Effect of urbanization and parasitism on the gut microbiota of Darwin’s finch nestlings

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Abstract

Host-associated microbiota can be affected by factors related to environmental change, such as urbanization and invasive species. For example, urban areas often affect food availability for animals, which can change their gut microbiota. Invasive parasites can also influence microbiota through either competition or indirectly through a change in the host immune response. These interacting factors can have complex effects on host fitness, but few studies have disentangled the relationship between urbanization and parasitism on an organism’s gut microbial composition. To address this gap in knowledge, we investigated the effects of urbanization and parasitism by the invasive avian vampire fly (Philornis downsi) on the gut microbiota of nestling small ground finches (Geospiza fuliginosa) on San Cristóbal Island, Galápagos. We conducted a factorial study in which we experimentally manipulated parasite presence in an urban and non-urban area. Feces were then collected when nestlings to characterize the gut microbiota (i.e., alpha and beta diversity, community composition). Although we did not find an interactive effect of urbanization and parasitism on the microbiota, we did find main effects of each variable. Urban and parasitized nestlings had lower bacterial diversity and differences in relative abundance of bacterial phyla and genera compared to non-urban non-parasitized nestlings, respectively. Overall, this study advances our understanding of the complex effects of anthropogenic stressors on the gut microbiota of birds.

Introduction

The gut microbiota is a community of microorganisms inhabiting an organism’s digestive tract that can affect host physiology and health (Gomaa, 2020; Grond et al., 2018). For example, specific gut bacterial taxa, such as *Ruminococcus*, can aid in host digestion by breaking down indigestible food compounds, such as cellulose, and increase digestive efficiency in humans (Arumugam et al., 2011; Thursby & Juge, 2017). Furthermore, variation in diet, such as a plant-based diet versus a meat-based diet, can shape host gut microbiota as it shifts in response to nutrient availability (Gomaa, 2020; Zhu et al., 2015). Additionally, the host’s immune system can interact with the gut microbiota, specifically by shaping its community composition (Hooper et al., 2012). For example, Kato et al. (2014) found that knockout mice who are deficient in B-cell production have lower gut bacterial diversity compared to non-knockout mice. The gut microbiota can also initiate the production of immune cells to help maintain or develop an effective immune response (Zhang et al., 2017). Studying the dynamic relationships between physiological processes and gut microbiota will be continually important, especially in light of increasing anthropogenic change which can directly and indirectly affect these factors.

Parasites and pathogens, which also affect host health, can directly and indirectly affect microbial communities in the gut (Stensvold & Van Der Giezen, 2018). Within the gut, parasites can directly affect the microbiota by competing with or consuming commensal bacteria (Abt & Pamer, 2014). Parasites might also indirectly
affect the gut microbiota via the host’s immune system. For example, gut parasites (e.g., *Heligmosomoides polygyrus*) can activate a non-specific mucosal immune response (e.g., T and Th2 cells), which can select for or against bacterial taxa, such as an increase in gram negative Enterobacteriaceae and a decrease in *Enterococcus faecium* (Rausch et al., 2018). However, parasites located outside of the gut might also elicit a systemic immune response that could affect the bacterial community in the gut. For example, fish with ectoparasitic fluke (*Dactylogyrus lamellatus*) infections have lower gut bacterial diversity compared to uninfected fish, which was likely mediated by elevated expression of immune genes related to the IgM antibody, toll-like receptor 3, and Major histocompatibility factor II responses (Wang et al., 2023). Knutie, (2020) found that parasitic nest fly abundance is negatively correlated with gut bacterial diversity and positively correlated with IgY antibody levels, in eastern bluebirds (*Sialia sialis*). Overall, most of these non-model organism studies are correlational and thus the causal effects of parasitism on gut microbiota are not well understood.

Factors related to urbanization, such as changes in food availability, can also influence the gut microbiota of wild hosts (Berlow et al., 2021; Phillips et al., 2018; Teyssier et al., 2020). Studies have found that urban hosts have a larger diet breadth, which can result in greater diversity of the gut bacterial community (Littleford-Colquhoun et al., 2017). For example, non-urban water dragons (*Intellagama lesueurii*) feed primarily on invertebrates but urban water dragons feed on invertebrates and plant material, which results in higher gut bacterial diversity. These effects are either because plants introduce additional bacterial taxa or a larger diet breadth selects for different bacteria to aid in digestion. If the latter is supported, a more diverse gut microbial community could help hosts be more equipped to deal with environmental change (Littleford-Colquhoun et al., 2017). In contrast, the gut of house sparrows (*Passer domesticus*) consuming a non-urban diet, which is rich in protein, is correlated with higher bacterial diversity compared to urban sparrows whose diet is poor in protein (Teyssier et al., 2020). While increased gut bacterial diversity is generally assumed to be beneficial to hosts, in some cases, such as in organisms with highly specialized diets, lower bacterial diversity can be associated with better health outcomes (Shade et al., 2017). For example, urban coyotes consume carbohydrate-rich anthropogenic food items and have higher bacterial diversity compared to their rural counterparts, but are in poorer body condition and have increased prevalence of the parasite *Echinococcus multilocularis* (Sugden et al., 2020). Ultimately, these effects of urbanization and other stressors related to human activity (e.g., invasive parasites) are complex and could result in positive or negative implications for host health.

Given the complex effects of human activity on hosts, few studies have examined the influence of multiple synergistic anthropogenic factors, such as urbanization and invasive parasitism, on host gut microbiota. The Galápagos Islands of Ecuador provide an ideal study system to investigate these complex effects. Since 1979, the number of residents and tourists have increased, leading to changes in the natural habitat. These changes include the introduction of non-native species, including parasites (Kerr et al., 2004; Wikelski et al., 2004). For example, the avian vampire fly (*Philornis downsi*; hereon, vampire fly) was introduced to the Galápagos in the past several decades and is found on nearly all islands, including human-inhabited islands such as San Cristóbal. Adult flies are non-parasitic but lay their eggs in birds’ nests where the hematophagous larvae feed on nestling hosts and brooding mothers (Fessl et al., 2001; Fessl & Tebbich, 2002). Several studies have found that the vampire fly can have detrimental effects on the survival of nestling Darwin’s finches (Fessl et al., 2010; Kleindorfer & Dudaniec, 2016; Knutie et al., 2016; Koop et al., 2011, 2013; McNew & Clayton, 2018; O’Connor et al., 2010). However, a recent study found that urban finches on San Cristóbal Island are less affected by and more resistant to the vampire fly than non-urban finches (Knutie et al., 2023) who suffer up to 100% mortality due to the fly (Koop et al., 2013; O’Connor et al., 2014). For non-urban finches, the vampire fly does not affect the gut microbiota (Addesso et al., 2020; Knutie, 2018; Knutie et al., 2019). However, because urban finches are more resistant to the fly, and immunological resistance can interact with gut microbiota, parasitism may cause a greater change on the microbiota of urban finches compared to non-urban finches. To date, no studies have causally explored whether parasitism and urbanization interact to affect the gut microbiota of hosts.

The goal of this study is to compare the effects of avian vampire flies and urbanization on the gut microbiota-
ta of nestling small ground finches (Geospiza fuliginosa) in 2018 and 2019. Specifically, we experimentally manipulated parasite abundance in urban and non-urban finch nests and then characterized the gut microbiota (i.e., alpha and beta diversity, community composition, relative abundance of taxa). Because diet can influence the gut microbiota (Davidson et al., 2020) and urban finches have a more diverse diet compared to non-urban finches (De León et al., 2019), we hypothesize that urban nestlings will have a different gut microbiota than non-urban nestlings (Loo et al., 2019). Because immunological resistance can be linked to the gut microbiota (Hooper et al., 2012), we predict parasitism will affect and ultimately change the gut microbiota of urban nestlings. Past studies have found that parasitism by vampire flies does not affect the gut microbiota of finch nestlings in non-urban areas (Addesso et al., 2020; Knutie, 2018; Knutie et al., 2019), but their diet is not supplemented. Therefore, we predict that parasitism will not affect the gut microbiota of non-urban nestlings. Further investigation will allow for a more thorough understanding of environmental change on the gut microbiota of small ground finch nestlings.

**Methods**

**Study system**

The study was conducted between February – May 2018 and 2019 (during the breeding season) in the arid lowlands of San Cristóbal (557 km$^2$) in the Galápagos Islands. The urban area was in the capital city of Puerto Baquerizo Moreno (0°54′9″S, 89°36′33″W) (hereon, urban area), which is the only large town on San Cristóbal and the second largest city in the Galápagos archipelago with a human population of 7,199 (INEC, 2016). The urban area primarily consists of impermeable concrete or stone surfaces and human built structures in altered landscapes. Our urban study area measured 0.79 km$^2$ (~1.2 km by 0.62 km) and included tourist and residential zones. The non-urban area was in Jardín de Opuntias (0°56′18.92″S, 89°32′54.93″W) (hereon, non-urban area), which is a Galápagos National Park site located 4.5 km southeast of the urban area. This site consisted of vegetated natural habitats with no unnatural impermeable surfaces present. Our non-urban study area measured 0.21 km$^2$ and covered 1.4 km of the main trail and 0.15 km to each side.

Small ground finches are abundant at both field sites (Harvey et al., 2021). Small ground finches build domed-shaped nests in native and non-native trees and shrubs as well as human-built structures, depending on the location. On San Cristóbal Island, they lay 1-4 eggs per clutch (mean = 2.84 eggs) and have an average of 2.83 nestlings per brood across urban and non-urban areas. However, in some years, nests in urban areas contained more eggs than non-urban areas, yielding higher nestling survival (Harvey et al., 2021).

**Experimental manipulation of parasites**

We searched field sites daily for evidence of nest building by small ground finches. Once eggs were laid, we checked nests every other day until the nestlings hatched. Prior to egg hatching, we experimentally manipulated fly abundance via adding approximately 10 mL of 1% permethrin solution (Permacap; hereon, fumigated) or water (hereon, sham-fumigated) to the nest. Within two days of nestling hatching, we treated nests again to ensure the removal of all parasites. Briefly, we removed the contents of the nests (including the nestlings, unhatched eggs, and the nest liner), and treated them with either a permethrin solution or water by spraying approximately 5 mL into the nest where the larvae live. After treatment, we returned the dry nest liner, and placed nestlings back into the nest. We used permethrin because it is highly effective at removing vampire flies from the nests (Kleindorfer & Dudaniec, 2016). However, studies have found that permethrin can have sublethal effects on nestling birds (Bulgarella et al., 2020); therefore, we ensured that nestlings did not come into contact with the insecticide. Additionally, adults returned to their nests with no cases of abandonment from the treatment (Koop et al., 2013; Knutie et al., 2016).

**Sample collection**

We banded nestlings at 6-8 days old with a unique color band combination and a numbered metal band (National Band and Tag, Kentucky, USA). We collected fecal samples from nestlings opportunistically at this time. To collect fecal samples, we removed nestlings from the nest and held them over a sterile weigh boat until they defecated. We then moved the fecal sample from the tray to a sterile tube, placed it on
ice in the field for up to 6 hours, and then stored it in a -20 °C freezer until the bacterial DNA was extracted. We transported the samples to the University of Connecticut and stored them in a -80 °C freezer for downstream 16S sequencing. Although studies show that the bacterial community in avian feces does not always represent the entire digesta of the host (e.g., in the cecum; Wilkinson et al., 2017), fecal samples are generally representative of the bacterial community in the large intestines (Videvall et al., 2018; Wilkinson et al., 2017) and are used when hosts cannot be euthanized. When nests were empty, they were collected and examined for P. downsi; the full data set was reported in Knutie et al., (2023).

**Bacterial DNA extraction and sequencing**

We extracted total DNA from feces using a ZymoBIONICS DNA kit and sent DNA extractions to the University of Connecticut Microbial Analysis, Resources and Services for sequencing with an Illumina MiSeq platform and v2 2x250 base pair kit (Illumina, Inc.). We also sequenced a laboratory extraction blank to control for kit contamination. We conducted bacterial inventories via amplification of the V4 region of the 16S rRNA gene using primers 515F and 806R and with Illumina adapters and dual indices (Kozich et al., 2013). We used the DADA2 (v. 1.22.0) pipeline (Callahan et al., 2016) in R (v. 4.2.0) to process sequence data. After quality assessment, we trimmed sequences to remove low quality read areas and chimeric reads. We classified amplicon sequence variant (ASV) taxonomies using RDP’s Naive Bayesian Classifier (Q. Wang et al., 2007) with the Silva reference database (v. 138.1) (Quast et al., 2012). After classification, we removed sequences identified as chloroplast and mitochondria from the dataset. We identified and removed likely bacterial contaminants with the package decontam (Davis et al., 2018) in R using the kit extraction blank that was processed in parallel with the other samples as a control. Sequences were aligned using the DECIPHER package (v. 2.22.0) in R (Wright, 2015), and a generalized time-reversible maximum likelihood tree of the remaining ASVs was constructed with the phangorn package version 2.9.0 (Schliep, 2011). The ASV table, taxonomic information, phylogeny, and sample metadata were joined for bacterial community analyses using the package phyloseq (McMurdie & Holmes, 2013). We filtered the feature table to retain samples with at least 1,500 total reads which reduced the dataset to 58 samples containing 2,526 unique ASVs. The resulting data set had an average of 51,056 ± 6,178 reads per sample (min: 1,732; max: 294,720). For alpha and beta diversity analyses, we rarefied samples to the sample with the lowest read count (1,732). The filtered dataset contained 1,448 ASVs after random subsampling.

**Statistical analyses**

**Alpha Diversity**

We used the R package vegan (Oksanen et al., 2022) to compute alpha diversity (observed ASV richness, Shannon diversity index) on the filtered dataset. Observed richness describes the number of observed species and evenness describes the distribution of abundance across the species. The Shannon diversity index is an estimator of species richness and species evenness. We ran generalized linear mixed effects models with a negative binomial error structure for observed ASV richness and a Gaussian distribution for Shannon diversity using the glmTMB package (Brooks et al., 2017). Location (urban, non-urban), parasite treatment (fumigated, sham-fumigated) and the interaction between location and parasite treatment were considered in all models. Because the microbiota of finches can vary across years (Michel et al., 2018), we also included year (2018, 2019) as a covariate in all models. Nest identification was included as a random effect in all models. We used the Anova function in the car package (Fox & Weisberg, 2018) to determine significance.

**Beta Diversity**

To deal with unequal sequence coverage and compositional variation within the data, we used Cumulative Sum Scaling (CSS) normalization on the filtered dataset using the R package metagenomeseq (Paulson et al., 2013) following the methods in Maraci et al., (2021). We then log (x + 0.0001) transformed the data and later corrected the transformed values by subtracting the log of the pseudo count (Thorsen et al., 2016). Dissimilarity matrices were computed based on Bray–Curtis (Bray & Curtis, 1957), unweighted UniFrac (C. Lozupone & Knight, 2005), and weighted UniFrac (Lozupone et al., 2007). To visualize the dissimilarities between nestlings based on parasite treatment and urbanization, we used a principal coordinate analysis
(PCoA) using the ordinate function in the *phyloseq* package (McMurdie & Holmes, 2013). To determine the effect of parasitism and urbanization on beta diversity metrics, we used PERMANOVAs with parasite treatment, location, year (2018, 2019), and the interaction between parasite treatment and location as fixed effects and beta diversity metrics as response variables. Nest identification was included as a random effect in all models. We used the adonis2 function with the *vegan* package for these analyses (Oksanen et al., 2022).

**Relative Abundance of Bacterial Taza**

We calculated the relative abundances of phyla and genera from the unrarefied dataset. For analyses, phyla and genera with mean abundances <1% were lumped into the “Other” category. This data stringency limited analyses to the top three most abundant phyla (Proteobacteria, Firmicutes, Actinobacteriota) and the top twelve most abundant genera (Table 1). We ran non-parametric Kruskal-Wallis tests using the kruskal.test function in R to compare abundances of bacterial taxa between locations (urban, non-urban), parasite treatments (sham-fumigated, fumigated), and locations within the parasitized (sham-fumigated) treatment group. *P*-values were adjusted for false discovery rate with a Benjamini–Hochberg correction where significance was determined as *P* adj < 0.05.

**Results**

**Bacterial diversity**

Nestlings from fumigated nests had higher bacterial richness than nestlings from sham-fumigated nests (Figure 1A, χ² = 4.04, *P* = 0.04). Shannon diversity index was not significantly affected by parasite treatment (Figure 1B, χ² = 1.10, *P* = 0.29). Urbanization did not significantly affect observed richness (Figure 1C, χ² = 2.30, *P* = 0.13), but did influence the Shannon index (Figure 1D, χ² = 7.50, *P* = 0.006). Non-urban nestlings had higher Shannon diversity index values compared to urban nestlings. Observed richness and Shannon index were not affected by the interaction between parasite treatment and urbanization (Figure S1, Observed richness: χ² = 0.28, *P* = 0.60, Shannon Index: χ² = 0.21, *P* = 0.65). The year that samples were collected (2018, 2019) did not significantly affect the observed richness (Table S1, χ² = 0.28, *P* = 0.60) or Shannon index (χ² = 0.38, *P* = 0.54).

**Bacterial community structure and membership**

Using the Bray-Curtis Dissimilarity distances, parasitise treatment, urbanization, and the interaction between the two variables did not significantly affect bacterial community structure (Figure S2A, parasite treatment: *F* = 1.52, *P* = 1.00, urbanization: *F* = 2.05, *P* = 1.00, Interaction: *F* = 1.24, *P* = 0.48). Using the unweighted UniFrac distances, parasitism, urbanization, and the interaction between the two variables did not significantly affect bacterial community membership (Figure S2B, parasite treatment: *F* = 1.45, *P* = 1.00, urbanization: *F* = 2.26, *P* = 0.52, Interaction: *F* = 1.77, *P* = 0.52). Using the weighted UniFrac distances, parasite treatment, urbanization, and the interaction between the two variables did not significantly affect bacterial community structure (Figure S2C, parasite treatment: *F* = 2.09, *P* = 1.00, urbanization: *F* = 2.73, *P* = 1.00, interaction: *F* = 3.14, *P* = 0.50). Year did not affect Bray-Curtis Dissimilarity, weighted UniFrac, or unweighted UniFrac (Table S2).

**Microbiota taxonomic composition**

There were 2,526 unique ASVs present in the samples. Twenty-four phyla were identified and samples were dominated by Firmicutes (52.6%), Proteobacteria (35.1%), and Actinobacteriota (8.5%; Fig. 2). All other phyla had mean relative abundances of <1%. Across the 58 samples, there were 551 genera identified and twelve genera had relative abundances >1%. The most commonly observed genera were Candidatus Arthromitus (22.3%), Escherichia-Shigella (13.8%), Enterooccus (13.5%), Ligilactobacillus (9.1%), Klebsiella (7.8%), Cronobacter (4.8%), Erysipelatostridium (2.3%), Rothia (2.2%), Clostridium sensu stricto I (1.9%), Campylobacter (1.6%), Corynebacterium (1.4%), and Kocuria (1.1%). At the phylum level, location significantly affected the relative abundance of Firmicutes and Proteobacteria (Table 1). Specifically, nestlings from urban sites had higher relative abundances of Firmicutes (χ² =
13.74, $P_{adj} = 0.008$) and lower abundances of Proteobacteria ($\chi^2 = 8.35, P_{adj} = 0.01$) compared to nestlings from non-urban field sites (Figure 2). At the genus level, location significantly affected the abundance of Candidatus Arthromitus, Klebsiella, Erysipelatoclostridium, and Rothia (Table 1, Figure S3). Candidatus Arthromitus was highly abundant in urban nestlings, but rarely observed in fecal samples collected from non-urban nestlings (Figure 3, $\chi^2 = 10.94, P_{adj} = 0.01$). Conversely, Klebsiella ($\chi^2 = 7.13, P_{adj} = 0.03$) and Erysipelatoclostridium ($\chi^2 = 6.62, P_{adj} = 0.03$) were observed at higher abundances in fecal samples from non-urban nestlings (Figure 3). The genus Rothia was more common in nestlings from urban nests and was rarely observed in non-urban nestlings ($\chi^2 = 7.29, P_{adj} = 0.03$). This trend was difficult to visualize due to two individuals that had very high relative abundance values (Figure S4: one non-urban nestling: 41.89% Rothia, and one urban nestling: 63.43% Rothia). Additional outliers were detected and removed using the boxplot.stats function in R (non-urban: 1.25%, urban nestlings: 8.36%, 2.77%, 2.15%, 0.73%). Location (urban, non-urban) still significantly affected the relative abundance of Rothia with the two greatest outliers removed (Figure S4: $\chi^2 = 10.11, P_{adj} = 0.01$) and with the five additional outliers identified by the boxplot.stats function removed (shown in Fig 3, $\chi^2 = 12.57, P_{adj} = 0.005$).

Parasite treatment did not significantly influence the relative abundance of the three most abundant phyla or the abundance of any of the twelve most abundant genera (Table 1). When analyzing only nestling samples from sham-fumigated nests, there was still a significant effect of location on the abundance of Firmicutes and Proteobacteria (Figure S5). However, there was no effect of urbanization on the abundance of the top twelve genera within the sham-fumigated nests (Table 1).

**Discussion**

Our study used a factorial experiment to determine the main and interactive effects of urbanization and parasitism on the gut microbiota of nesting small ground finches across two years. In contrast to a previous study (Michel et al., 2018), we did not find an effect of year on the gut microbiota of nesting small ground finches. Although we did not find an interactive effect of urbanization and parasitism on the microbiota, we did find main effects of each variable. Contrary to our prediction, urban nestlings had lower bacterial diversity (Shannon index) compared to non-urban nestlings. Parasitized (sham-fumigated) nestlings also had lower bacterial diversity (observed richness) compared to non-parasitized (fumigated) nestlings. However, parasitism did not affect Shannon index and urbanization did not affect observed richness, which suggests that parasitism affects the number of bacterial taxa but not evenness of those taxa, whereas urbanization influences gut microbiota evenness. This explanation is supported by our finding that urbanization, but not parasite treatment, affects the relative abundance of several bacterial phyla and genera. Although urbanization and parasitism did not have an overall effect on bacterial community membership and structure, changes in bacterial diversity or specific taxa can also affect physiological processes in hosts (Hooper et al., 2012; Round & Mazmanian, 2009), as discussed below.

We hypothesized that urban nestlings would have higher bacterial diversity because of their wide breadth of food items, including human-processed food, compared to a primarily insect-rich diet in non-urban nestlings. This hypothesis was based on several studies that found an increase in gut bacterial diversity in response to urbanization (Berlow et al., 2021; Knutie et al., 2019; Littleford-Colquhoun et al., 2019; Phillips et al., 2018) and because diet can influence the gut microbiota (Bodawatta et al., 2022). However, we found that urban nestlings had lower bacterial diversity than non-urban nestlings, as found in Teyssier et al., (2018). One possible explanation is that the human-related food items select for particular bacterial taxa that dominate the microbiota, leading to fewer taxa in the gut. Teyssier et al., (2020) found that adult house sparrows (Passer domesticus) that were experimentally fed an urban diet had lower gut bacterial diversity. Knutie, (2020) found that food supplementation with yellow mealworm beetle (Tenebrio molitor) larvae increased the gut bacterial diversity of eastern bluebirds. Thus, an insect-rich diet in non-urban nestlings might maintain high bacterial diversity compared to diets of human-based foods in urban nestlings. Another possible explanation is that the food itself is introducing bacteria into the gut of the nestlings (Grond et al., 2018; Videvall et al., 2019) because human food items have different microbiota (Jarvis et al., 2018). To test these hypotheses, future studies may consider sequencing the microbiota of specific diet items and...
comparing these results with the gut microbiota.

Urban living also affected the relative abundance of bacterial genera and phyla in nestlings. For example, urban nestlings had higher abundances of the phylum Firmicutes and genus Candidatus Arthromitus. These specific taxonomic changes in the gut microbiota can also facilitate functional changes to host physiology. For example, bacterial species from the phylum Firmicutes can aid in nutrient uptake and metabolism in chickens (Li et al., 2016; A. Zheng et al., 2016), which might be required for human-processed food. Candidatus Arthromitus is a well-studied, segmented filamentous bacterium that is non-pathogenic and attaches to the intestinal wall (Snell et al., 1995). Across host taxa, Candidatus Arthromitus influences the innate and adaptive immune responses in the gut (Macpherson & McCoy, 2015; Suzuki et al., 2004). Specifically, Liu et al., (2023) found that the relative abundance of Candidatus Arthromitus is positively correlated with the innate immune response (e.g., T-lymphocytes) during avian development. In our system, urban finch nestlings up-regulate the expression of genes related to the T-lymphocyte production (Knutie et al., 2023), which might explain why we observed higher relative abundance of Candidatus Arthromitus in urban nestlings.

Vertical transmission of microbiota from parent to nestling might also explain the observed differences in the microbiota of urban and non-urban nestlings. To feed their nestlings, finch parents regurgitate food from their crop into the mouth of the nestling (Koop et al., 2013; O’Connor et al., 2014; Price et al., 1983); thus, potentially transferring the crop and mouth microbiota from parent to offspring. Studies have found that crop microbiota can be transferred from parent to offspring through regurgitation in birds (Chen et al., 2020; Ding et al., 2020; Grond et al., 2018). However, the crop and fecal/cloacal microbiota of birds can differ in beta but not alpha diversity (Bodawatta et al., 2022; Wilkinson et al., 2017). To test whether our results are due, at least in part, to vertical transmission of microbiota, a future study could compare the crop and mouth microbiota to the gut microbiota of finch parents and nestlings.

Physiological stress associated with urban environments could also be a mechanism through which urban environments affect nestling gut microbiota. A study comparing urban and rural great tits (Parus major) found that urban birds had an upregulation of several genes linked to stress responses, innate and adaptive immunity, and detoxification and repair systems (Watson et al., 2017). Physiological changes in immunity associated with urban stress likely impact gut microbiota composition, as both innate and adaptive immune responses can regulate and respond to shifts in gut microbiota (Zheng et al., 2020). Additionally, hormones associated with the stress response, such as glucocorticoids, can alter gut microbiota. For example, higher glucocorticoid levels are associated with lower bacterial diversity in squirrels (Petrullo et al., 2022) and gulls (Noguera et al., 2018). However, the effect of urbanization on glucocorticoids is variable across avian studies (Brodin & Watson, 2023; Deviche et al., 2023), and the link between physiological stress and gut microbial taxa likely varies based on the metric being investigated and type of sample used (e.g., glucocorticoids measured from blood vs. feathers/hair; Stothart et al., 2019). Future work should explore whether physiological stress mediates the effects of urbanization on nestling gut microbiota diversity and the relative abundance of gut bacterial taxa in this system.

Given that past studies have not found an effect of parasitism on gut microbiota of non-urban finches (Addesso et al., 2020; Knutie, 2018), we did not expect to find different results in our non-urban nestlings. However, we found an overall effect of parasitism on the gut microbiota across both locations, with parasitized nestlings having lower bacterial diversity (via observed richness) compared to non-parasitized nestlings. One possible explanation for the contradictory results is that other species of Darwin’s finches (e.g., medium ground finch [Geospiza fortis] and common cactus finch [Geospiza scandens]), host different gut microbial communities due to their different diets (e.g., cactus flower pollen, different seed types; De León et al., 2014). The interactions between the immune system and gut microbiota can be determined by which microbes are recognized by immune molecules. Thus, a change in the gut microbiota in small ground finches, but not medium ground finches or common cactus finches, in response to parasitism could be because their specific members of the microbiota are recognized and removed by the immune system. We also hypothesized that only urban nestlings would be affected by parasitism since they are more resistant to parasites compared to non-urban nestlings (Knutie et al., 2023). This resistance is potentially related to expression of type 1
interferon (IFN) genes, which can activate natural killer cells and macrophages that can destroy bacteria (Perry et al., 2005). Since the bacterial diversity metrics of both urban and non-urban nestlings were affected by parasitism, this suggests that both populations are having a general response to the parasite that interacts with the gut microbes. To establish a causal relationship, further investigation is required to understand the interaction between gut microbiota and immune response.

Overall, our study suggests that both parasitism and urbanization affect the gut microbiota of small ground finches. Since these anthropogenic factors also affect the health of finches in the Galápagos Islands (Harvey et al., 2021; Knutie et al., 2023), the next question is whether the microbiota are mediating these effects or influencing other traits, such as the immune system, in developing finches. To causally test these interactions, an experimental manipulation of the gut microbiota is necessary, either with the introduction of relevant bacterial taxa or a disruption of the gut microbiota with antibiotics. Although birds of the Galápagos Islands have experienced many direct effects of human presence, such as the introduction of parasites and changes in diet (De León et al., 2019; Wikelski et al., 2004), many indirect effects that are more difficult to study, such as those on the gut microbiota, could have important implications for the fitness of many endemic birds.

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Data accessibility: Supporting information has been made available online. Data are available at FigShare (doi: available upon acceptance) and sequences have been uploaded to GenBank (BioProject accession number: available upon acceptance).

Authors’ contributions: Conceptualization: SAK; Experimental Methodology: SAK, GJV; Analyses: AL; Investigation: GS, AL, GJV, JH, TBV, KC, SS, LA, SAK; Visualization: AL; Funding acquisition: SAK; Project administration: JC, SAK; Supervision: SAK; Writing – original draft: GS, AL, SAK; Writing – review & editing: All authors.

Conflict of Interest: The authors declare that they have no conflict of interest.

Literature Cited


**Figure Legends**

**Figure 1.** Effect of parasite treatment (sham-fumigated, fumigated) on (a) observed ASV richness and (b) Shannon diversity of the microbiota in nestling small ground finches. Individuals from fumigated nests are represented by red circles and individuals from sham-fumigated nests are represented by blue circles. Effect of location (urban, non-urban) on (c) the observed ASV richness and (d) Shannon diversity of the microbiota in nestling small ground finches. Individuals from non-urban nests are represented by green circles and individuals from urban nests are represented by gray circles. Black circles denote the mean values (±SE) of birds from each treatment.

**Figure 2.** (a) Proportional abundance of bacterial phyla across location (urban, non-urban) and parasite treatment (fumigated, sham-fumigated). Each bar represents a sample from an individual bird. Samples are divided into treatment groups as follows: non-urban, fumigated: n = 7 nestlings; non-urban, sham-fumigated: n = 7; urban, fumigated: n = 23; urban, sham-fumigated: n = 21. Phyla with <1% relative abundance are collapsed into the category “<1% abundance.” (b) Relative abundance of the three most common phyla separated by location (urban, non-urban). Individual points represent the relative abundance of each phylum from an individual nesting. Black circles denote the mean (±SE) relative abundances across treatments.

**Figure 3.** Relative abundance of the four genera (*Candidatus Arthromitus*, *Klebsiella*, *Erysipelatoclostridium*, *Rothia*) that significantly varied by location (urban, non-urban). Individual points represent the relative abundance of each genus from an individual nesting. Black circles denote the mean (±SE) across treatments.

**Tables**

**Table 1.** Kruskal-Wallis chi-square ($\chi^2$) test statistics and p-values for comparisons of bacterial taxa across location (urban, non-urban), parasite treatment (sham-fumigated, fumigated), and location (urban, non-urban) across parasitized (sham-fumigated only) nests. $P$-values were adjusted for false discovery rate with a Benjamini–Hochberg correction where significance was determined as $P_{adj} < 0.05$. Significant differences between groups are represented in bold.

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<th>Taxonomic Group</th>
<th>Location</th>
<th>Location</th>
<th>Parasite Treatment</th>
<th>Parasite Treatment</th>
<th>Location (Sham-fumigated only)</th>
<th>Location (Sham-fumigated only)</th>
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<td>$\chi^2$</td>
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**Fig. 1**

**Fig. 2**
Fig. 3