visual inspection versus metabarcoding). Increasing specificity within diet determination by pairing visual inspection with non-visual methods, such as metabarcoding, provides a better overall picture of diet and can lead to more accurate associations of diet beta diversity with gut microbial diversity.

Percentage of specific prey items (mammals, passerines, arthropods, and annelids) was not related to microbial alpha diversity, but the relative abundances of specific bacterial genera were correlated in variable directions with the percentage of certain prey types. For example, the relative abundance of *Enterococcus* was positively correlated with the percentage of passerines but negatively correlated with the percentage of mammals in the diet. Thus, the overall effect of diet alpha diversity on microbial alpha diversity may be attributed to a combination of associations between microbial taxa and specific prey hosts (Bodawatta et al. 2022). Surprisingly, we found no associations between bacterial genera and the percentage of annelids in the diet despite the fact that annelids function as decomposers in the soil, potentially exposing them to diverse soil microbiota that are absent in other prey species (Thompson et al. 2017). Dion-Phénix et al. (2021) found that hosts of adjacent trophic levels (i.e. primary consumer caterpillar and secondary consumer blue tit) had a more similar bacterial microbiota than hosts separated by two trophic levels (i.e. primary producer plants and secondary consumer blue tits). Though we found correlations between some bacterial genera and specific prey types, we cannot claim causation without diet manipulations. Future studies should continue to investigate trophic transmission of microbiota (Dion-Phénix et al. 2021) and employ experimentally manipulated diets of different prey types to disentangle cause and effect relationships between gut microbial diversity and host diet (Davidson et al. 2020, Teyssier et al. 2020, Bodawatta et al. 2021a).

Gut microbial alpha diversity is negatively correlated with body mass in both contemporary and time-lagged manners

On a contemporary timescale, gut microbial alpha diversity (log Faith's PD) was negatively correlated with host body mass on nestling day 14, but not on day 7 or day 21. The negative, contemporary association between gut microbial diversity and host mass observed on day 14 may be explained by host–microbiota competition (Wasielewski et al. 2016). Nestling birds exhibit the fastest growth rates among terrestrial vertebrates (Case 1978), requiring them to direct their energy and metabolic resources to growth. Nestlings in our population experienced the most rapid growth from day 7 to day 14 (Cornell et al. 2021). Though we cannot claim causal links, the presence of particular microbial taxa or overall number of different microbial taxa may either promote or constrain the capacity of the host to absorb nutrients from the diet and divert energy to growth. For example, lactic acid bacteria (e.g. order Lactobacillales) can promote mass gain when given as a probiotic (Angelakis and Raoult 2010). Though microbiota provide clear benefits, hosts incur an energetic cost when resident microbial taxa utilize resources, making them unavailable to the host. Lactic acid bacteria ferment glucose to lactic acid, which reduces the energy available to the host gut epithelium (Saunders and Sillery 1982). An unassigned genus of order Lactobacillales was the third most abundant genus among our samples, but future studies should either supplement the amount of Lactobacillales spp. or reduce it with targeted antibiotics to validate its potential to promote or constrain nestling growth.

Gut microbial alpha diversity early in development was also negatively correlated with body mass later in development. Our findings that log Chao1 index and log Faith's PD at day 7 negatively predicted mass at day 14, and Shannon index at day 14 negatively predicted mass at day 21, are consistent with recent studies in which nestling great tits (Davidson et al. 2021) and juvenile ostriches (Videvall et al. 2019) with high gut microbial diversity early in development exhibited reduced mass later in development. The negative, time-lagged associations could be explained by differences in provisioning rate (Dawson and Bortolotti 2003) or shifts in diet diversity (Dawson and Bortolotti 2000) throughout the developmental period in addition to host–microbiota competition (above). As discussed in Davidson et al. (2021), time-lagged associations could be evidence that a specific gut microbial diversity at a critical early life period could prime the metabolic efficiency of the host for increased growth needs through development. High gut microbial alpha diversity is commonly linked with greater health of host organisms (reviewed by Heiman and Greenway 2016), but in some cases uncommon microbial species, such as opportunistic pathogens that increase overall alpha diversity, may have negative impacts on host health due to the diversion of energy from morphological growth to less-pertinent functions related to gut microbiota management (Krams et al. 2017). As space is limited within the gut tract, unbeneficial species may also

be outcompeting other microbial species during colonization that may provide more valuable functions to host development such as degradation of complex polysaccharides. Some studies have noted that gut microbial community composition, rather than diversity, is more influential for host health and survival as alpha diversity does not account for functional diversity or unbeneficial species within gut microbial communities (Worsley et al. 2021). We found variable associations between phylum-level relative abundances of Firmicutes and Proteobacteria and nestling body mass in both contemporary and time-lagged manners. In contemporary relationships, an increased relative abundance of Proteobacteria and decreased relative abundance of Firmicutes promote increased nestling body mass, whereas the opposite pattern exists for the time-lagged relationship. These findings suggest that the ratios of phylum-level microbiota may be temporally and context-dependent. For example, increased ratios of Firmicutes to Bacteroidetes have been positively correlated with obesity and Type II diabetes in humans (Magne et al. 2020). Though a high relative abundance of Firmicutes may be unbeneficial to humans, developing nestlings may benefit from Firmicute-mediated degradation of fiber into energy-rich fatty acids early in development that could be utilized by the host for tissue growth later in development (Flint et al. 2008).

Gut microbial alpha diversity is positively correlated with blood glucose in a time-lagged manner

Gut microbial alpha diversity at seven days post-hatch was positively correlated with blood glucose concentrations at 14 days post-hatch. Like morphological traits such as body mass, high gut microbial alpha diversity may have negative developmental consequences in the context of host metabolism. Elevated glucose concentrations are known to be a typical response to physiological stress, though not always in a straightforward way (Taff et al. 2022). The positive correlation between gut microbial alpha diversity and glucose concentration may further reveal that high alpha diversity has negative consequences for host health. In fact, significant increases in circulating blood glucose in wild vertebrates including birds are driven by a decrease in glucose utilization rather than increased glucose production (Romero and Beattie 2021). Studies with germ-free chickens have indeed shown that colonization by microbiota decreases total absorption of glucose and vitamins (Ford and Coates 1971). Thus, maintaining and provisioning fewer gut microbial taxa may allow the host to absorb and utilize circulating blood glucose more effectively, though dependent on the specific microbial taxa present.

We also explored potential correlations between nestling gut microbial diversity and physiology underlying aerobic capacity (e.g. hematocrit levels), as hematocrit is predictive of nestling body condition (Lill et al. 2013) and post-fledging survival (Bowers et al. 2014). Since physiology underlying aerobic capacity has been linked to fledgling flight ability (Cornell et al. 2017) and nestling survival (Nadolski et al. 2006, Kaliński et al. 2015), it is essential for nestlings to develop and maintain high hematocrit levels. Low hematocrit levels can be caused by a low supply of healthy red blood cells. Bacterial pathogens possess transferrin-binding proteins that compete with human transferrin for access to iron (Barber and Elde 2014). Microbial iron theft could subsequently lead to host iron deficiency, which in turn decreases the production of red blood cells (Rusu et al. 2020). Though we did not find any associations between hematocrit and gut microbial alpha diversity or phylum-level relative abundances, it still provides a fruitful area for future inquiry as an eco-physiological mechanism connecting host ecology and aerobic physiology. To our knowledge, no other studies have focused on how natural gut microbial diversity in free-living hosts affects physiology underlying aerobic capacity in a wild bird, leaving a sizable knowledge gap in the avian gut microbiota literature.

Conclusions and future directions

We found interactions among gut microbial diversity, diet diversity, and host morphological and physiological traits in developing American kestrel nestlings. The positive relationship between microbial alpha diversity on nestling day 21 and diet alpha diversity must be interpreted with caution, as the diet metrics presented in this study are representative of the entire sampling period (days 5–20 of nestling development). Within our study population, it was not possible to estimate diet at a finer scale, particularly for nests with brood sizes of 1–2 nestlings (3/10 nests). Therefore, grouping the prey items throughout the nestling period is the best way to accurately address the question of how diet during nestling development relates to American kestrel nestling gut microbial diversity. We hope future studies can measure diet at a finer temporal scale, but we also caution researchers to consider the restraints of observational diet characterization given their study system.

We found time-lagged relationships with gut microbial alpha diversity in early nestling development and host physiological (e.g. blood glucose) and morphological parameters (e.g. body mass) in late nestling development, though our low sample size may have prevented us from uncovering other relationships. Recent work found that baseline glucose is negatively correlated with body mass across 30 passerine species (Tomasek et al. 2019); however, in our dataset of a single raptor species, blood glucose and body mass are not correlated (Cornell unpublished), and the most rapid growth in our nestlings occurred during days 7 and 14 (Cornell et al. 2021). Therefore, we suspect the time-lagged relationships between gut microbial alpha diversity and both blood glucose and body mass demonstrate that high microbial alpha diversity individuals may have unbeneficial bacterial species competing with beneficial bacteria, or potentially increased stress that leads to independent effects on circulating glucose (Romero and Beattie 2021) and body mass (Almasi et al. 2015). These relationships and their potential trade-offs should be tested for causality using experimental approaches such as antibiotic

administration (Potti et al. 2002, Kohl et al. 2018) or nutrient/food supplementation (Davidson et al. 2020, Knutie 2020).

These time-lagged findings from the present and other studies (e.g. Davidson et al. 2021) underscore the importance of longitudinal sampling within individuals when investigating connections between gut microbial diversity and host developmental trajectory. Descriptive microbiome studies lack the ability to directly demonstrate an influence of gut microbiota on host phenotype that can be shown with manipulative studies; however, sampling gut microbiota of wild populations allows us to uncover unexplored host–microbiota relationships and provide baseline data that lead to hypothesis-driven, manipulative studies. We highlight the potential for gut microbial diversity to serve as an eco-physiological mediator between the host's ecological pressures, such as diet, and host physiology, but call for manipulative follow-up experiments to confirm these relationships.

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Supporting information

The Supporting information associated with this article is available with the online version.

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